GENE EXPRESSION UNDERLYING FERRET HAIR GROWTH INITIATION INDUCED BY MELATONIN AND MODIFIED BY STEROIDS

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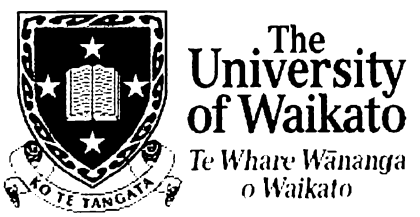

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ABSTRACT

Synchronised ferret winter pelage development induced by melatonin was utilised as an *in vivo* model for studying gene expression associated with the hair growth cycle. The effects of melatonin and steroids on hair follicle growth initiation in flank skin samples were monitored by histology and immunocytochemistry. Melatonin implantation initiated hair growth within 10 days. Exogenous steroids delivered by slow-release subcutaneous implants had mainly inhibitory effects on spontaneous and melatonin-initiated hair growth, either when given simultaneously or prior to melatonin administration. 17β-oestradiol totally suppressed hair follicle growth in all circumstances, while the inhibitory effects of dexamethasone, testosterone and deoxycorticosterone were weaker and attenuated by melatonin. Progesterone given simultaneously with melatonin inhibited hair growth in males, but did not significantly affect female hair growth initiated by melatonin.

Gene expression underlying ferret hair growth was studied using an optimised differential display technique using two sets of complementary skin samples covering all growth initiation stages. The differential display band patterns for approximately 8% of all skin transcripts suggested that more than a thousand genes are required for hair follicle growth. Of the differentially expressed genes detected in a set of flank skin samples covering all proanagen stages, up to a half are likely to be specifically associated with hair growth.

The 150 expressed sequence tags (ESTs) discovered represented 112 unique sequences. Forty-two of these were aligned by sequence homology to known genes, while the remainder are likely to represent novel genes. Differential expression through the follicle growth cycle or after the treatments was confirmed for 21 out of 23 genes whose mRNAs were detectable by Northern blot hybridisation. Overall, expression alteration for most of these ESTs occurred prior to morphological changes in the skin associated with hair follicle reactivation. All the ESTs whose expression sites were detected by *in situ* hybridisation were localised to hair follicles. Some were detected only in certain stages of the hair follicle cycle, others in all growth stages, but at different levels. Classification of the identified ESTs suggested that apoptosis as well as fatty acid synthesis and cholesterol metabolism were closely

associated with hair growth initiation, while cell interaction and motility were also particular prominent processes in hair follicle regrowth.

Additional DNA sequence information was obtained for ferret hair acidic keratin 8 (fHa8) and ferret cyclin D-interacting myb-like protein 1 (fDMP1). Both of these genes were expressed only in cortical cells in the keratogenous zone from mid-proanagen. fHa8 was expressed only in one side of the cortex, a feature has not previously been reported for other type I intermediate hair keratins. The restricted expression of fDMP1 and its smaller size suggested that it was an alternatively spliced form of the human and mouse homologues with a specialised function in the regulation of cortical cell proliferation and differentiation. Further studies on the expression regulation and function of the genes represented by the ESTs identified in this study promise to considerably advance our understanding of hair follicle growth control.

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°C	degrees Celsius
aa	amino acid
AAP	abridged anchor primer
ABR	arbitrary primer
ACL	ATP citrate lyase
ACTH	adrenocorticotrophic hormone
ADX	adrenalectomy
AMH	anti-Mullerian hormone
AP-1	activating protein-1
ARF locus	alternative reading frame locus
ATP	adenosine triphosphate
BCIP	5-bromo-4-chloro-3-indolyl-phosphate
β-HSD	β-hydroxysteroid dehydrogenase
BLAST	basic local alignment search tool
BMP	bone morphogenic protein
bp	base pair
BrdU	5-bromo-2-deoxyuridine
BSA	bovine serum albumin
BTEB1	basic transcription element binding protein 1
CAM	cell adhesion molecule
CDC	cell division cycle
CDK(s)	cyclin-dependent kinase(s)
cDNA	complementary DNA
Ci	Curies
CIR	CBF1-interacting corepressor
CoA ligase	xenobiotic/medium-chain fatty acid: CoA ligase form XL-III
Colgn α2I	type I collagen α-2 chain
CPI	carboxypeptidase inhibitor
CRH	corticotrophin releasing hormone
CRIP	cysteine-rich intestinal protein
CRHP	cysteine-rich heart protein
CS	chondroitin sulfate
CTP	cytosine triphosphate
D	day
DBest	GenBank EST database
dCTP	deoxycytosine triphosphate
DEPC	diethyl pyrocarbonate
Dex	dexamethasone
dGTP	deoxyguanosine triphosphate
DHEA	dehydroepiandrosterone
DHT	dihydrotestosterone
DIF	differentiation gene
DIG	digoxigenin
DMP1	cyclin D-interacting myb-like protein
DMSO	dimethyl sulphoxide
DNA	deoxyribonucleic acid
DOC	deoxycorticosterone
DNase	deoxyribonuclease
dNTPs	an equal molar mixture of dATP, dCTP, dGTP and dTTP

Drg	differentiation regulated gene
DHT	dihydrotestosterone
dTTP	deoxythymidine triphosphate
E2	oestradiol
EB	ethidium bromide
ECM	extracellular matrix
E. Coli	*Esherichia coli*
EDTA	ethylene diaminetetraacetic acid
EGF	epidermal growth factor
ER	endoplasmic reticulum or oestrogen receptor
ERK	extracellular signal-regulated kinase
ERM	ezrin/radixin/moesin
EST	expressed sequence tag
EVA	epithelial V-like antigen
F	female
f	ferret
FGF	fibroblast growth factor
FSH	follicle stimulating hormone
g	gram
G1	gap1 (pre DNA synthesis) phase
G2	gap 2 (post DNA synthesis) phase
GAKIN	guanylate kinase associated kinesin
GAPDH	glyceraldehyde-3-phosphate dehydrogenase
GnRH	gonadotrophin-releasing hormone
GnTI	mannosyl-glycoprotein acetylglucosaminyltransferase
GTP	guanosine triphosphate
GTPase	guanosine triphosphatase
HSCC1	*Homo sapiens* T84 colon carcinoma cell IL-1β regulated mRNA
HCHO	formaldehyde
h	human
HCG	human chorionic gonadotrophin
Ha	hair acidic keratin
Hb	hair basic keratin
HC	heavy chain
Hr(s)	hour(s)
HRP	horse radish peroxidase
HSPC	*Homo sapiens* proteasome subunit C
ICC	immunocytochemistry
IEX1	radiation early response gene
IEX1L	apoptosis inhibitor gene
IF(s)	intermediate filament(s)
IGSF	immunoglobulin superfamily
IPTG	isopropylthio-β-galactosidase
JNK	c-jun terminal kinase
kb	kilobase pair
KGF	keratinocyte growth factor
L	litre
LAR	leukocyte common antigen related protein
LB	Luria broth
LH	luteinizing hormone
LIM	Lin-1, isl-1 and mec-3 genes
LPA	lysophosphatidic acid

LMWG	low molecular weight GTP binding protein
M	male/moles/mitosis phase
MAGUKs	membrane-associated guanylate kinase homologues
MAP	mitogen activated protein
MAPK	MAP kinase
MEK	MAP/ERK kinase
MEKK	MAP/ERK kinase kinase
m	mouse
mg	milligram
MGP	matrix G1a protein
MHP	mitochondrial hinge protein
Min	minute
ml	millilitre
MLT	melatonin
mm	millimetre
mM	millimolar
MOPS	3-N-morpholinopropane sulfonic acid
mRNA	messenger RNA
MSSP	c-Myc single stranded DNA binding protein
MyHC	myosin heavy chain
N or n	nucleotide
NBT	nitroblue tetrazolium
Ndr	N-myc-dependent regulation gene
NF	neural factor
µg	microgram
µl	microlitre
µm	micrometre
NCAM	neural cell adhesion molecule
ng	nanogramme
nm	nanometre
NSS	Normal sheep serum
NTPs	an equal molar mixture of ATP,CTP, GTP and TTP
Oligo dT	deoxythymidine oligonucleotide
OSCP	osteoblast specific cysteine-rich protein
P	Proanagen/phosphorus
p	protein
PB	phosphate buffer
PBS	phosphate buffer salt
PCNA	proliferating cell nuclear antigen
PCR	polymerase chain reaction
PFU	plaque forming unit
PGs	proteoglycans
PMI	days post-melatonin implantation
pRb	retinoblastoma protein
PKC	protein kinase C
Prog	progesterone
PTPase(s)	protein tyrosine phosphatase(s)
PVA	polyvinyl alcohol
RACE	rapid amplification of cDNA ends
RDA	representational difference analysis
RNA	ribonucleic acid

RNase	ribonuclease
RNP	RNA polymerase
RPL	ribosomal protein for large subunit
rpm	revolution per minute
RT-PCR	reverse transcription-polymerase chain reaction
RTP	tunicamycin-responsive protein
S	DNA synthesis phase
SAE1C	SUMO-1-activating enzyme E1C subunit
SAGE	serial analysis of gene expression
SCD	stearoyl-coA desaturase
SDS	sodium dodecyl sulphate
sec	second
SEM	Standard error mean
seq	sequence
SF	steroidogenic factor
SRE	steroid response element
SREBP	sterol regulatory element binding protein
SRP72	signal recognition particle
SUMO	small ubiquitin-related modifier
SSC	sodium chloride and sodium citrate solution
TAF	TBP associated factor
TBP	TATA box binding protein
TCA	trichloroacetic acid
TdT	terminal deoxynucleotidyl transferase
TF	transcription factor
TGF	transforming growth factor
TIED	protein with ten integrin-like EGF repeated domains
TM9SF3	transmembrane protein 9 superfamily member 3
TMAC	tetramethylammonium chloride
tRNA	transfer RNA
TMF	TATA element modulatory factor
TNF	tumour necrosis factor
TRF	TAP-related proteins
Tris	tris(hydroxymethyl) aminomethane
TST	testosterone
TTP	thymidine triphosphate
TUNEL	terminal deoxynucleotidyl transferase-mediated dUTP nick end-labelling
U	unit
UBA	ubiquitin-like protein activating enzyme
UBL	ubiquitin-like (protein)
UTP	uridine triphosphate
UTR	untranslated region
UV	ultraviolet
V	volts
v	volume
vs	versus
XAR	x-ray autoradiography
X-GAL	5-bromo-4-chloro-3-indolyl-β-D-galactosidase

CHAPTER 1 INTRODUCTION

1.1 The hair follicle — a model for biological studies

Hair follicles are the skin appendages which produce hair or wool fibres. To fulfil the functions of hair in protection, insulation, sensation, cleansing and communication, tightly controlled developmental and growth processes have evolved. While most of the developmental stages of this tiny organ are completed before birth (Lyne, 1966; Holbrook *et al.*, 1989; Moore *et al.*, 1998; Widelitz and Chuong, 1999), a unique feature of hair follicles is their cyclic growth throughout the lifetime of the organism.

Four major aspects of hair growth control are recognised (Stenn *et al.*, 1996). The first is the heterogeneity of hair in different parts of the body, which is largely determined during foetal and early post-natal development. The second is the cycling of each hair follicle, resulting in the development of successive hair generations. The third aspect is the switch between normal (terminal) and vestigial (vellus) hair growth seen in many species while the fourth aspect controls hair pigmentation. These different processes are interrelated. The terminal/vellus switch and pigmentation are closely linked to cycling control, growth and differentiation of follicle cells. Furthermore, the growth initiation for each subsequent growth cycle is believed to repeat at least some of the developmental processes (Hardy, 1992; Stenn *et al.*, 1996; Widelitz and Chuong, 1999). Therefore, understanding the genetic events controlling the initiation of a new cycle of hair follicle growth is likely to provide insights into other hair growth processes.

As many biological processes in hair follicles and their molecular components are also present in other organ systems (Stenn *et al.*, 1994; Stenn *et al.*, 1996; Cotsarelis, 1997), hair follicles are good models for a variety of physiological and developmental studies. The easy accessibility of hair follicles and their comparative short growth cycles constitute an attractive model in studying mesenchymal-epithelial interactions, cell patterning, cell proliferation, cell differentiation, apoptosis, exogenous gene delivery and gene therapy.

1.2 Hair follicle gene expression and the medical and agricultural implications

As human hair has an important social function, people with hair growth disorders can suffer significant stress. The most common disorders of hair growth include androgenic alopecia, alopecia areata and hirsutism (Paus and Costarelis, 1994). Development of products aimed at correcting these conditions and for purely cosmetic purposes has become a multi-billion dollar industry with an increasing demand for effective treatments. However, the lack of understanding of the molecular mechanisms of hair growth in general, and these diseases in particular, has impeded the development of effective products which are free from side effects.

From the agricultural point of view, production of wool and fur with the features suitable for today's textile and garment industries are also of increasing importance. Farmed fur production requires a high level of consistency of fibre growth and priming, as well as a variety of colour and fibre characteristics. These are genetically controlled features of the hair cycle. The value of wool depends on fibre diameter, curvature, staple length and colour, which are all determined by genotype-specific gene expression. Understanding gene expression associated with these characters will allow future manipulation of quality and the development of new wool or fur products with enhanced yield and value. This is particularly important for countries like New Zealand, where fibre production and export are a significant component of the economy.

1.3 Background and research objectives for the current investigation

Differential display is a commonly used method for detecting the expressed genes associated with many biological processes (Liang and Pardee, 1992; Wan *et al.*, 1996; Sager, 1997). It has also been successfully used in this laboratory to study gene expression during the regressing phase of wool follicle cycle in sheep (Rufaut *et al.*, 1999a; Rufaut *et al.*, 1999b). Rufaut *et al.* isolated nine expressed sequence tags (ESTs) which showed variable expression in sheep skin samples during the transition from anagen to telogen (see Section 2.1 for description of hair cycle). Four of those were identified with previously known genes and the other five represented novel genes (Rufaut, 1997). However, progress was impeded by a lack of highly-synchronised wool follicle growth in sheep and the lower efficiency of the original differential display protocols.

A high degree of synchrony between follicles in their growth phase is important because it enables identification of gene expression associated with specific stages of the hair growth cycle. The adoption of new primer designs and two-stage PCR has increased the efficiency and specificity of differential display (Liang *et al.*, 1994; Ayala *et al.*, 1995; Linskens *et al.*, 1995). Furthermore, the juvenile ferret winter hair growth cycle experimentally initiated by melatonin has been found to be particularly well synchronised (Valtonen and Blomstedt, 1988; Nixon *et al.*, 1992; Nixon *et al.*, 1995). In this study, the combination of the ferret animal model and an improved differential display methodology was applied to identify genes associated with the hair growth cycle.

1.3.1 Effects of melatonin and steroids on ferret winter hair growth

Hair follicle renewal is mainly controlled by rhythms inherent within the follicles. However, these intrinsic rhythms are modulated by external factors, including hormones and photoperiod. For example, melatonin mediated by photoperiod initiates and synchronises winter pelage development in ferrets and other animals. Although this effect is associated with suppressed levels of prolactin (Rose *et al.*, 1987; Nixon *et al.*, 1992), the molecular mechanism is not well understood.

The effects of the steroid hormones, including testosterone, 17β-oestradiol, glucocorticoids, mineralocorticoids and progesterone, on hair growth have been recognised to various degrees in humans and other species. Although glucocorticoids and oestradiol have been studied extensively and shown to inhibit hair growth, their effects on different stages of pelage hair growth are not well defined. Contradictory effects have been reported for testosterone, mineralocorticoids and, in particular, for progesterone. The various outcomes suggest different mechanisms of action depending on the hair growth cycle stage and/or sex. Hence, the first objective of the present study was to investigate the effects of selected steroid hormones on spontaneous and exogenous melatonin-initiated winter hair growth in ferrets.

1.3.2 Gene expression associated with hair growth initiation

During hair follicle growth initiation, follicle stem cells proliferate and ultimately differentiate into up to ten different cell types to form the mature anagen hair follicle. A large number of genes are likely to participate in regulating these

complex processes (Stenn *et al.*, 1994; Stenn *et al.*, 1996). However, no systematic experiment has been reported to investigate the genes involved in hair growth initiation. The well-synchronised ferret hair growth initiated by melatonin in autumn provides a potential model to study gene expression associated with specific stages of hair growth initiation (Pearson *et al.*, 1989; Nixon *et al.*, 1992; Nixon *et al.*, 1995). Genes which are expressed in a growth stage-specific manner will include both those with regulatory functions and others associated with stage-specific components of the hair follicle. In principle, their expression patterns should be able to serve as markers for the stages of the hair cycle. Therefore, generating information on gene expression patterns, particularly for early follicle growth stages, was the second objective of this study. In addition, the altered patterns of gene expression associated with inhibitory and stimulatory steroid treatments were anticipated to provide information on potential key genes responsible for hair growth initiation. Differential display was chosen due to its high sensitivity in detecting both up- and down-regulation of novel and known transcripts and ease of application (Liang and Pardee, 1992; Bauer *et al.*, 1993; Linskens *et al.*, 1995; Wan *et al.*, 1996; Sager, 1997).

1.3.3 Gene identification and further study of selected ESTs

Although gene expression patterns associated with hair growth provide useful information, the cloning and identification of differentially displayed gene fragments by homology searches was the third and primary objective of this study. Cloning of the ESTs enables confirmation of differential expression by independent methods and localisation of their expression sites to skin structures, including hair follicles. Hence, confirmation of differential expression and establishment of a hair growth association for a subset of the ESTs was the fourth objective of this research. Generation of longer sequences for one or two of these ESTs would further corroborate the results. Finally, it was anticipated that the functions of identified ESTs in other tissues or cell culture systems would lead to a preliminary appreciation of the biological processes underlying hair growth initiation.

CHAPTER 2 LITERATURE REVIEW

2.0 Overview

In this chapter the basic biology of hair follicles and the effects of melatonin and prolactin on hair growth in general and ferret hair growth in particular, are introduced. This is followed by an introduction to steroid biochemistry, effects of steroids on hair growth and their general mechanisms of action. The current techniques for gene discovery are also introduced and compared to differential display, together with relevant results from some large-scale studies on gene expression.

2.1 Hair growth

2.1.1 Hair follicle anatomy and follicle growth cycles

Hairs are hard, keratinous, epithelial structures anchored in tubular invaginations of the skin epidermis, called hair follicles. Between different species, the thickness of a hair fibre ranges from less than 10 to more than 500 μm. Growing follicles are comprised of multiple concentric layers throughout most of their length and terminate in an enlargement, termed the follicle bulb, consisting of a germinal matrix and a dermal papilla. Matrix cells are located around an ovoid cavity containing the dermal papilla, which is in contact with surrounding connective tissue via an aperture at the base of the bulb (Fig. 2-1 and Fig. 2-3c).

In the centre of the follicle is the hair shaft surrounded by concentric structures called the inner and outer root sheaths (Figs. 2-1). The fibre is composed of three major cell types, the fibre cuticle, the cortex and the medulla. The latter is usually present in the coarse fibres of many species. In mammals, hair follicles grow in bundles, with the central follicles attached to a small strand of smooth muscle, the erector pili. Usually one or more sebaceous glands are situated at the level of the muscle. Together with the follicles, these structures comprise the pilosebaceous unit (Chase, 1954; Lyne, 1966).

In an actively growing follicle, germinal matrix cells continue to proliferate. The newly derived cells move upwards through the bulb and begin differentiation into various cell types which eventually form the hair and inner root sheath. During

this process, cells destined to form the fibre become keratinised in a keratinisation zone located between the lower and middle third of the follicle.

The three cellular layers of the inner root sheath are keratinised and form trichohyalin. They merge into a hyaline layer above the middle of the follicle and shed into the hair canal at the level of the sebaceous gland opening. The outer root sheath represents a continuation of the stratum basale and spinosum of the epidermis. It extends all the way to the tip of the bulb, around which are one or two layers of highly flattened cells. The hair follicle is enclosed by a glassy membrane which is very thin in the bulb region. Finally, the dermal sheath forms the outermost layer, which provides a barrier, as well as a connection to the dermal surroundings (Chase, 1954; Silver *et al.*, 1969; Holbrook *et al.*, 1989).

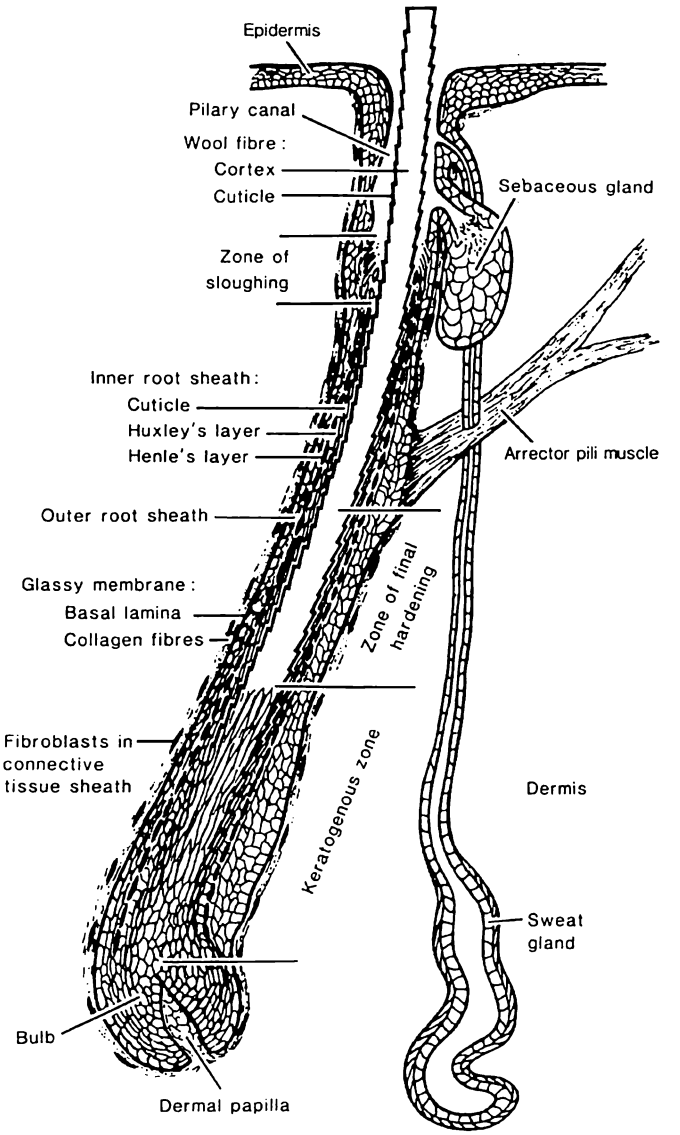


Fig. 2.1 An original follicle with a non-medulated wool fibre. Reproduced from Chapman & Ward, 1979.

The follicle structure described above is that observed in the growth phase, termed anagen (Figs. 2-1 and 2-3c) (Chase, 1954; Parry *et al.*, 1995). However, the growing follicle is not a permanent structure of skin, as hair follicles recycle

through resting and growing phases (Fig 2-2). After the cessation of growth, a follicle passes through a transitional regressive phase, catagen, before transformation into the resting stage, telogen (Figs 2-2 and 2-3a). During catagen, germinal matrix cells stop dividing, the cells derived from the matrix undergo keratinisation and follicles are shortening later in the stage. Apoptosis is also involved at this stage (Lindner *et al.*, 1997; Matsuo *et al.*, 1998; Muller-Rover *et al.*, 1999). These processes transform the bulb into a club-like mass which eventually separates from the shrunken papilla and ascends, forming a "club end" and a much shortened follicle, typical morphological features of the telogen follicle. The club hair is eventually shed or pushed out by the newly growing hair originating in the vicinity of the old dermal papilla (Straile *et al.*, 1961).

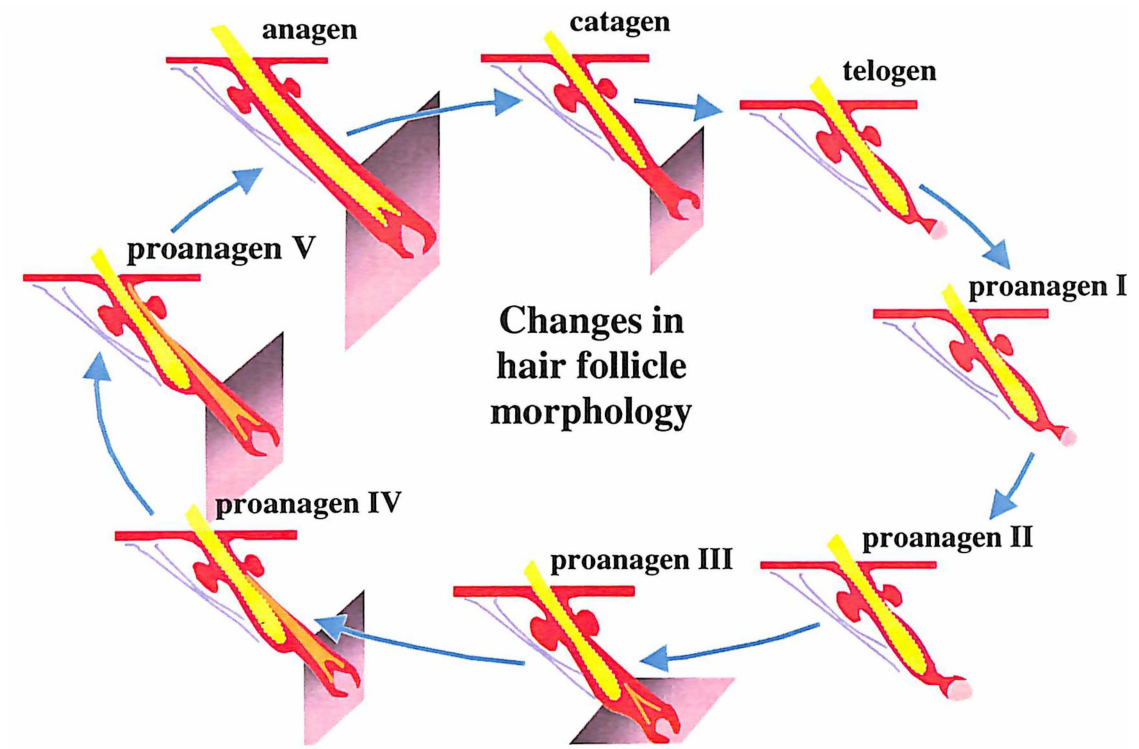


Fig. 2-2 Hair follicle growth cycle

Basic structure of hair follicles at various stages. yellow: fibre; red: inner- and outer-root sheath, sebaceous gland and epidermis; pink: dermal papilla; purple: erector pili; light brown: hair cone of a newly growing follicle.

2.1.2 Regrowth of the hair follicle (proanagen)

The initiation of hair follicle regrowth has been subdivided into 6 distinct stages (Fig. 2-2) (Chase, 1954; Nixon, 1993a; Nixon *et al.*, 1993b). Proanagen I is the period when the resting hair germ resumes mitotic activity (Silver and Chase,

1970). Proanagen II is the first stage in which the growth of a follicle can be identified by follicle histology, as the hair germ cells begin to grow down around the papilla. By proanagen III (Fig. 2-3b), the bulb is completely formed, the new follicle has significantly increased in size and a cone-shaped inner root sheath first appears. During proanagen IV, the inner root sheath has extended to the level of sebaceous gland. By proanagen V, the tip of the hair has broken through the inner root sheath and has reached the level of epidermis. Emergence of the hair from the epidermis marks completion of the growth initiation process and the establishment of a state of relatively constant fibre growth (proanagen VI or anagen) (Chase, 1954; Nixon *et al.*, 1992; Nixon *et al.*, 1993b; Parry *et al.*, 1995). This is accompanied by down-growth of the follicle into subcutaneous tissues. The duration of growth initiation varies between species and even different generations of hair within the same species, but typically lasts between a few days and several weeks.

It is generally believed that signals from the dermal papilla initiate hair regrowth (Oliver and Jahoda, 1989). As a response to these signals, it has been hypothesised that stem cells existing in the bulge region of a hair follicle (Cotsarelis *et al.*, 1990; Wilson *et al.*, 1994; Taylor *et al.*, 2000) migrate to the hair bulb. Other sources of stem cells have been identified in the lower part of the germinal matrix (Reynolds and Jahoda, 1991). Proliferation of the cells in the germinal matrix around the dermal papilla produces transient amplifying cells which form a hair cone in early proanagen. These cells differentiate into approximately 10 different cell types and eventually establish the different structures of the hair follicle. Hence, hair follicle regrowth and fibre formation require the delicate regulation of cell proliferation, pattern formation, differentiation, apoptosis, and other processes. As the hair follicle cycle is readily generated by depilation and other manipulations, and hair follicles can be microdissected and isolated, the hair follicle is an excellent model for identifying genes and gene expression associated with these processes. Understanding the mechanisms of growth initiation control and identification of associated genes is not only scientifically interesting, but also has important potential applications both in medicine and agriculture (Section 1.2). However, no systematic study of gene expression underlying hair growth initiation has yet been conducted.

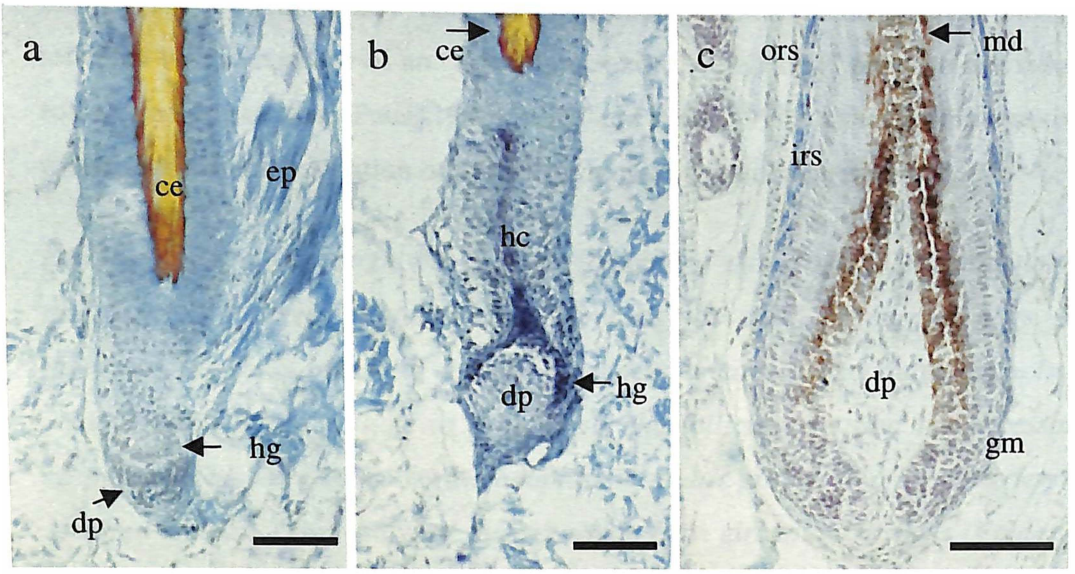


Fig. 2-3 Ferret hair follicle growth initiation

a: telogen; b: proanagen III; c: anagen; ce: club end; dp: dermal papilla; ep: erector pili; gm: germinal matrix; hc: hair cone; hg: hair germ; irs: inner root sheath; ors: outer root sheath.

2.1.3 Hair growth regulation

The initiation of a hair growth cycle is strictly controlled by a variety of internal and external factors (Ebling, 1965; Ebling, 1981; Stenn *et al.*, 1988; Hardy, 1992). In mice and rats, cycles of pelage hair growth occur several times a year, independent of temperature and daylight. However, in most wild animals, the seasonal cycles are associated with daylight length and endocrine activity. In other species such as guinea pigs and humans, each follicle follows independent growth rhythms.

Intrinsic regulation: The pattern in which a follicle grows is inherent to that follicle, as transplanted follicles remain true to their site of origin (Johnson, 1965; Jahoda *et al.*, 1996). In many species, the original follicles which produce guard hair and the derived follicles which produce finer under-fur differ in both the length of different growth stages and their responses to exogenous hormones (Jackson and Ebling, 1972; Ansari-Renani and Hynd, 1996; Pearson *et al.*, 1999). It is well known that hair in different body sites can respond to androgens in very different ways (Ebling, 1981; Randall, 1994). The levels of androgens needed for the growth of hair at different parts of the body are also different (Ebling *et al.*, 1991). Such observations demonstrate that hair growth is primarily controlled

intrinsically by genetically and developmentally determined settings. This is reflected by the concept of an "autonomous hair cycle clock" for each individual follicle located in skin (Paus, 1996; Stenn and Paus, 1999), although the working mechanisms and the location of this clock remain to be deciphered.

External factors: Although the core periodicity of this clock is intrinsic to the follicle, it is believed to respond to input signals from the surroundings. By incorporating these incoming signals, the clocks in turn release output signals to various parts of the follicle to orchestrate the follicle cells and the necessary responses. This is reflected by the adaptation of hair growth to a large variety of external stimuli. These external factors range from circadian rhythms (Foldes et al., 1991), neurotransmitters (Botchkarev et al., 1997; Botchkarev et al., 1999; Peters et al., 1999), cytokines (Danilenko et al., 1996; Mahe et al., 1996), cell adhesion molecules (Combates et al., 1997), growth factors (Danilenko et al., 1996; Lachgar et al., 1996a; Nixon and Moore, 1998; Nixon et al., 1999b) and a variety of hormones. Not only do these factors have varying effects on hair growth, but their effects are sometimes dependent on the growth stages of the follicle (Nixon et al., 1992; Nixon et al., 1995; Pearson et al., 1997; Nixon and Moore, 1998; Nixon et al., 1999b), indicating the extremely complicated regulating processes.

2.1.4 Identification of genes or gene expression patterns associated with hair growth

Growth initiation is a pivotal event in the control of the hair follicle as it commences a whole cycle of processes in hair growth. A number of genes which are expressed in hair follicles during development and cycling have been identified (Stenn *et al.*, 1994; Paus, 1996; Stenn *et al.*, 1996). Inhibition or over-expression of some of these genes leads to abnormal hair growth. Despite the ever increasing number of molecules identified and the establishment of important roles of some growth factors in follicle growth initiation (Jindo *et al.*, 1994; Danilenko *et al.*, 1995; Shimaoka *et al.*, 1995; Lachgar *et al.*, 1996b), the regulation of hair follicle growth initiation is largely unknown. Thus, determining the molecular mechanisms that orchestrate the hair follicle growth cycle remains a key challenge in hair biology. Given the complexity of hair follicle formation, the number of genes involved is potentially large and the expression of many of these

genes is expected to differ during the follicle growth cycle. Furthermore, the control of growth initiation may not be determined by the expression of one or two genes alone. It is more likely that changes in the expression of a group of genes and their subsequent regulation of other genes paves the way for hair growth reinitiation. As it is presently difficult to identify the initial genes responsible for proanagen, investigating the patterns of gene expression, especially for the early stage before structural formation, may be an alternative. If sufficiently large-scale screening is conducted, the patterns of gene expression can provide information on the number of genes associated with the process and the interactions between them, as well as possible candidates for the initiating genes. Furthermore, if treatments causing opposite effects are compared and analysed for the genes involved, it may be helpful not only in understanding the mechanisms of these treatments, but also in suggesting key genes involved in hair follicle growth or inhibition (McClelland *et al.*, 1994). To achieve these goals, a well-synchronised and easily controlled system of hair growth initiation is essential. Ferret winter hair growth, initiated and synchronised by exogenous melatonin appears an attractive model for this purpose (Nixon *et al.*, 1992; Nixon *et al.*, 1995).

2.1.5 Hair growth in the ferret

2.1.5.1 Postnatal hair growth

As in other members of the mustelid family, ferrets have seasonally dependent cycles of hair growth (Kondo *et al.*, 1988; Blomstedt, 1989). Ferrets are usually born in mid to late spring and the body of the new-born ferret is covered only with fine hair of first generation original (primary) follicles. Although the second generation of original follicles already exists, follicles are still at the primordial stage. Derived (secondary) hair follicles develop as branching of the original follicles. Original and derived follicles form compound follicles, usually in groupings of three original and their derived follicles (trios) (Nixon *et al.*, 1995). These original and derived follicles develop into the guard hair and underfur of juvenile summer coat (whelch). At 9 weeks of age, the number of underfur fibres is approximately 7 follicles/bundle, with both growing and resting follicles present (Blomstedt, 1995; Nixon *et al.*, 1995). Both original and derived follicles continue to enter the resting phase which gradually becomes dominant. Juvenile

hair growth takes about 12 to 14 weeks to complete, which occurs in early to mid summer (Fig. 2-4) (Blomstedt, 1995; Nixon *et al.*, 1995). At this stage, all hair follicles are in the resting phase. This is followed almost immediately by the growth of first winter fur and the shedding of the juvenile hair in a tail-to-head direction (Blomstedt, 1995). Original follicles begin regrowth and enter anagen earlier than derived follicles (Fig. 2-5A) (Nixon *et al.*, 1995), although they are also reported to complete the growth of this first winter pelage simultaneously (Blomstedt, 1995). New follicles initially grow alongside and eventually replace the previous fibres. The number of derived follicles increases as more follicles underfur peaks after two to two and half months of growth, with an average density of 16 follicles/bundle. The winter pelage lasts for approximately 6 months for females and seven and a half months for males before moulting (Fig. 2-4). Spring pelage commences growth in waves in a head-to-tail direction (Blomstedt, 1995).

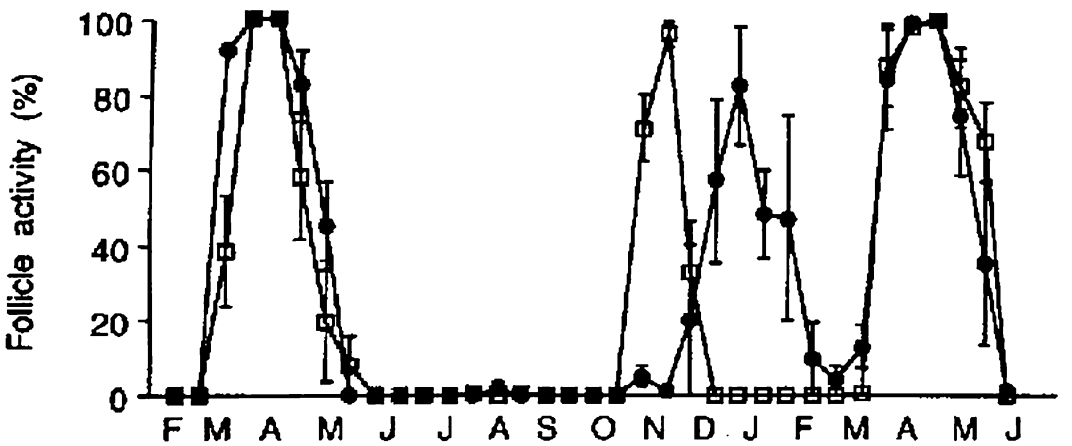


Fig. 2-4 Original hair growth cycle of ferrets

Lines with solid circles and hollow squares represent the percentages of growing male and female hair follicles, respectively. Vertical bars show standard errors. The ferrets were born in late November and follicle activity was monitored for 17 months from 3 months of age. Reproduced from Nixon *et al.*, 1995.

2.1.5.2 Effects of photoperiod on hair growth

Photoperiodism affects hair growth. In sheep, if the light cycle is reversed, the moulting cycle shifts 6 months out of phase (Rougeot *et al.*, 1984). Exposure of shedding sheep to long day light photoperiod, or to short day light, followed by long day light treatment induces a synchronised out-of-season follicle cycle, characterised by initial cessation of follicle growth and then resumption of growth

(Craven *et al.*, 1994; Parry *et al.*, 1995). This results from a short-term inhibitory effect of natural or artificial increases in daylength on growing wool follicles (Pearson *et al.*, 1996). When mink are subjected to short day light treatment (6L: 18D) in summer, pelage becomes prime nearly a month earlier than controls (Weiss *et al.*, 1980). This is consistent with the observation from others (Stout *et al.*, 1969) that abruptly reduced light to a constant amount of artificial light during summer to autumn, rather than to a gradually reduced light was necessary to promote early winter pelage. Ferret hair growth was found to be controlled by day length in the same way: decreased photoperiod length stimulated hair growth while increased day length halted hair growth (Harvey and MacFarlane, 1958). These effects of photoperiod on hair growth are achieved via the modulation of the levels of melatonin, a pineal hormone.

2.1.5.3 Synchronisation of autumn hair growth by melatonin

The stimulatory effects of melatonin on hair growth have been confirmed in various species (Rust and Meyer, 1969; Rose *et al.*, 1984; Rougeot *et al.*, 1984; Nixon *et al.*, 1993b; Nixon *et al.*, 1995; Gebbie *et al.*, 1999). The time frame and doses needed to promote winter fur growth in ferrets, mink and other mustelids have also been reported (Rose *et al.*, 1984; Rougeot *et al.*, 1984; Rose *et al.*, 1985; Rose *et al.*, 1987; Pearson *et al.*, 1989; Nixon *et al.*, 1995). Melatonin at 10 to 120 mg dose ranges stimulates winter fur growth in mink with the effectiveness increasing with dose (Rose *et al.*, 1987).

However, neither the duration of the growth phase, nor the growth rate was altered (Rose *et al.*, 1987). The advancement in prime time is due to the quicker and better synchronised growth between follicles compared with natural winter hair growth (Fig. 2-5B) (Valtonen and Blomstedt, 1988; Nixon *et al.*, 1995). By labelling with the thymidine analogue 5-bromo-2'-deoxyuridine (BrdU) (Dean *et al.*, 1984; Holle and Harris, 1992), it was found that DNA synthesis in the dermal matrix started 2-4 days after melatonin treatment and 2 days before morphological changes could be identified (Nixon *et al.*, 1992; Saywell and Nixon, 1992). Proanagen II was predominant at day 8-10, proanagen III and IV at day 12 and anagen at day 14.

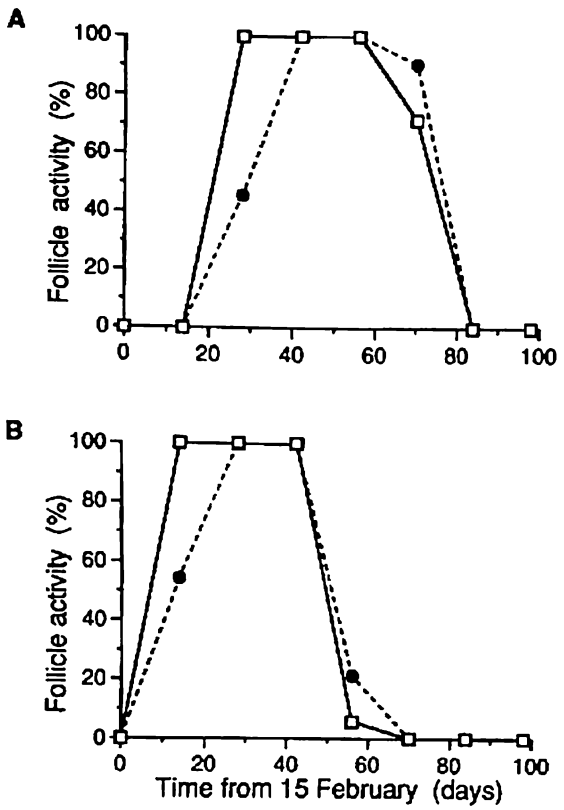


Fig. 2-5 Effects of melatonin on ferret winter pelage follicle growth

Lines with hollow squares and solid circle represent original and derived hair follicles, respectively. A: untreated ferrets; B: ferrets administered with melatonin on day 0. Reproduced from Nixon *et al.*, 1995.

In summary, synchronised hair growth occurs within a matter of days after the administration of exogenous melatonin in the autumn (Nixon *et al.*, 1992; Nixon *et al.*, 1995). This high level of synchronisation is useful in dissecting the different hair growth stages and the associated patterns of gene expression. In comparison, the lack of synchrony in spontaneous hair growth in most species complicates the identification of genes related to specific growth stages. In contrast to animal models in which hair growth is initiated by plucking, the ferret model does not involve wounding effects. Plucking-initiated hair growth differs from spontaneous hair growth due to the different pathological and physiological processes involved (Silver *et al.*, 1969).

2.2 Effects of melatonin and prolactin on hair growth

2.2.1 Melatonin

Melatonin, N-acetyl-5-methoxytryptamine, is synthesised mainly in the pineal gland, by N-acetylation of serotonin followed by methylation of the 5-hydroxy moiety. The primary function of melatonin is photic regulation, including adaptation to light intensity, daily changes of light, darkness and seasonal changes of day length (Vanecek, 1998). Darkness stimulates the synthesis of melatonin,

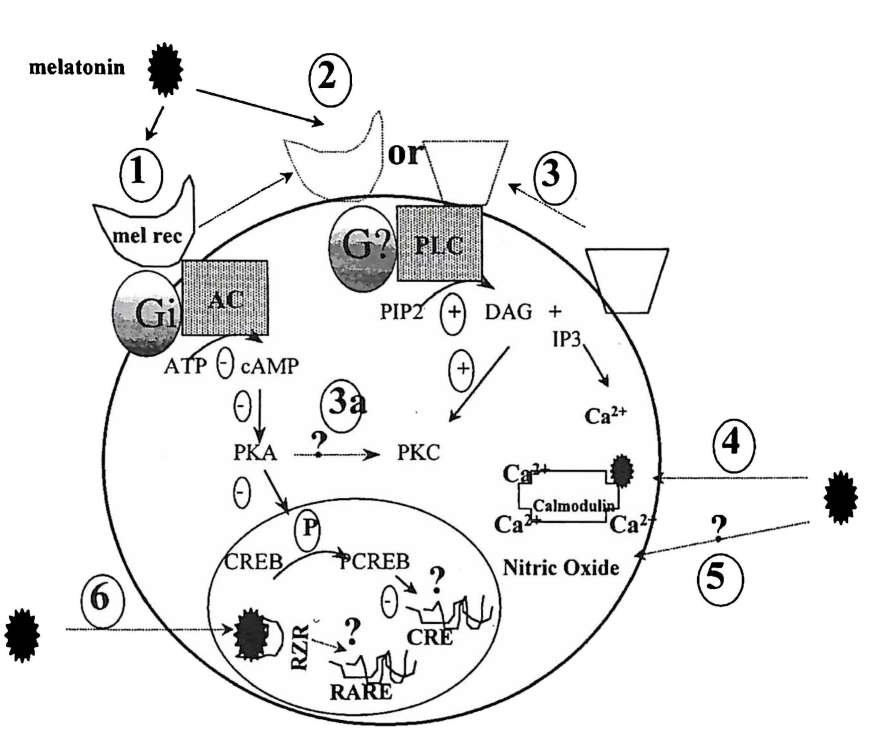


Fig. 2-6 Proposed melatonin signalling pathways at the cellular level

Melatonin can work through different classes of receptors (1, 2 or 3 indicated by different shapes), either by decreasing adenyl cyclase (AC) activity (1) or coupling to phospholipase C (PLC)-dependent pathway (2 and 3). Melatonin can also act independently of receptors by direct binding to cytosolic proteins (4 and 5) or nuclear proteins (6). Reproduced from Witt-Enderby *et al.*, 1995.

while light represses it. Therefore, melatonin synthesis in the pineal gland and other organs shows a marked daily rhythm with low values during the day and a large increase at night. Under long-day photoperiod, the duration of melatonin secretion decreases and the melatonin pulse is short. The target organs are hence informed of the seasons of the year by the changing length of the melatonin pulse.

The effects of melatonin are often mediated through binding to specific cell membrane melatonin receptors. Being highly lipophilic, melatonin can also enter cells through the cell membrane to act independently of receptors. Melatonin receptors are GTP-binding protein coupled receptors characterised by a seven membrane-spanning domain. On binding to the receptor, melatonin can achieve its various effects through diversified signal transduction pathways (Fig. 2-6) (Williams *et al.*, 1996; Witt-Enderby and Li, 2000). Besides entraining circadian and diurnal rhythms, melatonin also influences reproduction and sexual maturation (Smith *et al.*, 1987; Connor, 1988), among many other functions (Vanecek, 1998; Wetterberg, 1999). While melatonin affects gonads directly (Kitay and Altschule, 1954), it also modulates steroid hormone production (Maywood and Hastings, 1995; Lincoln, 1999), their receptors (Slayden and Stormshak, 1992; Rose *et al.*, 1996; Sainz *et al.*, 1999), prolactin production (Lincoln and Clarke, 1994; Lincoln, 1999) and its receptors (Slayden and Stormshak, 1992; Rose *et al.*, 1996). It also inhibits GnRH pulse and luteinizing hormone (LH) (Nakazawa *et al.*, 1991). Taking melatonin orally (1.0 to 1.3 mg/kg) significantly decreased serum oestradiol, testosterone and dehydroepiandrosterone (DHEA) concentrations over 28 days in female dogs and caused a significant decrease in serum oestradiol and 17-hydroxyprogesterone concentrations in male dogs (Ashley *et al.*, 1999). However, oestradiol concentration was increased (Slayden and Stormshak, 1992; Rose *et al.*, 1996), while progesterone levels were reduced by giving melatonin in mink (Rose *et al.*, 1996).

Exogenous melatonin administered during the autumn causes earlier hair growth initiation in mink (Rougeot *et al.*, 1984; Rose *et al.*, 1985; Rose *et al.*, 1987; Rose *et al.*, 1998), ferrets (Nixon *et al.*, 1995), silver fox (Connor, 1988) and blue fox (Smith *et al.*, 1987). Goat spring fibre growth is also advanced by melatonin administration (Nixon *et al.*, 1993b; Dicks *et al.*, 1995). However, melatonin administered in spring shows inhibitory effects on hair growth in mink (Rougeot *et al.*, 1984; Rose *et al.*, 1998) and blue fox (Smith *et al.*, 1987). As melatonin receptors have not been found in skin (Dicks *et al.*, 1996), it is likely that its effect is through regulation of other hormones, such as prolactin, whose levels are associated with hair follicle growth and regression (Section 2.2.2).

2.2.2 Prolactin

Prolactin is primarily a pituitary hormone that is present in all vertebrates. With the exception of fish prolactin, all characterised prolactin molecules are comprised of 197-199 amino acids and contain six cysteines forming intra-molecular disulfide bonds (Bole-Feysot *et al.*, 1998). Pituitary prolactin acts via a classic endocrine pathway, secreted by a gland, transported by the circulation system, and acting on specific target cells through receptors located on the plasma membrane. The prolactin receptor belongs to the class I cytokine receptor family. The binding of prolactin to the binding sites of the receptor induces dimerisation of the receptor and stimulates signal transduction pathways leading to various gene expression responses in different target tissues (Fig. 2-7) (Goffin and Kelly, 1997; Bole-Feysot *et al.*, 1998). Isoforms of the prolactin receptor have been found in some species and their respective functions have attracted much attention (Krown *et al.*, 1992; Clevenger *et al.*, 1998; Kline *et al.*, 1999). Prolactin receptors are not only expressed in skin (Ouhtit *et al.*, 1993), but are also expressed in hair follicles in a cycle-dependent manner (Choy *et al.*, 1995; Nixon *et al.*, 1999b). These observations allow for the actions of prolactin on fibre growth suggested by a variety of other endocrine studies (Martinet *et al.*, 1983; Duncan and Goldman, 1984; Martinet *et al.*, 1984; Rose *et al.*, 1987; Curlewis *et al.*, 1988; Pearson *et al.*, 1999).

Prolactin has numerous functions, some 200 or more, covering many aspects of biological processes in almost all mammal tissues (Bole-Feysot *et al.*, 1998). Effects on hair follicle growth are also evident. Administration of exogenous prolactin induces spring moult in mink (Rougeot *et al.*, 1984), while the local infusion of prolactin enhances localised summer coat development in red deer (Thomas, 1994). In contrast, administration of prolactin or dopamine antagonists to elevate circulating prolactin level prevents the growth of the winter fur in hamsters (Duncan and Goldman, 1984; Badura and Goldman, 1992) and meadow voles (Smale *et al.*, 1990). In New Zealand Wiltshire sheep, rising prolactin levels due to either photoperiod transition or the administration of exogenous prolactin, are associated with regression of both original and derived follicles (Pearson *et al.*, 1993; Pearson *et al.*, 1996; Pearson *et al.*, 1999). Similarly, suppression of

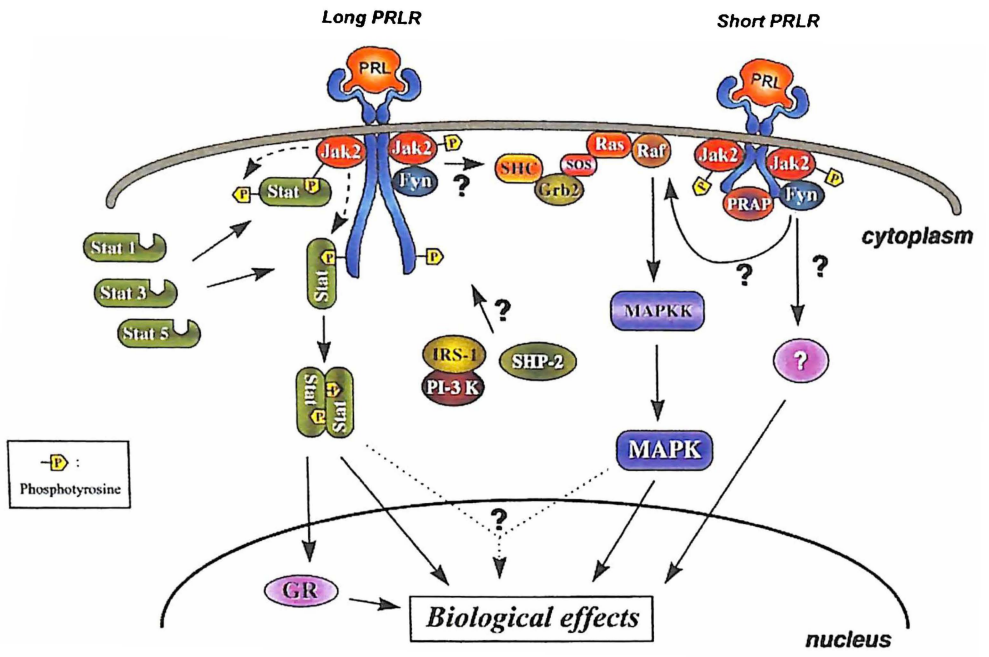


Fig. 2-7 Prolactin receptor signalling pathways

Prolactin induces receptor dimerisation by binding to either long or short form receptors. Activation of JAK-Stat and MAP kinase pathways relay the signal into nucleus to modulate gene expression. Connections between the JAK-Stat and MAPK pathways have been suggested. Reproduced from Bole-Feysot *et al.*, 1998.

prolactin levels by bromocriptine, a prolactin antagonist, maintains hair follicle growth, while the rising level of prolactin resultant from bromocriptine pump removal is linked initially to hair follicle regression (Pearson *et al.*, 1993; Nixon *et al.*, 1997), followed by synchronised out of season wool growth (Nixon *et al.*, 1997). The suppression of prolactin secretion with bromocriptine during summer or autumn telogen induces winter pelage development in both mink (Martinet *et al.*, 1984; Rose *et al.*, 1987) and red deer (Heydon *et al.*, 1995). However, it has also been reported that changes in serum levels of prolactin are not requisite to onset of summer or winter hair growth in mink (Rose *et al.*, 1998; Jonston *et al.*, 1999).

The effect of photoperiod on hair growth is considered to be mediated through melatonin via its inhibitory effects on pituitary prolactin secretion (Rougeot *et al.*, 1984). Prolactin decreases in autumn and increases in spring are associated with hair growth of the winter and summer pelage, respectively (Martinet *et al.*, 1984; Rougeot *et al.*, 1984). Experimental disruption of these seasonal changes also disrupts the corresponding seasonally dependent cycles of hair follicle growth.

Constant-release melatonin implants act as a short day signal (O'Callaghan *et al.*, 1991) to suppress prolactin secretion in a dopamine-related manner (Lincoln and Tortonese, 1995). The effects of melatonin may be mediated indirectly at the pars tuberalis through regulating tuberalin, an unidentified factor (Morgan, 2000). Administration of melatonin to animals in summer telogen inhibits prolactin secretion and induces winter hair growth in mink (Rose *et al.*, 1984; Rougeot *et al.*, 1984; Rose *et al.*, 1985; Rose *et al.*, 1998), ferrets (Nixon *et al.*, 1992; Nixon *et al.*, 1995), and goats (Nixon *et al.*, 1993b). Moreover, spring moult occurs when plasma prolactin levels are increasing (Rougeot *et al.*, 1984). Inhibition of the increasing levels of prolactin in spring delays hair growth in mink (Martinet *et al.*, 1982; Martinet *et al.*, 1983) and blue fox (Smith *et al.*, 1987).

The findings of extrapituitary prolactin production (Healy *et al.*, 1978; Ben-Jonathan *et al.*, 1996; Imaoka *et al.*, 1998) indicate that the locally produced hormone can act in a paracrine or autocrine manner. Thus, it is possible that many of the functions associated with prolactin can occur without involving the circulating hormone. Production of prolactin mRNA in skin has also been confirmed (Nixon *et al.*, 1999a), but whether the hormone is made locally and affects hair growth is not known.

Prolactin also affects steroid production, metabolism and their receptor concentrations. It increases steroidogenesis (Glasow *et al.*, 1996), adrenal androgens (Higuchi *et al.*, 1984), cortisol and aldosterone (Glasow *et al.*, 1996) levels, 21-hydroxylase activity in adrenal gland (Albertson *et al.*, 1987)) and the activity of steroidogenic enzyme 3-β-hydroxysteroid dehydrogenase (3-β-HSD) in skin (Couet *et al.*, 1994; Martel *et al.*, 1994). Prolactin also increases the nuclear uptake of dihydrotestosterone (DHT) (Prins and Lee, 1982) and androgen receptor concentration in prostate (Prins, 1987). Similarly, it enhances the concentration of progesterone receptor and oestrogen receptor in the uterus (Saiduddin and Zassenhaus, 1977; Chilton and Daniel, 1987). However, it is not known whether these effects of prolactin have any association with its functions in the hair growth cycle.

2.3 Steroid biochemistry

2.3.1 Structure and functions of steroids

Steroid hormones are members of large family of compounds derived from the cyclopentanoperhydrophenanthrene ring structure, which consist of three cyclohexane rings and one cyclopentane ring (Fig. 2-8). This group of compounds has many different biological effects depending on the nature of the modifications to the basic steroid nucleus. The unsaturated 17-carbon ring structure is termed gonane. Steroids with 18 carbons, derived from adding a methyl group at C-13, are estranes. Steroids derived by adding another methyl group at C10 are androstanes. Further addition of an ethyl group at C-17 generates a pregnane (Grodsky, 1973).

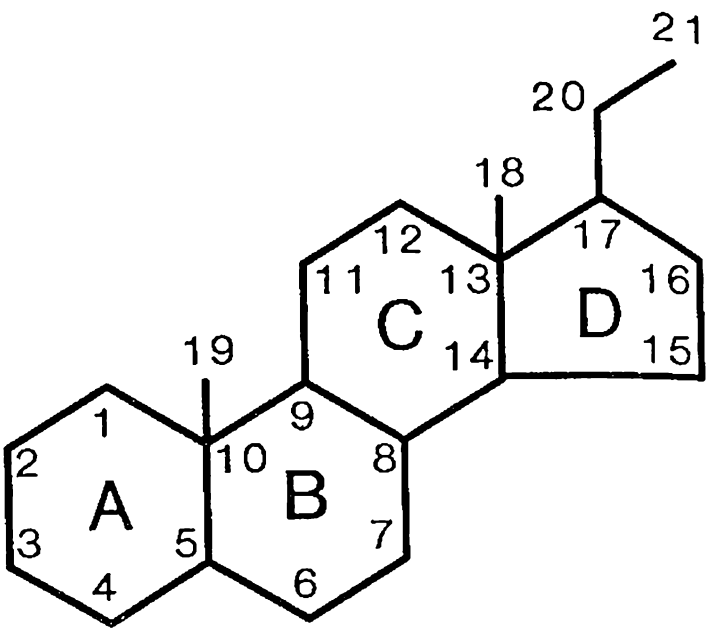


Fig. 2-8 Cyclopentanoperhydrophenanthrene ring structure

Androgens: Androgens are derived from androstane and the main androgens include testosterone (TST), dihydrotestosterone (DHT), androsteneione, androstenedione, androstane, androsterone, epiandrosterone and dehydroepiandrosterone (DHEA). Testosterone is the principal active circulating androgen and it is predominantly secreted by the testis in adult males (Grodsky, 1973).

The adrenal cortex also secrets androstenedione which is the major circulating androgen in females and pre-pubertal males. However, the androgenic effects of androstenedione and DHEA possibly rely on enzymatic conversion to testosterone in peripheral tissues by the enzyme 17β-hydroxysteroid dehydroxygenase (17β-HSD) (Fig. 2-12). In most tissues, intracellular testosterone is reduced by the enzyme 5α-reductase to DHT before it associates with the receptor and subsequent translocation into the cell nucleus. Androgens have diverse effects in many organs and tissues (Moradian *et al.*, 1987), including regulation of the growth and the physiological function of the prostate and seminal vesicles, spermatogenesis in concert with follicle-stimulating hormone (FSH) and the development, growth and the activities of the kidney, liver, muscle and other organs. Testosterone is also converted in the brain into oestrogens that function via the oestrogen receptor (Naftolin *et al.*, 1972; Sisk and DonCarlos, 1995). This metabolic conversion is of utmost importance in developing the male-type brain. Androgens also affect the growth and development of skin and skin appendages (Moradian *et al.*, 1987).

Oestrogen: Oestrogens are C-18 steroids, differing from androgens in lacking the C-19 methyl group at C10. In contrast to all other natural steroids, ring A is aromatic (Fig. 2-9). The three main natural oestrogens: oestradiol, oestrone and oestriol are produced predominantly by the developing graafian follicle cells in the ovary. 17β-oestradiol is the principal circulating and biologically most active form of oestrogen (Orth *et al.*, 1992). Oestriol is mainly present in the placenta and the urine of pregnant women and oestrone is in metabolic equilibrium with oestradiol (Grodsky, 1973). Oestrogen is essential for the normal development, growth and maintenance of most female tissues and their activities. These include the preparation of uterine mucosa for the later action of the progesterone, proliferation and vascularity of many uterine and mammary tissues and the maintenance of female secondary sex characters. Oestrogen actions in the brain might contribute to the differential regulation of oestrogen-dependent functions in the two sexes (Tobet *et al.*, 1993).

Progestins: Progesterone is a C21 steroid hormone and the principal progestational hormone produced mainly in the corpus luteum, the ovarian

structure that develops from the ruptured follicle (Grodsky, 1973). It is also produced in large quantities in the placenta in late pregnancy. In the adrenal cortex, progesterone is also synthesised as a precursor to both glucocorticoids and mineralocorticoids (Orth *et al.*, 1992). Progesterone and oestrogen interact to control the growth, development and physiology of the female reproductive tract and other organ systems. It is important for the establishment and maintenance of pregnancy, and the stimulation of mammary gland cell growth and differentiation.

Glucocorticoids and mineralocorticoids: These are the C21 steroid hormones produced in the adrenal cortex. C-21 hydroxylation is generally necessary for both glucocorticoid and mineralocorticoid activities. Glucocorticoids influence virtually every organ and tissue in the body. Their effects span the physiological spectrum from behaviour to metabolism of carbohydrates (Orth *et al.*, 1992). Mineralocorticoids maintain the electrolyte balance by regulating the activity of kidney, salivary glands, sweat glands and gastrointestinal tract. In general, those with an additional hydroxyl or carbonyl group at C11 position and an hydroxyl at C17 position have greater glucocorticoid and lesser mineralocorticoid activities. Examples of glucocorticoids are cortisone, hydrocortisone (cortisol) and corticosterone. The most potent mineralocorticoid is aldosterone, which has the same structure as corticosterone except that an aldehyde group replaces the methyl group at position 18 of corticosterone (Fig. 2-9). Synthetic 11-deoxycorticosterone (DOC) is widely used, although it is only 4% as potent as aldosterone. In contrast, the often-used synthetic glucocorticoid, dexamethasone (Dex), is more potent than many natural glucocorticoids.

2.3.2 Production and regulation of steroids

Cholesterol is the precursor for steroid biosynthesis (Fig. 2-9). Hydroxylation reactions play a very important role in the synthesis of cholesterol and in the conversion of cholesterol into steroid hormones. Hydroxylation requires the activation of oxygen, which is accomplished by the reduced form of P-450, a specialised cytochrome containing multiple enzymes (Orth *et al.*, 1992).

The first stage in the synthesis of steroid hormones is to remove the cholesterol side chain and form pregnenolone. This step is stimulated by a pituitary hormone, adrenocorticotrophic hormone (ACTH). Via oxidation and isomerisation,

pregnenolone is transformed into progesterone, a precursor for many other steroids (Grodsky, 1973; Orth *et al.*, 1992). Cortisol is synthesised from progesterone by hydroxylation at C-17, C-11 and C-21. The hydroxylation at C-21 generates deoxycorticosterone. Further hydroxylation at C-11 and the oxidation of the C-18 angular methyl group to an aldehyde produces the mineralocorticoid, adolsterol. Hydroxylation at C-17 generates 17-α-hydroxyprogesterone, which becomes androstenedione, an androgen, by removal of the side chain. Reduction of the 17-keto group produces testosterone which can also be converted back to androstenedione. These two androgens can be converted into oestrone or oestradiol, respectively, by aromatase (Fig. 2-12). As an alternative to progesterone as a precursor, pregnenolone can be hydroxylated into 17α-hydroxypregnenolone, which is then reduced to DHEA (Fig. 2-9).

Steroid hormone secretion is regulated by hormonal interactions with the hypothalamus, the pituitary, and the gonads or adrenal glands as well as by neural and other stimuli (Orth *et al.*, 1992). The synthesis and secretion of glucocorticoids is controlled by ACTH from the pituitary, which is in turn regulated by corticotrophin releasing hormone (CRH) produced in the hypothalamus. Secretion of aldosterone, the main mineralocorticoid, correlates with cortisol and is also ACTH-dependent. However, unlike that of glucocorticoids, its secretion is tightly controlled by a multiple factor regulatory system involving mainly renin-angiotensin and potassium concentrations (Orth *et al.*, 1992).

The synthesis and secretion of androgens, oestrogen and progesterone are regulated by follicle stimulating hormone (FSH), luteinizing hormone (LH) from pituitary and the respective releasing hormones of FSH and LH from the hypothalamus (Orth *et al.*, 1992). The production of sex hormones in the adrenal cortex is also controlled by ACTH and other factors. In simple terms, there are long and short loops that control steroid hormone secretion. High concentrations of the hormones inhibit the production and secretion of gonadotrophin or corticotrophin from the pituitary gland. Simultaneously, high levels of the hormones also send signals to the hypothalamus to reduce the synthesis and secretion of trophin releasing hormones, which in turn reduces the production and

release of gonadotrophin and corticotrophin from the pituitary. Both these mechanisms result in lower production and secretion by hormone producing cells, and lower steroid hormone concentrations in circulation (long loop). Similarly, high concentrations of trophins signal the hypothalamus to suppress the synthesis and secretion of the relevant trophin releasing hormones (short loop). Low circulating steroid hormones work in the reverse direction to increase their levels. However, oestrogen productions in males and testosterone production in females are not regulated in this manner. In both cases, gonadotrophin production is controlled by the predominant steroid in that sex. Feedback control mechanisms also do not appear to operate in the secretion of placental hormones.

Steroid sulphonation catalysed by steroid sulphontransferases are no longer considered simply a metabolic step preparatory to eventual elimination. Sulphonation converts steroids from essentially hydrophobic compounds to hydrophilic ones by insertion of a highly charged sulphonate (SO_3^-) group (Strott, 1996). The high concentration of circulating steroid sulphonates creates a reservoir of precursors for eventual conversion to unconjugated hormones. Therefore, steroid sulphonation can influence the action of steroid hormones on transcription regulation by controlling the level of the unconjugated hormone capable of receptor binding (Strott, 1996; Song and Melner, 2000).

Peripheral conversions between some sex hormones have been confirmed in skin and hair follicles. 5α-DHT and other steroids can be produced from the circulating androgens (Hay and Hodgins, 1973; Schweikert *et al.*, 1974; Randall and Ebling, 1981; Schweikert and Wilson, 1981). The metabolic pathways and enzymes involved are exactly the same as in the adrenal cortex and testis (Fig. 2-12).

2.3.3 General mechanisms of steroid effects

After secretion, steroid hormones are transported from their production sites to target cells through binding to plasma proteins: corticosteroid-binding globulin (CBG), sex hormone-binding globulin (SHBG) and albumin. Only the free hormones are available to act on target cells. These hormones primarily exert their effects by diffusing into cytoplasm and binding to their specific steroid receptors.

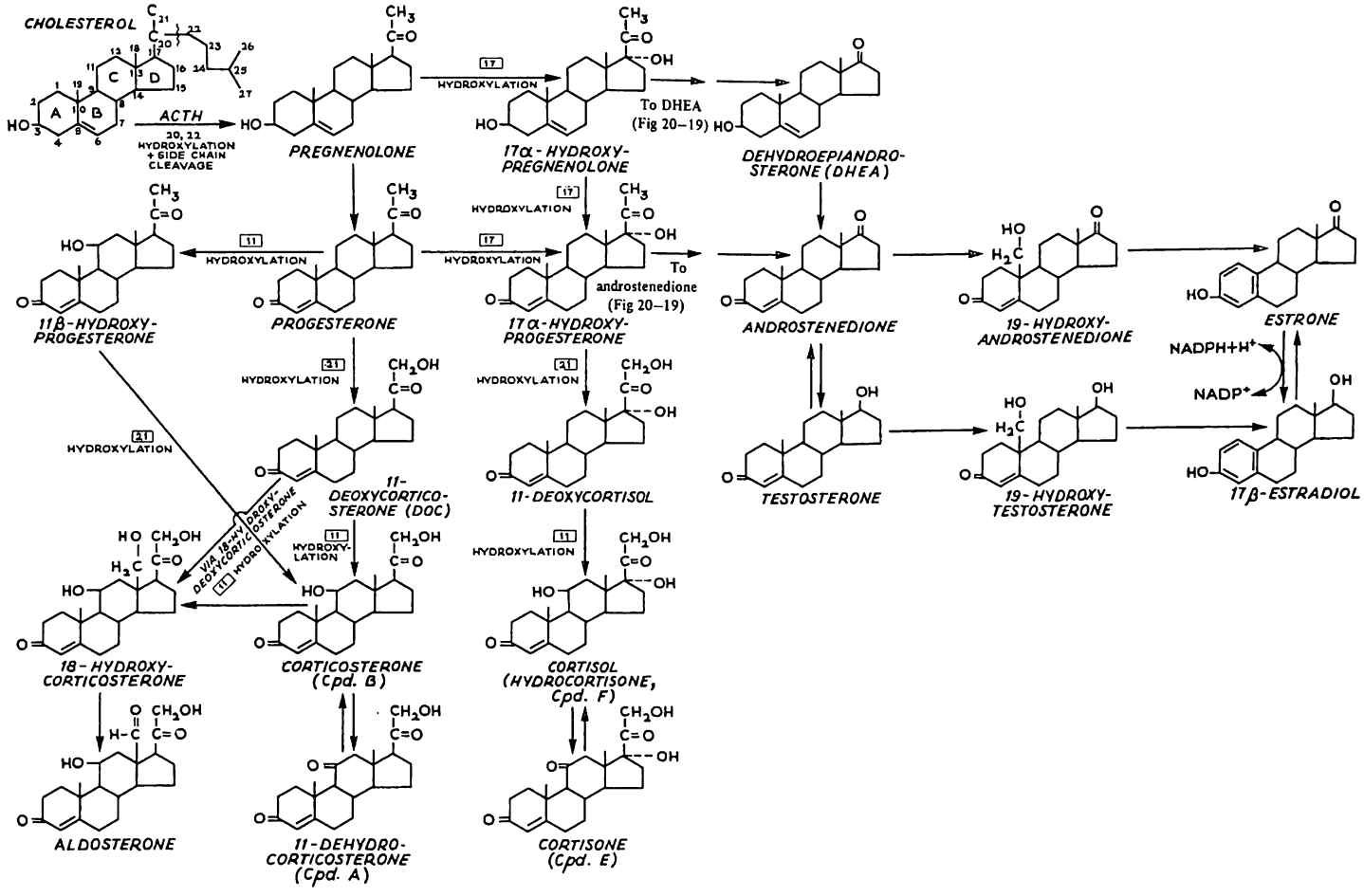


Fig. 2-9 Steroid biosynthesis pathways. Adapted from Grodsky, 1973.

The binding of the hormone (ligand) causes dimerisation of their receptors and the translocation into the nucleus, where the receptor-ligand complex binds to steroid response elements (SRE) of target genes. The interactions of steroid receptors with DNA, together with transcription regulators and co-factors up-regulates or down-regulates the transcription of these genes (Yamamoto, 1985; Truss and Beato, 1993; Beato *et al.*, 1996). Two other mechanisms of steroid action have also been discovered in the past few years. Steroids can function without entering the target cells (Grazzini *et al.*, 1998; Revell *et al.*, 1998; Rosner *et al.*, 1998), which explains some of their rapid effects (from seconds to a few minutes) after administration. The identification of SHBG specific receptor on the cell membrane has also led to the confirmation of SHBG as an active regulator of steroid hormone action in target cells (Fortunati, 1999; Rosner *et al.*, 1999).

Steroid hormone receptors are ligand-activated transcription factors and members of a large family of nuclear hormone receptors. They all have a DNA binding domain, a hormone binding domain, a nuclear localisation signal and several transactivation domains (Carson-Jurica *et al.*, 1990; Grandien *et al.*, 1997). High sequence homology has been preserved in the DNA binding domain of different steroid receptors. Before binding to their ligands, steroid hormone receptors are associated with a large multi-protein complex which maintains the receptors in a conformation able to bind to ligands (Pratt, 1993). Four mechanisms for steroid actions have been suggested after receptor-ligand complexes enter the nucleus (Beato *et al.*, 1996; Beato and Sanchez-Pacheco, 1996; Thackray *et al.*, 1998; McKay and Cidlowski, 1999; Webster and Cidlowski, 1999).

(1) Binding of steroid hormone-receptor complex to SREs may enhance unwinding of the double helix and permit attachment of other transcription factors. Besides binding to the SRE via two zinc fingers within the DNA binding domain of the steroid receptor, the complexed steroid hormone itself also inserts into the unwound DNA/receptor interface (Truss and Beato, 1993; Hendry *et al.*, 1998). The insertion is facilitated by conformational changes in the receptor induced by the ligand. The specificity of the steroid hormone response is largely the result of steroid hormone-receptor

interactions, whereas the magnitude of responses is governed by ligand insertion.

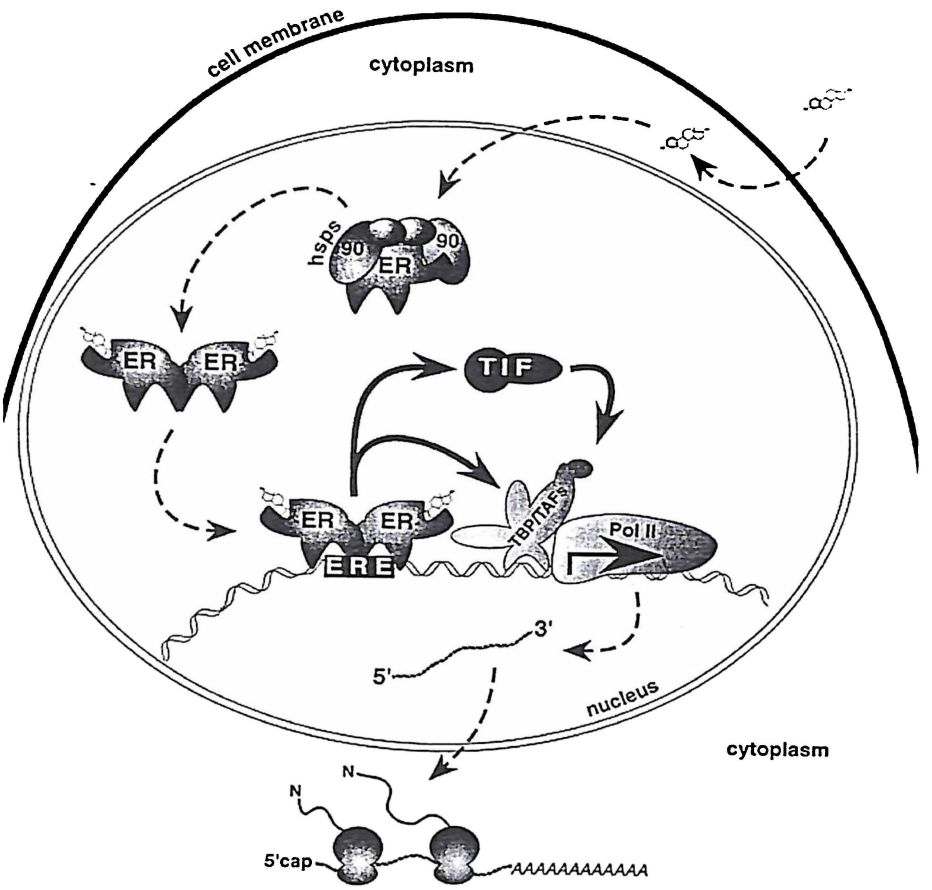


Fig. 2-10 Oestrogen functions through binding to cognate receptors

After diffusing into cytoplasm, oestrogen binds to its receptors (ER) and activates dimerisation of the receptors which then translocate into nuclear and bind to oestrogen element binding element (ERE). The interactions between the hormone, its receptor and transcription machinery activate or inhibit the expression of target genes. Reproduced from Gradient *et al.*, 1997.

(2) Interaction of the steroid hormone receptor to other protein transcription factors, like the Fos-Jun complex can remove transcription factors from AP-1 (activating protein-1) sites on the DNA (Konig *et al.*, 1992).

(3) Steroid hormone receptors compete for binding sites in the promoter regions with general transcription machinery. For example, oestrogen receptor transactivation is enhanced in response to overexpression of the TATA box

binding protein (TBP), and the two proteins interact in vitro. Other components of the transcription factor IID (TFIID) complex, which are essential for transcription and direct promoter selectivity for RNA polymerase II, have also been identified as the targets of hormone receptors (Beato and Sanchez-Pacheco, 1996).

(4) Steroid hormones have been shown to interact with a number of transcription cofactors (Beato and Sanchez-Pacheco, 1996; Freedman, 1999). Some of these proteins enhance or enable the transcription activity of ligand-activated hormone receptors (coactivators), others have opposite actions (corepressor). Several of these could interact with the ligand-binding domain of a steroid receptor in a ligand-dependent manner (McKay and Cidlowski, 1999).

It is important to realise that the effects of steroids are not limited by their direct actions on target genes or other direct effects. The indirect effects exerted by the up or down regulation of target genes magnify their actions. For example, oestrogen increases the expression of the proto-oncogene c-myc and cyclin D1 sequentially. The activation of cyclin D1 transcription and the resultant accumulation of cyclin D1 is accompanied by the formation of cyclin D1-CDK4 complex with kinase activity to hyperphosphorylate pRb (retina blastoma protein) (Fig. 2-11). On the other hand, via the regulation of the expression of other genes, including cyclin-dependent kinase (CDK) inhibitor p21, c-myc also promotes hyperphosphorylation of pRb. This leads to the release of E2F and other transcription factors from pRb (Sutherland *et al.*, 1992; Sutherland *et al.*, 1998). The subsequent actions of these transcription factors on gene expression enables the cells to enter the S phase (Sherr, 1996; Weinberg, 1996).

The finding of a new type of oestrogen receptor, oestrogen receptor β indicates a more complicated mechanism of oestrogen action (Saunders, 1998). The two oestrogen receptors (α and β) can form both homodimers and heterodimers. They also show different patterns of affinity for naturally occurring hormone response elements (Giguere *et al.*, 1998; Jones *et al.*, 1999; Lau *et al.*, 2000), revealing new pathways for oestrogen regulation of gene transcription.

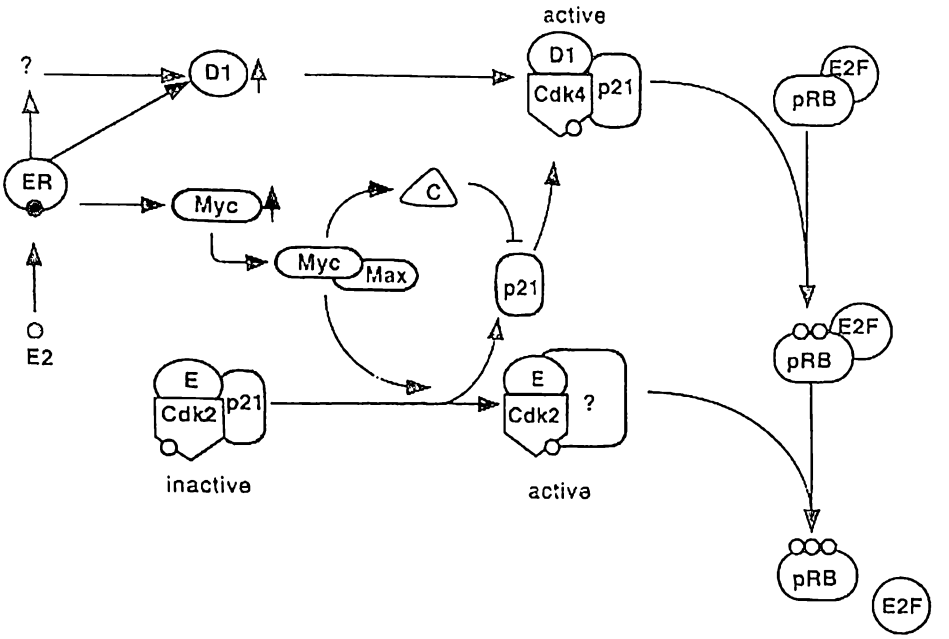


Fig. 2-11 Cell cycle progression regulated by oestrogen receptor pathway

Binding of oestrogen to its receptors regulates the expression of c-myc and cyclin D1. Their subsequent actions release the cells into S phase (see text). Reproduced from Sutherland *et al.*, 1998.

2.4 Effects of steroids on hair growth and their mechanisms

Receptors for androgens, oestrogen, glucocorticoids, mineralocorticoids and progesterone have been located in skin and, all but the progesterone receptor, have been identified on hair follicle populations (Randall *et al.*, 1993; Kenouch *et al.*, 1994; Oh and Smart, 1996; Hendry *et al.*, 1998; Uotinen *et al.*, 1999). Therefore, it appears that steroid hormones play an important role in regulating hair growth through direct binding to their receptors in the dermal papilla or other parts of the hair follicle.

2.4.1 Glucocorticoids

Early studies indicated that adrenalectomy stimulates hair growth and adrenocortical extracts inhibit hair growth in the rat (Mohn, 1958). Inhibitory effects of the administration of exogenous glucocorticoids on hair growth have also been reported in mice (Stenn *et al.*, 1993; Paus *et al.*, 1994a), sheep (Wallace, 1979; Chapman *et al.*, 1982; Ansari-Renani and Hynd, 1996), and mink (Rust *et al.*, 1965). When several synthetic analogues of cortisol were compared to cortisol in their inhibition of hair growth waves in castrated male C3H/Ep mice,

a correlation between the glucocorticoid potency and hair inhibition was found (Houssay *et al.*, 1978).

In sheep, low doses of cortisol given intramuscularly stimulated the growth rate of fibre around the site of injection, in contrast to high doses which caused no change or a decrease in wool growth (Downes and Wallace, 1965). Elevation of plasma concentrations of cortisol from 5-8 ng/ml to 12 ng/ml over a three week period by the injection of cortisol, enhanced wool growth (Chapman and Bassett, 1970). Acute changes in plasma cortisol concentration did not produce acute changes in wool growth in adrenalectomised sheep. However, a sustained elevation of plasma cortisol concentration reduced the rate of wool growth by reducing the mitotic rate in wool follicles, evidenced by cells arrested at metaphase of mitosis two hours after a intradermal injection of colchicine (Scobie and Hynd, 1995). These results led to the hypothesis that the presence of cortisol may be necessary for maintaining normal hair growth in animals (Scobie and Hynd, 1995).

In line with this, fur growth in mink was found to be positively correlated with the total serum glucocorticoid concentrations induced by short artificial light treatment (6L:18D) during the period from June to December (Weiss *et al.*, 1980). On the other hand, adrenalectomy in mink advances both summer and winter fur growth (Rose and Sterner, 1992; Rose, 1995) and this was not caused by DHEA or its metabolite (Rose *et al.*, 1998). Furthermore, exogenous glucocorticoids, including dexamethasone, when administered both locally and systemically induced regression of growing follicles (Chapman *et al.*, 1982; Paus *et al.*, 1994a) and inhibited growth of new follicles (Mohn, 1958; Rust *et al.*, 1965; Stenn *et al.*, 1990; Stenn *et al.*, 1993). However, for induction of hair follicle growth from telogen, only the initiation stage (Mohn, 1958), or the inducing signal (Stenn *et al.*, 1993) appears to be affected. This is indicated by the observation that removal of topical application of glucocorticoid is followed by hair growth in depilation induced hair follicles, but not in the control (Stenn *et al.*, 1993).

Glucocorticoid receptors are not only located in skin, but also in hair follicles (Karstila *et al.*, 1994). As similar effects were obtained by applying dexamethasone both topically and systematically, it appears that the actions of the

steroid are exerted through its receptors in the hair follicles. Dexamethasone induced hair follicle regression has been shown to link to its suppression of α-melanocyte-stimulating hormone (MSH) activity (Ermak and Slominski, 1997), by decreasing its production and receptor concentration. Dexamethasone is also a potent inducer of connective tissue growth factor (CTGF), an important player for fibroblast proliferation and extracellular matrix deposition (Dammeier *et al.*, 1998). It is not known whether this growth factor has a role in hair follicle growth. Serum- and glucocorticoid-inducible protein kinase (Sgk) is a novel serine/threonine protein kinase expressed in mammary tumour cells. The shuttle of Sgk between nuclear and cytoplasmic compartments is a requirement for cell cycle progression (Webster *et al.*, 1993; Buse *et al.*, 1999). Dexamethasone has been found to arrest Sgk in the perinuclear or cytoplasmic compartment in a hypophosphorylated form, which suppresses the growth and DNA synthesis of cells (Buse *et al.*, 1999). Sgk has been identified to be expressed in sheep whisker dermal papilla cells (N.W. Rufaut, personal communication, 2000). If this mechanism also works in hair follicle cells *in vivo*, it may represent an important pathway for steroids to control hair growth.

2.4.2 Mineralocorticoids

Mineralocorticoids are important steroid hormones secreted by the adrenal cortex. Surprisingly, very little information has accumulated concerning the effects of mineralocorticoids on hair growth. In contrast to the inhibitory effects of glucocorticoids, aldosterone does not have inhibitory effects on hair growth initiated by plucking, and monitored by thymidine incorporation in the dermis (Stenn *et al.*, 1993). Mink summer fur growth was also found not to be affected by this hormone (Rose and Sterner, 1992). No hair inhibitory effect of deoxycorticosterone acetate was found in castrated mice (Houssay *et al.*, 1978). In contrast, bilateral adrenalectomised mink treated with deoxycorticosterone showed summer fur growth 5 weeks earlier than intact controls, while intact mink treated with deoxycorticosterone exhibited hair growth 2 weeks earlier than the controls (Rose, 1995). However, the time between the first observed hair growth and the attainment of maximal guard hair length was approximately 10 days longer for normal and 13 days longer for adrenalectomised mink treated with deoxycorticosterone.

2.4.3 Androgens

In humans, male pattern baldness and female hirsutism both result from androgen actions on hair follicles (Ebling, 1981; Randall, 1994). The effect of androgens depends on the body site, varying from stimulation of beard, non-response in eyelashes, and regression on the scalp, although all follicles are presumably exposed to the same level of circulating androgens. Hence, androgens have two opposing effects: gradually transforming vellus hair (short, fine, unpigmented and non-medulated hair) to terminal hair (long, thick, dark and often medulated) in many parts of the body while inducing regression of terminal follicles to vellus ones on parts of the scalp (Ebling, 1981; Randall, 1994). The levels of androgens needed for the growth of hair at different parts of the body are also different. For instance, follicles in pubic and auxiliary areas respond to female levels of androgens, while beard growth requires male levels of androgen. The androgenic response of the hair follicle requires not only the initial stimulation by the hormone, but continuing presence of androgens for maintenance of the effect.

Testosterone and DHT inhibit the growth rate in organ cultures of human scalp hair follicles. The minimum effective dose to suppress hair growth was approximately 5 ng/ml (Kondo *et al.*, 1990; Williams and Kealey, 1993), which corresponds well to normal plasma levels of testosterone in the adult male. However, the concentrations of DHT needed to produce a significant fall in the rate of human scalp hair follicle growth is 5.8 ng/ml, which is approximately 10 times higher than its circulating level in men (Williams and Kealey, 1993). High concentrations of testosterone and DHT (99.5 ng/ml) significantly reduce the proliferation of scalp dermal papilla cells (Kiesewetter *et al.*, 1993). Consistent with these data, low doses of two anti-androgens, cyproterone and 17α-propylmesterolone induced enhancement of dermal papilla cell and outer root sheath keratinocyte growth (Kiesewetter *et al.*, 1993). However, high doses of this antiandrogen suppressed their growth.

The effects of androgens on animal hair growth are not as well understood. The advanced spontaneous new hair in female adrenalectomised rats, and acceleration of the growth wave over the body in adrenalectomised rats of both sexes, were considered partially due to reduced androgen levels (Johnson, 1958a). In female

mink, bilateral adrenalectomy (ADX) resulted in a six-week earlier onset of winter fur growth (Rose and Sterner, 1992; Rose, 1995). As the adrenal gland is the main androgen secreting tissue in females, it appears that androgens are associated with the suppressed hair growth in these mink. However, no change in the concentration of DHEA, the main androgen secretion from adrenal gland, was observed during the onset of mink summer and winter fur growth (Rose *et al.*, 1998). Administration of this hormone or its metablite Δ^5DIOL did not alter mink winter fur growth (Johnston and Rose, 1999).

Mixed effects of androgen administration on rodent hair growth were recorded (Mohn, 1958), varying from no effects on the length of hair or the rate at which club hair was lost in the rat, to inhibitory effects in mice. The effects of testosterone in sheep are also contradictory. Injection of 175 mg of testosterone intramuscularly at two week intervals increased wool production, while a lower dose (125mg) did not have an effect on the weight of clean wool in intact yearlings (Slen and Connel, 1958). However, administration of testosterone reduced the average fibre lengths in thyroidectomised ewes or wethers, but not in intact animals (Slen and Connel, 1960). Interestingly, exogenous androgen administered by implantation stimulates hair growth in Syrian hamster flank organ (Lucky *et al.*, 1986), but suppresses hair growth in the area surrounding the flank organ (Mezick *et al.*, 1999). The inhibitory effect of long term elevated levels of the hormone was overcome by unknown factors. In adult red deer, androgen-responding hair follicles from the neck mane and non-responding hair follicles from the flank from either breeding or non-breeding seasons cultured *in vitro* showed different responses to testosterone. The growth of neither flank follicle nor non-breeding season neck follicles was altered. However, the neck mane hair follicles during the breeding season grew faster in the presence of testosterone (Thornton *et al.*, 1994). Adding the antiandrogen, cyproterone acetate, blocks the stimulating effect of testosterone. Therefore, as in humans, testosterone and DHT appear to be the effective androgens and they can exert stimulatory and inhibitory effects on hair growth depending on the body site of the animal.

Androgen receptor has not only been identified in the dermal papilla, but its abundance is higher in androgen-dependent hair follicles than in androgen-

independent follicles (Randall *et al.*, 1992b; Randall *et al.*, 1993). Conversion of testosterone into DHT is considered to have some relevance to the various effects of androgen on hair follicles from different body sites. Studies of testosterone metabolism using beard and scalp hair follicle dermal papilla cells demonstrated that significant amounts of 5α-DHT were recovered from beard, but not scalp cells, whereas androstenedione was identified in both (Thornton *et al.*, 1993).

Similarly, 5α-DHT was found only in the culture medium of beard dermal papilla cells, but androstenedione was present in a similar amount in both dermal papilla cells and fibroblast cells. In addition, dermal papilla cells from beard and other androgen-dependent hair follicles showed a higher activity of 5α-reductase than dermal papilla cells from occipital scalp, axillary and reticular fibroblasts (Itami *et al.*, 1991b; Itami *et al.*, 1991a). Considering that 5α-DHT is the major androgen complexed to androgen receptors, and the poor beard growth in men with 5α-reductase deficiency (Leshin and Wilson, 1981), these results clearly indicate that 5α-reductase mediates androgen effects on human androgen-dependent hair follicles through the dermal papilla (Randall *et al.*, 1994; Thornton *et al.*, 1998). The administration of finasteride, a 5α-reductase inhibitor, reverses the balding process and enhances hair growth in the monkey, thus confirming this mechanism (Diani *et al.*, 1992). Although the conversion of testosterone to DHT obviously can not explain the effect of androgens in all human hair follicles, or hair follicles in other species, it does indicate that the conversion between the steroids (Fig. 2-12) can be important for their hair growth effects.

2.4.4 Oestrogen

Removal of the ovaries from female rats accelerates the passage of the moult, both increasing the rate of hair growth and the terminal length of the hair and accelerating the loss of club hairs (Johnson, 1958b; Mohn, 1958; Chanda *et al.*, 2000). The biologically active 17β-oestradiol is found to be a powerful hair growth inhibitor in most species. High doses of the oestradiol (3.7 mg/day) (Johnson, 1958b) or 10 mg implants (Hale and Ebling, 1975) inhibited hair growth in both neutralised male and female rats. When 17β-oestradiol (0.3 mg/kg/day) was administered subcutaneously to rats for 13 weeks, retarded hair growth or alopecia was observed (Attia and Zayed, 1989). Hair growth in guinea-

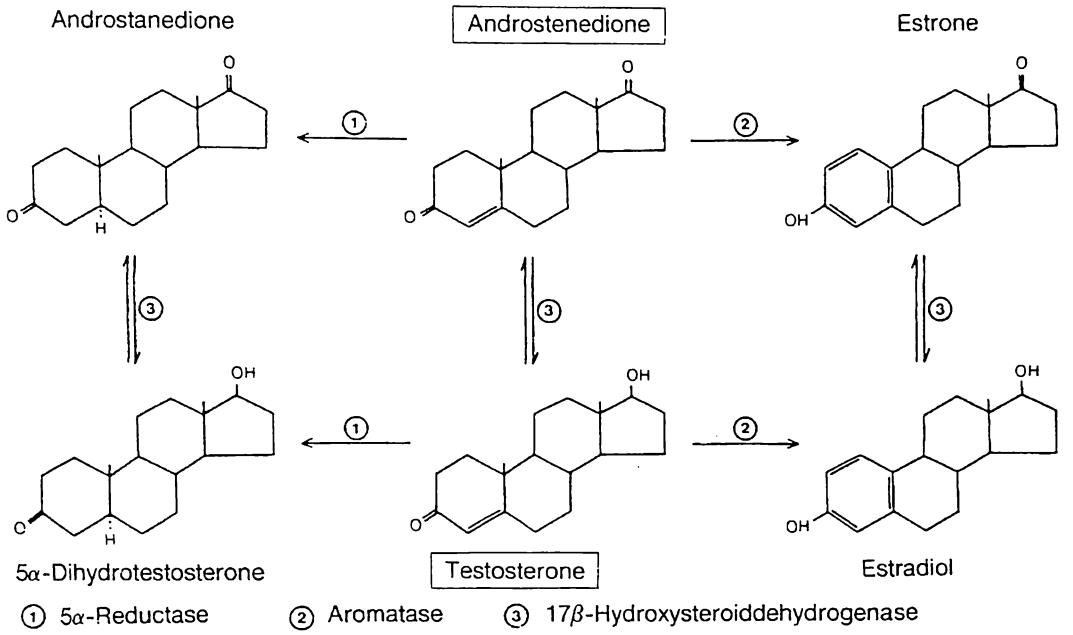


Fig. 2-12 Conversion of steroids in hair follicles

The main circulating androgen, androstenedione can be converted into DHT though both testosterone and androstanedione by 5α-reductase and 17β-HSD. It can also be transformed into oestradiol by aromatase and 17β-HSD. Reproduced from Schweikert *et al.*, 1981.

pigs was also inhibited by the hormone (Jackson and Ebling, 1972), but it did not reduce the length of the active phase, differing from the effect in rats (Hale and Ebling, 1975). In sheep, intramuscular administration of 5 mg of oestradiol at 14 day intervals caused a significant reduction in the average weight of clean wool and fibre length in both normal sheep and thyroidectomised ewes, but not in wethers (Slen and Connel, 1958; Slen and Connel, 1960). In ferrets, alopecia was associated with high oestradiol concentration caused by adrenal gland tumors (Wagner and Dorn, 1994). More recently, topical application of 17β-oestradiol arrested mice hair follicles in telogen for more than three weeks (Oh and Smart, 1996) and prevented the hair growth in ovariectomised mice (Chanda *et al.*, 2000). The effects of oestradiol were dose-dependent and localised (Oh and Smart, 1996; Chanda *et al.*, 2000). More importantly, oestrogen receptor expression was found to be mainly expressed in the dermal papilla and was follicle growth stage-related, with the highest concentration in telogen follicles (Oh and Smart, 1996). Twice weekly topical application with the oestrogen receptor antagonist, ICI 182780, resulted in a four-week advancement of hair growth (Oh and Smart, 1996; Chanda *et al.*, 2000). The oestrogen converting

enzyme, aromatase was also expressed in a follicle growth stage-related manner (Messenger, 1997). Furthermore, the inhibitory effects of oestradiol on hair growth have also been confirmed by *in vitro* studies (Kondo *et al.*, 1990). The minimum effective dose to suppress growth rate of human scalp hair follicles was around 5 ng/ml, approximately 20 times higher than the concentration during the ovulatory surge. More importantly, an oestrogen receptor antagonist blocks the effect of oestrogen on hair follicles. Topical treatment with oestrogen receptor antagonists causes the hair follicles to exit telogen and enter anagen (Oh and Smart, 1996; Chanda *et al.*, 2000). These data indicate that oestrogen can not only act directly in hair follicles, but may also play a role in regulating follicle cycle phase transitions.

2.4.5 Progesterone

Hair growth is often retarded during pregnancy and lactation in humans and animals (Mohn, 1958; Franz and Bosse, 1975; Ebling, 1981). However, progesterone injected at the dose of 125 mg or 250 mg at 14 day intervals for 5 months had no effect on wool growth (Slen and Connel, 1958). Daily injection of 50-200 μg of progesterone in oil had little effect in female rats and moderately large doses in dogs (sex unknown) also had no effect (Mohn, 1958). Furthermore, physiological concentrations of progesterone fail to show significant effects on the growth of human hair follicles *in vitro* (Williams and Kealey, 1993).

In contrast, administration of progesterone inhibited the replacement of hair in mice (sex unknown) (Mohn, 1958). Some synthetic progesterone analogues with very low glucocorticoid activity show strong or intermediate inhibitory effects in castrated male mice (Houssay *et al.*, 1978), depending on the specific analogue. Topical application of progesterone stimulated hair growth in female mice (Chanda *et al.*, 2000). Progesterone levels were correlated with growth and shedding of fur in female mink (Harvey and MacFarlane, 1958) and ferrets (Martinet *et al.*, 1983). Growth and shedding of hair also has been noticed following progesterone treatment during oestrus (Harvey and MacFarlane, 1958). Hence, it appears that progesterone may have different effects on hair growth in males and females and that these effects can be either inhibitory or stimulatory.

2.5 Gene expression identification

2.5.1 Techniques for studying gene expression profiles

The phenotype of an organism is determined by the genes expressed within it. The number of expressed genes and their expression levels form a transcriptome for an organism or a defined population of cells. Unlike the genome, which is essentially a static entity, the transcriptome can be modulated by both external and internal factors (Velculescu *et al.*, 1997). Thus, the transcriptome serves as a dynamic link between genotype and phenotype. Although the traditional approaches of examining the expression of a single gene have provided a great deal of information, they are limited in their ability to understand the overall effects of a treatment, or the interactions between genes. The description of the expression patterns of numerous genes simultaneously is more informative in both revealing the genes involved and in mapping their functions, thus allowing for a more comprehensive understanding of a biological process. Although subtractive library screening can be used for studying gene expression, it is biased towards abundant transcripts and limited to a single comparison (Rothstein *et al.*, 1993; Wan *et al.*, 1996). The era of simultaneous study of multiple gene expression really arrived with the invention of differential display (Liang and Pardee, 1992) and arbitrary primed RNA fingerprinting (Welsh *et al.*, 1992). Two other methods, representational difference analysis (RDA) (Lisitsyn and Wigler, 1993; Hubank and Schatz, 1994) and serial analysis of gene expression (SAGE) (Velculescu *et al.*, 1995), sparked by differential display, were published shortly afterwards. RDA was originally used for the analysis of the difference between two complex genomes (Lisitsyn and Wigler, 1993). When used in studying the difference between two population of cDNAs, it is similar to subtractive library screening in that it detects only the increased cDNAs remaining between the two populations, but it is more sensitive (Hubank and Schatz, 1994). SAGE is based on two principles. The first is that short nucleotide sequence tags of 9-10 bp from a defined location within the transcript contain adequate information to uniquely identify that transcript. The second is that concatenation of short sequence tags enables efficient analysis of transcripts in a systematic manner by sequencing multiple tags within a single clone. An added advantage is that the relative number of the transcripts being sequenced accurately reflects the abundance of the

transcript, as no PCR amplification is involved (Velculescu *et al.*, 1995). However, it is biased to abundant transcripts, thus less sensitive to rare transcripts which often code for regulatory molecules. Similar to subtractive screening, large numbers of concatemer cDNAs must be sequenced to detect rare transcripts, a relatively expensive exercise, although this can be partially overcome by a micro-SAGE method (Datson *et al.*, 1999; Neilson *et al.*, 2000).

Microarray technologies for gene discovery have developed rapidly in recent years (Lemieux *et al.*, 1998; Spellman *et al.*, 1998; Watson *et al.*, 1998; Wang *et al.*, 1999; Elek *et al.*, 2000; Kelly and Rizzino, 2000). These are based on the relative levels of hybridisation of probes and target genes arrayed on solid matrix, such as slides and nylon membranes. Both cDNA array (Duggan *et al.*, 1999) and oligonucleotide arrays (Lipshutz *et al.*, 1999) are efficient methods of measuring expression of a large number of genes. As this method is basically dependent on known sequences, it is particularly suited for human, mouse and other extensively studied species. While these methods are presently comparatively expensive, automation in data collection, storage and analysis have made microarray technologies extremely powerful tools for gene expression profiling.

2.5.2 Differential display

Differential display has been a powerful tool for gene discovery since its invention in early 1990s (Liang and Pardee, 1992) and is still widely used (Sager, 1997; Too, 1997; Amara and Satchidanandam, 2000; Darden *et al.*, 2000; De Vries *et al.*, 2000; Melichar *et al.*, 2000). The method is based on the visual comparisons of relative levels of mRNA species in different biological processes. A subset of eukaryotic mRNA populations, defined by an anchor base and the multiple thymidine of the oligo dT anchor primer, are reverse transcribed. This primer and an arbitrary primer which binds to an upstream region of the mRNA, are then used to amplify the subset of mRNA by PCR. The PCR products are separated and displayed in a denaturing acrylamide gel. Each band in the gel represents a 3' fragment of a transcript. The presence or absence, or density difference of the same band associated with different treatments or growth stages suggests differences in the relative concentrations of the original mRNA species. The differentially displayed bands are isolated, eluted and then re-amplified by PCR with the same set of primers. Amplified target bands are cloned, sequenced,

and compared with the database sequences for identification by sequence homology. The cloned sequences can also be used as probes in Northern blot hybridisation or other quantitation methods for confirmation of differential gene expression.

A number of improvements have been made to the method since its introduction. The most important modifications are the adoption of the one-base oligo dT anchor primer (Liang *et al.*, 1993), longer primers and a two-stage PCR program (Ayala *et al.*, 1995; Linskens *et al.*, 1995) with a reduced cycle number (Linskens *et al.*, 1995). Replacing degenerate two-base anchored oligo dT primer with one base anchored primer enhances the signal and reduces the false positive bands by increasing the specificity. The adoption of a low stringency, followed by a high stringency PCR program and longer primers improves the sensitivity and reproducibility of the method. A lower cycle number in amplification aids in reducing the saturation of abundant species and better maintains the relationship between band densities and the relative mRNA concentrations in the original samples.

Differential display has some advantages over other methods. It can be performed with small amounts of total mRNA, which makes this method applicable to situations with limited samples. It detects up-regulated as well as down-regulated gene transcription simultaneously. As only genes in which changes in levels of expression are identified, the number of gene candidates for further investigation is substantially reduced, compared with random sequencing (electronic subtraction) (Okubo *et al.*, 1992; Adam *et al.*, 1993). Low and high abundance mRNA are detected with similar probability, while other methods are biased to identifying abundant mRNA (Wan *et al.*, 1996; Sleeman *et al.*, 2000). The use of oligo dT anchor primer designed to target mRNA poly A tail region in both reverse transcription and PCR amplification, theoretically exclude ribosomal RNA (rRNA) transcripts, which constitute the majority of total RNA species. Finally, differential display is readily set up and is relatively inexpensive.

Many novel genes have been identified and a large number of known genes have been detected in new systems by differential display (Benito *et al.*, 1996; Harris, 1996; Hsiang *et al.*, 1996; Joshi *et al.*, 1996; Bertram *et al.*, 1998; Franz *et al.*,

1998; Srivastava *et al.*, 1998; Jorgensen *et al.*, 1999; Darden *et al.*, 2000). In principle, by assessing gene expression patterns, differential display can also provide estimates of the numbers of genes required for various developmental and growth processes, or responses to environmental cues and manipulations. Provided that the reactions are conducted under the same conditions and displayed in parallel, the method also allows comparison of gene expression patterns responding to different experimental treatments, indicating shared or antagonistic regulatory pathways (Liang and Pardee, 1992; McClelland *et al.*, 1994; McClelland *et al.*, 1995). However, compared with its wide application for identifying new genes, the potential of differential display to investigate regulatory pathways perturbed by different treatments has not been fully exploited.

In this laboratory, differential display has proved to be an effective method of identifying genes associated with wool growth (Rufaut *et al.*, 1999a; Rufaut *et al.*, 1999b). The regressive phase of the hair cycle was induced by manipulating circulating prolactin levels in New Zealand Wiltshire sheep and the altered patterns of gene expression were examined. Nine sequence tags for differentially expressed genes were isolated. Four of these tags were identified as fragments of known genes, encoding a wool keratin (KRTAP3.2), a desmosome component (desmoglein 1), an epithelial cell marker (stratifin) and a protein kinase (Clk3). All four genes were shown to be down-regulated in telogen compared with anagen skin. The other five tags, including two representing genes that were up-regulated during catagen, could not be identified by sequence homology at the time of the study.

However, differential display also has disadvantages. As the anchor primer targets the mRNA poly A tail, most of the cDNA fragments displayed and cloned are at the 3' end. In most cases, these fragments are not in the coding region, making the identification of the molecule more difficult. Another disadvantage is the relatively high number of false positives reported by some investigators who found that differential expression of some gene tags could not subsequently be confirmed (Liang *et al.*, 1993; Linskens *et al.*, 1995; Hsiang *et al.*, 1996). Furthermore, a single differentially displayed band isolated from acrylamide gel

may consist of multiple cDNA fragments of similar sizes, but representing different sequences (Bauer *et al.*, 1993; Liang *et al.*, 1995). Therefore, cDNA clones obtained from one differentially displayed band may include both differentially and constitutively expressed mRNA. For this reason, it is important to confirm the identified genes are genuinely differentially expressed. This confirmation step is a time-consuming process, especially when a large number of clones are involved.

A number of methods have been applied to the confirmation process. These include Northern blot hybridisation (Liang *et al.*, 1993; Linskens *et al.*, 1995), nuclear run on analysis (Bauer *et al.*, 1994), Southern blot hybridisation (Hsiang *et al.*, 1996), dot (slot) hybridisation (Callard *et al.*, 1994; Vogeli-Lange *et al.*, 1996), or reverse RNA dot (slot) hybridisation (Zhang *et al.*, 1996a). Among these, Northern blot hybridisation is most often used. It gives, not only the expression pattern of the cloned gene, but also the size of the mRNA and the number of transcripts if there are more than one. Such attributes are also useful in confirmation of gene identity. However, it is laborious to screen a large number of clones by Northern blot hybridisation. The sensitivity of Northern blots is another limiting factor for genes expressed at low levels, such as some regulatory molecules.

2.5.3 Gene expression profiles in mammalian tissues

In an initial assessment of human gene diversity and expression patterns, ESTs from 30 human tissues, totalling 83 million nucleotides were analysed and categorised by function (Section 8-10) (Adams *et al.*, 1995). The percentages of known transcripts involved in different biological processes in each tissue and over all tissues were obtained. In a more recent, large scale study using SAGE, the expression levels of genes were found to range from 0.3 to 9417 transcripts per cell (Velculescu *et al.*, 1999). The transcript tags were matched to 4,300 genes with known functions and 41,000 genes with unknown functions, while the remaining transcripts (46%) had no matches. Moreover, only a small fraction of transcripts were expressed in all the tissues (Adams *et al.*, 1995; Velculescu *et al.*, 1999). However, the finding that as many as 43,500 genes can be expressed in a single cell type was much higher than the 15,000 generally recognised (Albert *et al.*, 1989) and previously estimated by the same (Velculescu *et al.*, 1997) or

different methods (Hereford and Rosbash, 1977) in yeast. A study of the human thyroid expression profile has also revealed a large number of novel genes (Pauws *et al.*, 2000). Furthermore, most unique transcripts are expressed at low levels, as 90% of the unique transcripts comprised fewer than 20% of the total mRNA species. In addition, variation in gene expression between physiological states of a particular cell type or between different samples of the same cell type were less than the variation of the transcriptome between cell types of different origins (Velculescu *et al.*, 1999).

Gene expression profiles for some specific biological processes, particularly cancer growth have also been characterised. More than 300,000 transcripts derived from at least 45,000 different genes were analysed between normal and gastrointestinal cancer cells in humans (Zhang *et al.* 1997). Although extensive similarity was noted between the expression profiles, more than 500 transcripts that were expressed at significantly different levels in normal and neoplastic cells were identified. Investigations of breast cancer gene expression also revealed numerous alterations in gene expression (Perou *et al.*, 1999; Sgroi *et al.*, 1999; Martin *et al.*, 2000a). However, only a small faction of these transcripts was found to be exclusively expressed in a particular normal or diseased tissue (Velculescu *et al.*, 1999).

Hair follicles are complicated structures comprised of cells of various embryonic lineages (Hardy, 1992; Widelitz and Chuong, 1999). Cells in telogen and anagen follicles also differ in their growth and differentiation status. In addition to their intrinsic growth regulation, follicle growth is modulated by a variety of external factors. Therefore, it can be expected that gene expression patterns during the hair follicle growth cycle will be complex. Over 100 different genes involved in hair growth cycles have already been identified (Powell *et al.*, 1992; Stenn *et al.*, 1994; Stenn *et al.*, 1996; Rogers *et al.*, 1998; Langbein *et al.*, 1999; Rogers *et al.*, 2000), but many more remain to be discovered. Even the expression patterns of many of these identified genes and their interrelationships are still obscure. For better understanding of hair growth controlling mechanisms, the identification of many more novel genes associated with key control points of the growth cycle, particularly hair growth initiation, is required.

CHAPTER 3 METHODS AND MATERIALS

3.0 Introduction

The general methods are described in this chapter. These protocols include ferret husbandry, sample collection, histology, steroid assays, total RNA extraction, poly A^+ RNA purification, differential display, Northern blot hybridisation, *in situ* hybridisation, cDNA library construction and rapid amplification of cDNA ends (RACE).

3.1 Ferret trials

Four ferret trials were conducted between February and March in 1996, 1997 and 1998. The trial in 1996 was designed to provide samples for establishment of the gene expression patterns induced by melatonin in association with hair growth initiation. The trials in 1997 and 1998 were conducted with two aims. The first was to investigate the effects on ferret winter hair growth of different steroids administered either alone or in combination with melatonin. The second aim was to collect additional skin samples to identify and confirm genes responsive to melatonin and/or steroids. The use of animals described in this thesis was conducted in a manner that fulfills the AgResearch animal ethics policy, and as outlined in animal ethics applications: FP010/01 to FP010/03. The work was approved and audited by an institutional animal ethics committee comprising representatives from the New Zealand Veterinary Association, the SPCA, MAF, and local authorities as required by New Zealand animal welfare legislation: Animals Protection Act 1960 and Animal Welfare Act 1999. Details of these trials are provided in Chapter 4.

3.2 Ferrets and their husbandry

Ferrets were purchased from a commercial fitch farm (Mr. Bruce Hollamby, Mystery Creek Road, Ohaupo, NZ). Juvenile male and female ferrets of a local strain between 98-114 days old were used in three trials between 1996 and 1998. Ferrets were housed in standard, single bank, wire fitch cages. Feed was prepared and frozen by the breeder. Chicken frames and by-products from a chicken processing plant were blended with fish meal, cereal and mineral/vitamin supplements. Rations appropriate to the number of animals were thawed and fed once or twice each day to the paired ferrets according to *ad-libitum* intake. Animal weights were recorded weekly. When

it became cold in March and April, straw was placed in the nesting box. Long day-light (16L: 8D) was applied to all the ferrets in the three trials, but was started at different ages.

3.3 Anaesthesia

All animals were anaesthetised with 1.0-1.2 ml/kg Saffan (Glaxovet, Palmerston North, NZ) before implanting with either melatonin or steroid pellets, blood sampling or collection of skin biopsies. Each ml of Saffan contained 9 mg alphaxalone and 3 mg alphadolone acetate solubilised in saline by polyoxyethylene castor oil. Tests with Saffan have failed to show evidence of mineralocorticoid, glucogenic or progestational activity, but it has been shown to possess weak anti-oestrogenic effects (Child *et al.*, 1971). When only skin biopsies were collected, the flank skin site was locally anaesthetised with Lopaine (2% Lignocaine hydrochloride, Ethical Agents Ltd., Auckland, NZ).

Ferrets sacrificed for the collection of large flank skin samples were euthanised by an intraperitoneal injection (1ml per 2kg body weight) of sodium pentobarbitone solution (pentobarb 300; Chemstock Animal Health Ltd., Hamilton, NZ).

3.4 Subcutaneous implants and administration

Melatonin: Two long-releasing melatonin 18 mg pellets (Regulin, Hoechst, UK) were administered to each ferret to stimulate winter pelage development. These implants were stored at 4°C before use.

Steroids: Slow-release implants designed to deliver steroids at a constant rate over 21 days (Innovative Research of America, Sarasota, FL, USA) were used in the trials. Testosterone, 17β-oestradiol, dexamethasone and deoxycorticosterone were each formulated as an acetate salt.

Administration of implants: The skin behind the ear was pierced with the needle of an implant gun while the animal was under general anaesthetic and pellets were inserted under the skin. The wound was sutured. If both melatonin and steroid pellets were used, they were administered to opposite sides of the neck.

3.5 Sample collection

3.5.1 Skin samples for histological analysis

Skin biopsies: Lens-shaped skin sections (6 x 12 mm) were collected from the mid-side under local anaesthesia. The skin was pinched with forceps and snipped with curved surgical scissors. Forceps and scissors were cleaned in 70% ethanol after sampling each animal. The wound were sutured and dusted with Terramycin powder (Pfizer Laboratories, Manukau city, NZ) before returning the animals to their cages.

Postmortem samples: When flank skin samples were collected from sacrificed ferrets, small strips from the same location were cut out for histological and immunocytochemical (ICC) studies.

3.5.2 Samples for RNA extraction

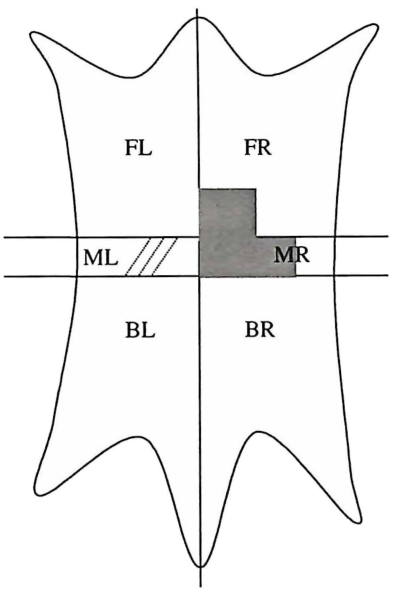


Fig. 3-1 Collection of skin samples from sacrificed animals

FL: front left; FR: front right; ML: middle left; MR: middle right; BL: back left; BR: back right; Strips of skin cut according to follicle growth orientation in ML were kept for histology and ICC and all the others were frozen for RNA extraction. The skin which was used for RNA extraction is shaded.

Skin biopsies: In the 1998 trial, skin biopsies (4 mm in diameter) were removed daily from alternate mid-sides using the biopsy technique described above. All skin samples were collected in mid-morning. Care was taken not to remove the skin with excess connective tissue or muscle attached.

Flank skin: For RNA extraction, the whole flank skin patches were removed immediately after the death of the sacrificed ferrets. The skin was then cut into six pieces according to Fig. 3-1. All the skin samples were snap frozen flat on an aluminium plate chilled with dry ice.

Internal organs and footpad skin: Portions of liver, kidney and whole adrenals were also collected from some animals and frozen on dry ice. Footpad skin, where no hair is present, was collected in the same way. All tissue samples used for RNA extraction were kept at –75 °C without thawing.

3.5.3 Blood samples

Three ml blood samples were collected by cardiac puncture (under general anaesthesia) into an EDTA vacutainer (Becton Dickinson Ltd., Franklin Lakes, NJ, USA) using a 20 gauge needle. Plasma was separated by centrifugation at 2000 rpm and stored at –20 °C.

3.6 Histological assessment of hair growth

3.6.1 Tissue and section preparation

Fixation and processing: Skin samples were divided into halves and preserved in either phosphate-buffered 10% formalin or in Bouin's fluid (Luna, 1968). After 24 hours in 10% formalin or 6 hours in Bouin's fluid, samples were transferred into 70% ethanol and processed within 3 days. All samples were processed to wax using a standard overnight protocol (LEICA TP 1050, Nussloch, Germany) as follows:

70% ethanol	1 hr	dehydration
80% ethanol	1 hr	
95% ethanol I	1 hr	
95% ethanol II	1 hr	
Absolute ethanol I	1 hr	
Absolute ethanol II	45 min	
Absolute ethanol III	45 min	
Absolute ethanol IV	45 min	
Absolute ethanol/toluene	1 hr	
Toluene I	1 hr	clearing
Toluene II	1 hr	
Paraffin wax I (60°C)	1 hr	infiltration
Paraffin wax II (60°C)	2 hrs	
Paraffin wax II (60°C)	2 hrs	

Tissue sectioning: Skin samples were embedded in wax according to follicle orientation. For longitudinal sections of follicles, series of 7 μm continuous sections were collected, mounted and dried by standard histological protocols. For transverse sectioning, two adjacent 7 μm sections at 30-50 μm intervals along the follicle shaft were collected.

3.6.2 Histological and immunocytochemical staining

All sections used for assessing follicle growth stages were stained with Sacpic as previously described (Nixon, 1993a).

For proliferating cell nuclear antigen (PCNA) staining, sections were deparaffinised and rehydrated, then washed three times in 50 mM phosphate buffered saline (PBS). The sections were blocked with 10% normal sheep serum (NSS) in 50mM phosphate buffer (PB) for 30 min. Diluted normal mouse IgG2a (DAKO, Carpinteria, CA, USA) or mouse anti-PCNA monoclonal antibody PC10 (DAKO, Carpinteria, CA, USA) was added to the control and test sections. Slides were put into a humidity box and incubated at 4-8°C overnight. After three washes with PBS, 500 times diluted, biotin-labelled sheep anti-mouse antibody (Amersham, Buckinghamshire, UK) was added to all the sections except the no-secondary-antibody control. Sections were incubated 30 min at 37°C and washed three times with PBS, 200 times diluted streptavidin-biotinated horseradish peroxidase (HRP) (Amersham, Buckinghamshire, UK) was added to all the sections except no-HRP control. After incubation for 20 min at room temperature and washing three time with PBS, diaminobenzidine (DAB; Sigma, St. Louis, MO, USA) in an intensifier solution (3% nickel cobalt) was applied to each section. When the colour was fully developed, the slide was rinsed with tap water, and then counter-stained with 0.2% eosin. Sections were dehydrated, cleared and mounted.

3.6.3 Assessment of follicle growth stages

The growth stages of hair follicles were assessed by examining the longitudinal sections under the Olympus BX50 light microscope (Olympus Optical Co. Ltd., Tokyo, Japan). The stages of growth were classified as described (Chase *et al.*, 1951; Nixon, 1993a). Seven to 24 original hair follicles (averaging 14) with complete bulb regions present were recorded in one of the following categories:

catagen, telogen (Section 2.1.1), proanagen II, proanagen IIIa, proanagen IIIb, proanagen IV, proanagen V and VI (anagen) (Section 2.1.2). For statistical analysis and graphing, follicles in catagen and telogen were classified as telogen follicles; follicles in proanagen II and III as early proanagen follicles and those in proanagen IV to VI as late proanagen follicles. For pretreatment hair growth assessments, transverse sections of both the original and derived follicles were classified into telogen, proanagen and anagen follicles.

Sections for PCNA staining were interpreted as described (Saywell and Nixon, 1992; Kitano *et al.*, 1996). Five to 14 hair follicles with dermal papilla and bulb region present were counted for each sample. Only cells exhibiting an intense and uniform red to deep-red nuclear staining were considered positively labelled. Hair follicles were recorded into four stages: telogen, proanagen I, proanagen II and proanagen III and more advanced. Follicles with telogen morphology and had two or more positive cells were assigned telogen. Those with telogen morphology and increased number of positive cells in germinal matrix, dermal papilla and dermal sheath were classified into proanagen I. Follicles with increased number of positive cells and morphology equivalent to proanagen II or proanagen III and more advanced stages (Section 2.1.2) were grouped into proanagen II and ≥ proanagen III stages, respectively.

3.6.4 Pretreatment hair growth assessment

To determine the optimum time to commence the treatments, the stage of fur growth was assessed by pelt scoring for the trials in 1996 and 1997. The pelt of each ferret was scored on a 6-point scale based on guard hair growth, skin colour and underfur thickness, whereby "prime" fur was allocated a score of 6. This subjective system for fur assessment was developed in conjunction with ferret breeders and with reference to grades used at the Copenhagen fur sales. For the 1998 ferrets, pretreatment hair growth was monitored using pelt scoring in conjunction with histological assessment of skin biopsies.

3.6.5 Image recording and analysis

Histological and ICC images were recorded either on film with a Olympus Bx50 microscope equipped with a MTI 3CCD camera (model DC330E), or by digital

image capture with a Scionimage™ system (Scioncorp, Frederick, MD, USA) and editing with Microsoft Photoeditor (Microsoft, Seattle, WA, USA).

3.7 Steroid radioimmunoassays

Plasma samples were thawed, agitated and assayed without extraction or dilution. When the level of a hormone was found to be so high as to be outside the detection range, it was diluted with zero calibrator and remeasured. To determine the interference of the Saffan anaesthetic on the steroid assays, additional plasma samples were collected from ferrets after first being anaesthetised with CO_2 and then again after Saffan injection.

All solid-phase radioimmunoassay kits used for detecting total testosterone, progesterone and oestradiol were from Diagnostic Products Corporation (DPC) (Los Angeles, CA, USA). Antibodies to the hormone were already immobilised to the wall of a polypropylene tube provided in the kits. Following incubation of ^{125}I labelled hormone and the serum samples in these antibody-coated tubes, in conditions stipulated for each kit, bound from free ^{125}I labelled hormone were separated and counted for 1 minute in a gamma counter (1261 multigamma, LKBwallac, Pharmacia, Turku, Finland). The level of a hormone in the sample was determined by comparison with a calibration curve derived from pre-prepared standards provided in the kit. The ranges of detection for oestradiol, testosterone and progesterone were 20-3,600 pg/ml, 200-16,000 pg/ml and 100-40,000 pg/ml, respectively.

3.8 Statistical analysis

All data were subject to examination of distribution to select the best-fit model. Data on hair growth initiation were analysed using generalised linear models with the binomial distribution using Genstat (Rothamsted Experimental Station, Harpenden, Herts, UK). Body weight gain and steroid concentration were analysed using analysis of variance (ANOVA), conducted with Minitab (Minitab Inc., State College, PA, USA), or Excel (Microsoft, Seattle, WA, USA). The variation in the data is expressed as ± standard error of the mean (SEM).

3.9 Total RNA extraction

All the solutions and glassware used for RNA work were treated with diethyl pyrocarbonate (DEPC) and autoclaved to reduce the risk of RNase degradation.

Most of the ferret skin RNA was extracted by using a method modified from Unit 4.2, of Current Protocols in Molecular Biology. A denaturing stock solution was prepared from guanidine thiocyanate (Sigma, St. Luis, MO, USA) according to the method reported (Chomczynski and Sacchi, 1987) and a working solution was prepared on the day of use. Skin samples were cut into small pieces, weighed and ground with a freezer mill (Spex 7700, Glen Creston Ltd, Middlesex, UK) under liquid nitrogen. The frozen skin powder was mixed into a denaturing solution and extracted with acid-equilibrated (pH 4.7) phenol/chloroform (5:1) (Sigma). RNA in the aqueous phase was precipitated with isopropanol and re-extracted with denaturing solution. The aqueous phase was then re-precipitated with isopropanol and washed with 75% ethanol. The RNA was resuspended in DEPC-H_2O. The quantity and quality of the total RNA was determined by UV absorbance at 260 nm and the ratio of the absorbance at 260 nm and 280 nm. The RNA integrity was further checked by running on 1.2% denaturing agarose gel (Unit 4.9.3, Current Protocols In Molecular Biology) and visualising 18s and 28s ribosomal RNA bands after staining with ethidium bromide (EB). In some experiments, skin RNA was extracted with TRIzol (Gibco BRL, Rockville, MD USA) according to the manufacturer's protocol. The RNA integrity was checked as described above. The total RNA samples were stored at –80 °C.

3.10 Differential display

3.10.1 DNase I treatment of total RNA

To ensure the RNA samples used for differential display were free of genomic DNA contamination, they were treated with RNase-free DNase I according to the method described in Unit 15.8 of Current Protocols in Molecular Biology. Re-suspended RNA was checked by spectrophotometry and electrophoresis before use as described in Section 3.9.

3.10.2 Primers

Three poly-dT primers with a 3' anchor base and six arbitrary primers were designed and synthesised. Poly-dT primers and arbitrary primers were 20 and 21

bp in length respectively, and all had a *Hind* III restriction site towards the 5' end (Table 3.1). Eighteen sets of primers, each consisting of one poly-dT anchor primer and one arbitrary primer, were used in differential display.

Table 3-1 Differential display primers and their sequences

Name	Function	Sequence
F08	3' reverse	5' GCGCAAGCTTTTTTTTTTTC 3'
F09	3' reverse	5' GCGCAAGCTTTTTTTTTTTG 3'
F10	3' reverse	5' GCGCAAGCTTTTTTTTTTTA 3'
F11	5' forward	5' CGGGAAGCTTATCGACTCCAAG 3'
F12	5' forward	5' CGGGAAGCTTTAGCTAGCATGG 3'
G01	5' forward	5' CGGGAAGCTTGCTAAGACTAGC 3'
G02	5' forward	5' CGGGAAGCTTTGCAGTGTGTGA 3'
G03	5' forward	5' CGGGAAGCTTGTGACCATTGCA 3'
G04	5' forward	5' CGGGAAGCTTGTCTGCTAGGTA 3'

3.10.3 Reverse transcription with poly T anchor primer

Reverse transcriptions (RT) were conducted at 41°C for 50 minutes with the Superscript II preamplification system for first strand cDNA synthesis (Gibco BRL, Rockville, MD, USA). For the convenience of working on a number of RT reactions simultaneously, a modification of the method was to add Superscript II reverse transcriptase into a cocktail of the reaction mixture instead of adding the enzyme to each tube individually after mixing the reaction mixture with RNA/primer. The poly-T anchor primer concentration was 0.5 μM in both the 20 μl or 50 μl reactions, while the amount of total RNA was 0.75 μg and 1.5 μg respectively. The concentrations of $MgCl_2$, DTT and dNTPs were 1.25 mM, 10 mM and 0.5 mM. One μl of Superscript II reverse transcriptase (200 units/μl) was added per reaction regardless of the volume and one μl RNase H (Gibco BRL, Rockville, MD USA) was used to degrade the RNA before the first strand cDNAs were subjected to PCR amplification.

3.10.4 PCR amplification

All the PCR reactions for differential display were conducted in 20 μl volumes, either with thin walled 0.2 ml tubes or standard 0.5 ml tubes. Thin walled 0.2 ml

tubes were used with the PC-960 Air Cooled Thermal Cycler (Corbett Research, NSW, Australia) and 0.5 ml tubes with either PTC-100™ Programmable Thermal Controller (MJ Research Inc., Watertown, MA, USA) or TouchDown (Hybaid Limited, Ashford, Middlesex, UK). Taq DNA polymerase was generally supplied by Life Technologies (Gibco BRL, Rockville, MD, USA). To minimise differences amongst the reactions, all PCR components, except templates, were prepared in cocktails. PCR reactions for amplification of first strand cDNA were identical except that different concentrations of dNTPs were used, namely 125-200 μM with the 1996 samples, but only 25 μM with the 1998 samples. Differential display reactions using the 1998 skin samples were amplified in duplicate. The protocols were as follows (for a 20 μl reaction):

1 μl RT products

1x PCR buffer

1.5 mM $MgCl_2$

125-200 μM (or 25 μM) 4 dNTPs mixture

1.0 μM poly T primer

0.5 μM arbitrary primer

0.2 μl ^{33}P-α-dATP (10 mCi/ml) (NEN™ Life Science Product Ltd., Boston, MA, USA)

1.25 units of Taq DNA polymerase (0.25 μl, 5U/μl)

The optimised PCR program was:

4 cycles:	94°C	30 min
	41°C	45 sec
	72°C	1 min, then
18 cycles:	94°C	30 sec
	55°C	45 sec
	72°C	2 min

3.10.5 Separation of PCR products and autoradiography

A 6% polyacrylamide gel (37 x 43 cm) was prepared from 30% acrylamide/bis (20:1) solution (Bio-Rad, Hercules, CA, USA) and run in 1x TBE. In most cases, 5 μl from each of 48–50 reactions were loaded in an order either reflecting the follicle growth stages determined histologically, or the days after melatonin

treatment. Usually the gel was run at 60 watts until the band of bromophenol blue dye had migrated to the bottom of the gel plate. If differentially displayed bands (differentials) were found to be crowded at the top of the gel, the samples were rerun in a 4% acrylamide gel over a longer period so as to resolve these higher molecular weight products. The polyacrylamide gel was dried and exposed to X-ray film (X-OMAT AR, Eastman-Kodak, Rochester, NY, USA) at –75 °C for one to two days, with orientation markers.

3.10.6 Recording differential display results

The relative strengths of a band across all the reactions were estimated subjectively and plotted as a histogram for the growth stages for all time points presented. When the density of a band observed in an X-ray film increased or decreased two fold or more, it was recorded as being differentially displayed or a differential.

3.11 Cloning and sequencing of differentials

3.11.1 Isolation and reamplification of differentials

The polyacrylamide gel sites corresponding to the differentials were isolated from the gel by overlapping the X-ray film and the gel over a light box. The isolated bands were kept in a –20°C freezer before being eluted into 20 µl of DEPC-H_2O. To reamplify the cDNA contained in the differentials, 4 µl of the eluted DNA was used as a template for PCR reamplification in a 40 µl reaction. The PCR program used was similar to that for the second stage amplification in differential display, except for a higher cycle number (35-40 cycles), slightly longer annealing time (1min), 2.5 to 10 times higher concentration of dNTPs and excluding ^{33}P-α-dATP. Reamplification results were checked by visualisation in UV light after running the PCR products in a 1.2% agarose gel and staining with EB.

3.11.2 Cloning into pGEM T vector

When only one prominent band was reamplified, or a dominant band of similar size to the template was present, it was ligated into pGEM T vector (Promega, WI, Madison, USA) directly. Otherwise, the bands of expected size were isolated by agarose gel purification with Qiaquick Gel Extraction kit (Qiagen, Hilden, Germany) prior to ligation. DH5α competent cells (Gibco BRL, Rockville, MD

USA) were transformed and recombinant cells were isolated by colour selection. Plasmid DNA of selected cells was prepared with a High Pure™ Plasmid Isolation Kit (Boehringer Mannheim, Germany). After digesting with *Hind* III (Gibco BRL, Rockville, MD USA or Boehringer Mannheim, Germany), the size of insert was checked in agarose gel. Occasionally, the presence of insert and size were checked by PCR reamplification with appropriate primer sets to select clones for miniprep. Two or more clones for each differential were stored in 50% glycerol at -75°C.

Some of the differentials derived from the 1996 skin samples were cloned by a "shotgun" strategy. Four to five reamplified differentials of distinct sizes were ligated together to pGEM T vector and used for transformation. Clones containing inserts of corresponding sizes to the original differentials were retained.

The creation of genetically modified organisms through cloning of the differentials and construction of cDNA library (Section 3.15.1) described in this thesis was conducted in a manner that fulfills the policy of the Environmental Risk Management Authority (ERMA) of New Zealand and approved in application GM099/ARO11.

3.11.3 Sequencing of cloned differentially displayed gene fragments

A minimum of one clone derived from each differential was sequenced through sequencing facilities at either University of Waikato, or University of Auckland. Sequences from the latter were provided from both strands. Some clones which gave low quality sequences were subsequently re-sequenced with higher concentration of templates or alternative clones.

3.12 Northern blot analyses

Sample arrangements: Ten μg of total RNA was denatured and separated by electrophoresis on a denaturing agarose gel (Unit 4.9.3, Current Protocols In Molecular Biology). RNA samples from skin collected in the 1996 trial were arranged according to follicle growth stages of the samples. The samples from the 1998 trial were arrayed according to days post melatonin treatment and melatonin plus steroid treatment.

Blot preparation: After electrophoresis, the gel was stained with EB and a photograph was taken to record the loading variations and the positions of 28S and 18S ribosomal RNA. The gel was blotted onto Hybond^{TM}-N^{+} (Amersham Pharmacia Biotech, Little Chalfont, Buckinghamshire, UK) in 10x SSC overnight and cross-linked with UV light (UV Stratalinker 1800, Stratagene, La Jolla, CA, USA) (Unit 4.9.5, Current Protocol In Molecular Biology). Occasionally, the loading and positions of the 28s and 18s rRNA were detected by methylene blue staining of the blot. The blots were then destained, rinsed, dried and kept in plastic bags at room temperature until use. For preliminary Northern blots using the 1996 samples, four sets of seven RNA samples, ranging from telogen to anagen, were loaded into a 14 x 11 cm agarose gel in two parallel rows (double-deck gel). After electrophoresis and blotting conducted as previously described (Unit 4.9.3 to 4.9.6, Current Protocols in Molecular Biology), the membrane was cut into four pieces and each used for screening different ESTs.

Probe stripping: Most of the Northern blots were used three times. After each hybridisation, they were stripped of the probes with 0.1x SSC containing 0.5% SDS at 70°C for 30 minutes, then checked for probe removal with a Geiger counter or by re-exposure to X–Omat AR film.

Probe preparation: Probes were prepared by random priming of 25 ng DNA template in the presence of 0.5 mM dATP, dGTP, dTTP and 2.5 µl of ^{32}P α-dCTP (3000ci/mmol) (NEN^{TM} Life Science Product Ltd., Boston, MA, USA), 2 µl of random hexanucleotide (Boehringer Mannheim, Germany) and 1 µl of Klenow enzyme (Boehringer Mannheim, Germany). The template DNA was purified from agarose gel after isolation of the inserts from miniprepped DNA. Incorporation rates of the probes were checked by dotting samples onto filter paper and comparing the radioactivity before and after washing with 5% TCA (trichloroacetic acid, BDH Laboratory Supplies, Dorset, UK). Probes were denatured with 0.4 N NaOH for ten minutes at room temperature before hybridisation.

Hybridisation and washing: Hybridisation was conducted at 65°C in Church and Gilbert solution (Unit 6.3.3 to 6.3.4 and Unit 4.9.7, Current Protocols In Molecular Biology). Blots were prehybridised for 30 min to an hour before hybridisation overnight with probes in 5ml of hybridisation solution in a rotating Hybaid bottle.

The background was reduced by successive washes of decreasing concentrations of SSC with 0.1 % SDS before exposing to X-Omat AR film at -75°C.

Size determination and image recording: The size of the mRNA was determined by either comparing with migration of RNA marker (Boehringer Mannheim, Germany), or plotting the relative mobility of the 28S, 18S rRNA and mRNA signal. The results were scanned into a computer using Photopaint and edited by Photoeditor (Microsoft, Seattle, WA, USA).

3.13 Reverse RNA dot blot analysis

DNA dot blot preparation: Inserts from the plasmid DNA (1 µl of a 1 to 1000 dilution) of the clones to be screened were amplified by PCR for 40 cycles in a 40 µl reaction. The primers used were the original sets from which they were derived by differential display. The amplification conditions were the same as described for differential amplification (Sections 3.10.4 and 3.11.1). Products and yields were checked by agarose gel electrophoresis and EB staining. Fifty nanograms of DNA was dotted onto a nylon membrane, cross-linked and washed as described (Vogeli-Lange *et al.*, 1997).

Probe synthesis: The first strand cDNA was produced as described for differential display. Probes were synthesised as previously described (Section 3.10.4), but with only 14 to 16 cycles of PCR for the second stage. Probes were labelled with ^{33}P-α-dATP or ^{32}P-α-dCTP (NEN™ Life Science Product Ltd., Boston, MA, USA).

Hybridisation and washing: Hybridisation was conducted as described for Northern blots (Section 3.12) at 60-65°C.

3.14 *In situ* hybridisation

3.14.1 Preparation of skin sections

Longitudinal sections were cut from selected samples as described in 3.5.1.2. However, DEPC-H_2O and polysine™ slides (Erie Scientific Company, Portsmouth, NH, USA) were used for mounting the sections. Skin samples, which were most likely to express high level of the transcripts according to the differential display pattern, or Northern blot hybridisation, results if available, were chosen for *in situ* hybridisation. Sections were deparaffinised and skin sections were outlined with a plastic pen. The sections were treated with 2 µg/ml

proteinase K and then with acetic anhydride before use (Miller *et al.*, 1993a; Oliver *et al.*, 1997).

3.14.2 Preparation of RNA probe

Non-radioactive (DIG-labelled) RNA probe: Plasmid DNA with the insert was linearised with *Nco* I or *Not* I (Boehringer Mannheim, Germany) and purified. SP6 and T7 RNA polymerases (Boehringer Mannheim, Germany) were used in the synthesis of the sense or anti-sense probes in the presence of DIG-labelled NTPs (Boehringer Mannheim, Germany). To compensate for the lower yield of SP6 RNA polymerase, an extra one µl of the enzyme was added after one hour of incubation at 37 °C and incubated for another hour. RNase-free DNase I (Boehringer Mannheim, Germany) was added to degrade DNA and DNase I was then destroyed by heating and RNA probes were precipitated with LiCl and tRNA as carrier. RNA probes resuspended in TE were checked for yield and size using a denaturing agarose gel as described previously.

Preparation of ^{35}S-labelled RNA probe: ^{35}S-labelled RNA probes were synthesised similarly, but with an ATP, CTP and GTP mixture, together with 4 µl ^{35}S-UTP (1175 Ci/mmol) (NEN™ Life Science Product Ltd., Boston, MA, USA) as substrates. RNA probes were precipitated and the incorporation rates of the probes were checked by TCA precipitation. The size and yield of the probe were checked by 6% acrylamide gel electrophoresis and autoradiography.

3.14.3 Hybridisation with DIG-labelled probes

Skin sections were often subjected to direct hybridisation, but some were prehybridised in premix hybridisation buffer (150 mM NaCl, 50% formamide, 2x SSC final concentration with 0.2 mg/ml tRNA, 1.0 mg/ml herring DNA). For hybridisation, DIG-labelled sense and anti-sense probes diluted with hybridisation solution (780 µl premix hybridisation buffer with 20 µl of 20 mg/ml BSA and 200 µl of 50% dextran sulfate) were added directly to the unprehybridised sections or after decanting the prehybridisation solution. In order to optimise results, the probes were usually diluted to 2-3 different concentrations between 1: 200 to 1: 2000 and hybridisation was often conducted at two different temperatures between 55-65°C in a chamber equilibrated with a 50% formamide solution.

3.14.4 Detection of antibody binding and colour development

The sections were washed twice with 2x SSC containing 50% formamide, then twice with 2x SSC. The sections were then treated with RNase A and RNase T1 (Sigma, St. Louis, MD, USA) solution of 35mg/ml and 3.5mg/ml respectively. The sections were again washed with 2x and then 0.2x SSC. The slides were rinsed with DIG buffer I (100mM Tris-HCl, 150mM NaCl, pH7.5), then incubated in a blocking solution (buffer I containing 0.1% triton X-100, 5% normal sheep serum). After decanting the blocking solution, a Fab fragment of sheep anti-DIG cross-linked with alkaline phosphatase (Boehringer Mannheim, Germany) was diluted to 1:500 in blocking solution and applied to the sections. The sections were incubated for 1 hour at 37 °C in a humid chamber. After the sections were washed three times with washing buffer (100mM Tris-HCl, 100mM NaCl, pH 7.0 with 0.1% Tween 20), they were soaked in detection buffer (100mM Tris-HCl, 100mM NaCl, 50mM $MgCl_2$, pH9.5) containing 1 mM levamisole (Sigma, St. Louis, MD, USA) to inhibit the internal enzyme activity. Substrate solution (4.5µl NBT +3.5 µl BCIP per ml of PVA buffer) was then added and a cover slip was placed on the sections and the slides were incubated at 37°C or room temperature in a humid box shielded from light for colour development. When colour development was optimal, the cover slip was removed and the slides were rinsed with warm tap water, then counter-stained with 0.2% eosin.

3.14.5 Hybridisation with ^{35}S-labelled RNA probe

Probes were diluted with the hybridisation buffer to approximately 10K cpm/µl before being added to the sections. Hybridisation was conducted at 58°C overnight. After the sections were washed, treated with RNase solution and washed again, they were dehydrated and exposed to X-ray film overnight to assess the results. Photographic emulsion (Eastman Kodak, Rochester, NY, USA) was then applied to the sections and exposed for 4-6 days before development (Miller *et al.*, 1993a). The slides were then counterstained and coverslips were mounted with DPX.

3.15 cDNA library construction and screening

3.15.1 cDNA library construction

Purification of mRNA: Poly A^+ RNA was isolated by affinity chromatography with poly T cellulose (Boehringer Mannheim, Germany). Total skin RNA samples representing different growth stages of hair follicles were pooled and purified with 50 µg oligo (dT) cellulose (Gibco BRL, Rockville, MD, USA) (Rufaut, 1997).

Construction of cDNA library: The cDNA library was made with a ZAP-cDNA^R Gigapack^{R}II Gold Cloning Kit (Stratagene, La Jolla, CA, USA), following the manufacturer's protocols. Five micrograms of poly A^+ RNA was used to synthesise the first strand cDNA, which was then used for the second strand synthesis. The cDNA was blunt-ended, ligated to adapters, restriction digested, size fractionated, quantified and then ligated into Uni-Zap^{TM} XR γ-phage vector. Lambda-phage vector with cDNA inserts were packaged into infective phage particles with Gigpack^{R}II Gold packaging extract. Packaged phage were titred and amplified using XL1-Blue MRF' *E coli* cells. The amplified library was resuspended, retitred and kept at 4°C in the presence of 0.3% chloroform before it was stored in aliquots in 7% (v/v) DMSO at –80 °C.

3.15.2 Library screening by DNA hybridisation

The process was conducted as described in the Stratagene Library Construction Manual, but on a larger scale. Duplicate plaque lifts were taken from a 22 x 22 cm plate with approximately $1.4x10^5$ PFU of phage from the amplified frozen library. Hybond^{TM}-N^+ Nylon membranes with the plaques were denatured, neutralised, washed, cross-linked and stored at 4°C until use.

DNA probes were made either by random priming (Section 3.12) or by PCR using gene specific primers. The 40 cycle PCR protocol was similar to the second stage of the program used for differential display amplification (Section 3.11.1), but with 0.75 µM primers and an annealing temperature of 57 °C. Hybridisation was done on both duplicate lifts from the same plates. Hybridising plaques were recovered and re-screened to isolate individual plaques. Lambda DNA was prepared with Lambda Miniprep Kits (Qiagen, Hilden, Germany) and the DNA was digested with *Nco* I and *Not* I to determine the sizes of the inserts. Bluescript

plasmid containing the inserts were isolated from the infective lambda virus according to Stratagene Library Construction Manual and were used to transform *E. coli* SOLR cells (Qiagen, Hilden, Germany). Plasmid DNA was prepared, cut with the same restriction enzymes to check the sizes of the inserts and the plasmids with inserts of expected sizes were sequenced.

3.16 RACE

3.16.1 Primer design

All of the gene specific primers (forward, forward nested, reverse and reverse nested) were made from the sequences generated by differential display or the extended sequences. They were designed in such a way that they would cover some existing sequence before extending into the upstream or downstream part of the transcripts (Sections 7.2.3 & 7.3.5).

3.16.2 5' RACE

5' RACE was carried out as described in the manufacturer's protocol (5' RACE system for rapid amplification of cDNA ends, Version 2, Gibco BRL, Rockville, MD, USA). Total RNA was reverse transcribed with a gene-specific reverse primer, then the RNA was degraded and the first strand cDNA was purified. This purified first strand cDNA was tailed by Terminal deoxynucleotidyl Transferase (TdT) with dCTP as substrate, which added a poly C tail to the first strand cDNA. The tailed cDNA was amplified by PCR with the same reverse primer or reverse nested primer and a 5' abridged anchor primer (AAP) designed to bind to the poly C tail. If necessary, this was further reamplified with a reverse nested primer and an abridged universal amplification primer (AUAP) that binds to the upper part of AAP to further increase the specificity of the product. The amplified DNA was gel purified before ligation or ligated directly into pGEM T vector for transformation. Plasmid DNA was prepared and clones with inserts of expected size were sequenced to ensure that the amplified DNA came from the target templates by showing overlapping sequence before extending into 5' known sequence.

3.16.3 3' RACE

A poly T adapter primer (AP) with adapting sequence was used to synthesise first strand cDNA from the 3' end of the mRNA. A universal amplification primer

(UAP) which binds to the adapting sequence of AP and a forward primer designed from known sequence were used to amplify the 3' end segment of the cDNA. When needed, the forward primer was replaced with a forward nested primer and 3' AUAP for further amplification. The amplified cDNA was cloned and sequenced as for 5' RACE products.

CHAPTER 4 EFFECTS OF MELATONIN AND OF STEROIDS ON FERRET WINTER PELAGE DEVELOPMENT

4.0 Abstract

Steroid effects on hair growth are known in many species but their actions at specific points of the hair growth cycle remain obscure. The main purposes of the experiments reported in this chapter were to assess steroid effects on proanagen and, to collect skin samples for the discovery of genes underlying the hair growth cycle.

Steroid effects were investigated in two trials conducted in 1997 and 1998 with pre-pubertal ferrets housed under long day light (16L:8D). During March, treated ferrets were implanted with 21-day slow release pellets, containing one of five different steroids, either alone or in combination with two 18 mg melatonin implants. Flank skin biopsies were collected at 4-7 day intervals for histological assessment of growth status of original follicle in longitudinal skin sections.

In untreated controls, hair follicles generally remained dormant, although some spontaneous hair growth was evident, especially in 1997. In the absence of exogenous melatonin, oestradiol (5 and 15 mg), dexamethasone (15 mg), testosterone (15 mg) progesterone (15 mg) and deoxycorticosterone (15 mg), all reduced spontaneous hair growth activity 15 days after subcutaneous implantation, by blocking hair follicle growth initiation and/or by inducing regression of existing anagen follicles. Although testosterone, progesterone, especially deoxycorticosterone, appeared to initially permit or even stimulate spontaneous hair growth, this effect was reversed by day 15.

Melatonin stimulated original follicle growth effectively in both male and female ferrets. When applied in combination with melatonin, oestradiol strongly inhibited follicle growth whether administered prior to, simultaneously or after melatonin. Dexamethasone was strongly inhibitory when administered before melatonin, but less effective when given simultaneously. Deoxycorticosterone had no effect. Testosterone slightly stimulated hair growth when given prior to melatonin, but had no effect or inhibited hair growth when administered simultaneously with melatonin. Progesterone implanted simultaneously with melatonin did not have significant effects in females, but inhibited follicle growth initiation in males.

4.1 Introduction

The influences of steroids on hair growth have been demonstrated by studies involving gonadalectomy, adrenalectomy, pregnancy and the administration of adrenal cortical extracts (Johnson, 1958b; Franz and Bosse, 1975; Rose, 1995). The effects have been further studied by the direct administration of steroid hormones, either alone or in combination with other hormones, *in vivo* and *in vitro* (Section 2.4) (Hooker and Pfeiffer, 1943; Johnson, 1958b; Mohn, 1958; Morill and Herrmann, 1961; Jackson and Ebling, 1972; Hale and Ebling, 1975; Oh and Smart, 1996; Chanda *et al.*, 2000). However, some of the studies have produced varying outcomes which may have arisen from the different animal models, or experimental protocols. Most of the investigations assessed hair growth by recording the emergence of hair above the skin. This is a straightforward, but insensitive measure of hair growth responses. On the other hand, histological monitoring of structural changes by Sacpic staining of skin biopsy longitudinal sections can provide much more precise data (Nixon, 1993a; Nixon *et al.*, 1995). In addition, the well-synchronised ferret winter hair growth initiated by exogenous melatonin could not only provide suitable materials for identification of genes associated with hair growth initiation (Sections 2.1.5.3 & 2.2), its value in addressing various issues of steroid effects on hair growth (Section 2.4) were investigated.

4.2 Materials and methods

The animal husbandry, pelt scoring, anaesthesia, euthanasia, steroid and melatonin implants, sample collection and steroid analyses were as described in Chapter 3.

Hair follicle growth was assessed by examining the original follicle stages after Sacpic staining of longitudinal sections, supplemented with immunocytochemistry (Section 3.6). Statistical analysis was conducted as described in Section 3.8.

4.2.1 1996 trial

The objectives were (1) to confirm the effect of melatonin in synchronising ferret winter hair growth and; (2) to provide skin samples for gene expression studies.

Ferrets born around 17 November 1995 were weaned in mid-January 1996 and caged in pairs at the same ferret farm for the duration of the trial. The animals were kept in natural daylight until 11 of March, 1996, when long day photoperiod

(16L:8D) was applied using artificial lighting to prevent spontaneous winter fur growth. The fur growth stage of each animal was monitored by pelt scoring. On 21 March 1996, 28 ferrets (20 males and 8 females at 125 days of age) were treated with two 18 mg melatonin implants (Table 4-1). Three males without implants were sacrificed at the time of melatonin administration (day 0) for collection of flank skin. Over the subsequent 14 days, groups of three (days 1-5) or two (days 6, 8, 10, 12 and 14) ferrets were euthanised for skin collection.

Table 4-1 Design of the 1996 ferret trial

No. of Ferrets	Sex	Melatonin	Skin collection
28	M/F	D0[1]	at slaughter (D0, D1-6, 8, 10, 12, 14)

[1] Day 0 corresponds to the day of melatonin implantation in all trials.

4.2.2 1997 trial

The objectives were (1) to collect serial biopsies, and large flank skin samples at slaughter over 6 days after melatonin administration for further gene expression studies and; (2) to examine, in serial skin samples, the effects of 5 different steroid hormones on the initiation of fibre growth when given alone, simultaneously, or seven days prior to melatonin treatment.

One hundred juvenile ferrets (90 males and 10 females) born around 21 November 1996 were weaned into male/female pairs in mid-January 1997. They were transported to the Ruakura Research Centre on 4 February and caged in pairs. From 18 February, and for the duration of the trial, they were exposed to long days (16L:8D). Pelt scoring was conducted to monitor autumn pelt prime development. Ninety-four ferrets were allocated into 19 groups (Table 4-2) balanced for litter source, pelt score and weight. On 3 March, at 102 days of age, all the ferrets in subgroup C of Groups 5-9 were administered one of the five steroids (Tables 4-2 and 4-3). Seven days later, ferrets from all groups except Group 3, were administered either two 18 mg subcutaneous implants of melatonin (Groups 1, 2, 4 and subgroup C of Groups 5-9), or one of five steroids (subgroup A of Groups 5-9), or melatonin plus one of the five steroids (subgroup B of Groups 5-9). Serial skin biopsies were collected daily from the mid-side for 6 days from each ferret of Group 1. Fourteen ferrets (Group 2) were euthanised with phenobarbitone (2 animals prior to melatonin administration and 2 more each day

for 6 days) to collect large skin samples. Three skin biopsies were collected from each ferret in Groups 3-9 at 4-day intervals, commencing seven days after melatonin implantation, in order to assess hair follicle growth. In winter (mid-July), three other ferrets were sacrificed at the ferret farm for samples of flank skin, footpad, liver, breast muscle and adrenal for use in differential display and Northern blots.

Table 4-2 Design of the 1997 ferret trial

Group	No.	Sex	Melatonin	Steroid	Skin collection
1	8	M	D0	-	D0-6
2	14	M	D0	-	D0-6[1]
3	6	M	-	-	D7, 11, 15
4	6	M	D0	-	D7, 11, 15
testosterone					
5A	4	M	-	D0	D7, 11, 15
5B	4	M	D0	D0	D7, 11, 15
5C	4	M	D0	D-7	D7, 11, 15
oestradiol					
6A	4	M	-	D0	D7, 11, 15
6B	4	M	D0	D0	D7, 11, 15
6C	4	M	D0	D-7[2]	D7, 11, 15
deoxycorticosterone					
7A	4	M	-	D0	D7, 11, 15
7B	4	M	D0	D0	D7, 11, 15
7C	4	M	D0	D-7	D7, 11, 15
dexamethasone					
8A	4	M	-	D0	D7, 11, 15
8B	4	M	D0	D0	D7, 11, 15
8C	4	M	D0	D-7	D7, 11, 15
progesterone					
9A	4	F	-	D0	D7, 11, 15
9B	4	F	D0	D0	D7, 11, 15
9C	4	F	D0	D-7	D7, 11, 15

[1] at slaughter. [2] 7 days before melatonin administration.

4.2.3 1998 trials

There were three objectives in the 1998 trials. The first was to corroborate the effects of the four steroid hormones on the initiation of fibre growth when administered simultaneously with melatonin. The second was to collect skin samples over 10 days from melatonin and steroid administration for immunocytochemical and gene expression studies. And the third was to investigate the effect of oestradiol on growing hair follicles previously initiated by exogenous melatonin.

Table 4-3 Amount of steroid in constant release implants in 1997 trial

Steroid	Group	Implant loading (mg)	Release duration (days)	Calculated dose (mg/day)
testosterone	5	15	21	0.72
oestradiol	6	15	21	0.72
deoxycorticosterone	7	25	21	1.19
dexamethasone	8	15	21	1.19
progesterone	9	15	21	0.72

One hundred juvenile ferrets (72 males and 28 females), born around 2 December 1997, were weaned on the 9th of January and transported to the Ruakura Research Centre four days later. They were housed in pairs and in long daylight (16L:8D) for the duration of the trial. The artificial light was stronger than that of the previous year by placing the lighting source closer to the cages. For the first month, half the feed ration was fed in the morning and the other half in the late afternoon. Subsequently, the ferrets were fed once daily in the late afternoon.

Pelt growth stage was visually assessed and skin biopsies were taken to monitor hair growth by histology. Only those ferrets with pelt scores equal to or greater than 5.5 were used in the trial. As the hair growth development was found to be uneven among the ferrets, the experiments were conducted in two time periods.

Ferrets which were closer to completion of their juvenile pelage were allocated into Groups 10-15, balanced for pelt scores, weight and litter source. On 10 March, ferrets in Group 10 were treated with melatonin, while ferrets in Groups 11-13 were implanted simultaneously with melatonin and oestradiol or

progesterone (Tables 4-4 and 4-5). Groups 14 and 15 were untreated males and females controls respectively. Two ferrets from Group 10, and one ferret from Groups 11 to 13 were sacrificed on each of days 0.5, 1, 1.5, 2, 2.5, 3, 6 and 10. Samples from the male and female untreated control ferrets (Groups 14 and 15) were collected at slaughter on day 0 (Table 4-4).

Table 4-4 Design of the 1998 ferret trials

Group	No.	Sex	Melatonin[1]	Steroid & administration day	Skin collection
Controls					
1	4	M	-	-	D0, 7, 11, 15
2	4	F	-	-	
3	4	M	D0	-	
4	4	F	D0	-	
Effect of steroids					
5	4	M	D0	testosterone, D0	D0, 7, 11, 15
6	4	M	D0	dexamethasone, D0	
7	4	M	D0	oestradiol, D0	
8	4	M	D0	progesterone, D0	
9	4	F	D0	progesterone, D0	
Slaughtered for skin samples					
10	16	M	D0	-	D0.5, 1, 1.5, 2, 2.5, 3, 6, 10
11	8	M	D0	oestradiol, D0	
12	8	M	D0	progesterone, D0	
13	8	F	D0	progesterone, D0	
14	4	M	-	-	D0
15	4	F	-	-	
Effect of oestradiol on growing follicles					
16	6	M	D0	-	D0, 7, 14, 18, 21
17	6	M	D0	oestradiol, D+7[2]	

[1] Day 0 for Groups 1-9 and 16, 17 was 19 March 1998, while that for Groups 10-15 was 10 March 1998.

[2] Seven days after melatonin administration.

Trials for Groups 1 to 9 and Groups 16 and 17 commenced 9 days later on 19 March 1998 when the animals were 107 days old. Treatments and steroid doses are outlined in Tables 4-4 and 4-5. For Groups 16 and 17, melatonin was

implanted on day 0. Oestradiol (5 mg) was administered seven days later to ferrets in Group 17.

Table 4-5 Amount of steroid in constant release implants in 1998 trial

Steroid	Group(s)	Implant loading (mg)	Release duration (days)	Calculated dose (mg/day)
Testosterone	5	15	21	0.72
Oestradiol	7, 12	5	21	0.24
Dexamethasone	6	15	21	0.72
Progesterone	8, 9, 13	15	21	0.72

4.2.4 Comparison of experimental factors for the three years

All trials over the three years were conducted under similar conditions (Table 4-6). Ferrets and their feed all came from the same farm. The trials commenced in March, and the timing of melatonin administration varied maximally by 11 days by date and 27 days by age. Mean pelt scores immediately before the trials were similar. However, ferrets were weaned earlier in 1998 than in the previous two years and the long day light treatment was applied earlier and at a stronger intensity.

4.3 Results

4.3.1 Body weight gain

Live weights increased over the course of the experiments in 1997 and 1998 ($P \leq 0.001$) (Figs. 4-1 and 4-2). In the 1997 trial, no significant difference in live weight gain was found between the treatments, except for the dexamethasone group where live weight declined within a week of administration (Fig. 4-1).

Two weeks after the treatment (24 March), the combined live weight gain for three groups of ferrets treated with dexamethasone was 19 ± 15g (SEM), compared with 181 ± 21g for the untreated controls and 155 ± 21g for the melatonin controls ($P \leq 0.001$). A reduction in live weight gain among the dexamethasone treated ferrets in the 1998 trial ($P \leq 0.05$) was consistent with that seen in 1997 (Fig. 4-2).

Table 4-6 Comparison of some experimental factors for the four trials

Trial year	1996	1997	1998[1]	1998[2]
Birth date (of the previous year)	Nov. 17	Nov. 21	Dec. 2	Dec. 2
Weaned age (days)	59	55	38	38
Experimental site	On farm	Ruakura	Ruakura	Ruakura
Long day light treatment (LT) (L:D)	16:8	16:8	16:8	16:8
Duration of LT before start (days)	10	13	48	58
Artificial light intensity	weak	weak	strong	strong
Age (days) implanted with MLT	125	109	98	107
Melatonin implant date (D0)	21 March	10 March	10 March	19 March
Duration of observation (days)	14	15	10	15-21
Mean initial body weight (g) (M/F)[3]	1128[4]	1133/1007	993/887	1088/937
Mean final body weight (g) (M/F)	-	1233/1084	-	1177/1011
Mean pelt score[5]	-	5.8	5.8	5.7
No. of pre-treatment skin biopsies	0	0	3	4

[1, 2] First and second trials in 1998. [3] Weight closest to D0. [4] Average of males and females. [5] Immediately prior to treatment.

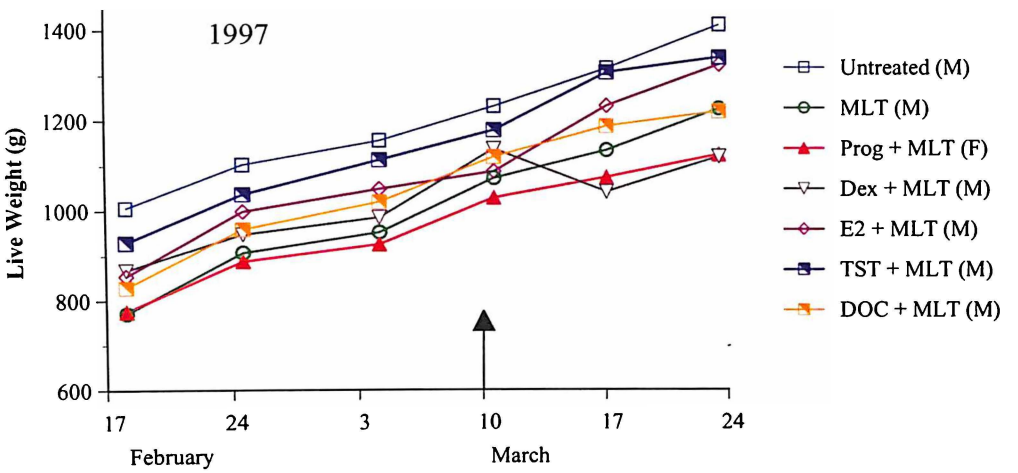


Fig. 4-1 Effect of the treatments on live weight gain in 1997 trial

Only the live weights of ferrets treated with melatonin and steroids simultaneously are presented. Arrow shows date of administration of the hormones (the same as in Fig. 4-2). Administration of dexamethasone alone or prior to melatonin also depressed live weight gain.

Fifteen mg of oestradiol did not affect body weight increments in 1997, but half of the animals in Group 6 died towards the end, or shortly after the experiment. However, a smaller oestradiol dose (5 mg) in 1998 was associated with increased

body weight gain 7 (26 March) (206 ± 35g) and 14 days (2 April) (247 ± 41g) after its co-administration with melatonin, compared with melatonin treated ferrets (68 ± 28g and 86 ± 33g respectively) (P≤0.05) (Fig. 4-2). Four out the 8 oestradiol treated ferrets died a week after the experiment was completed.

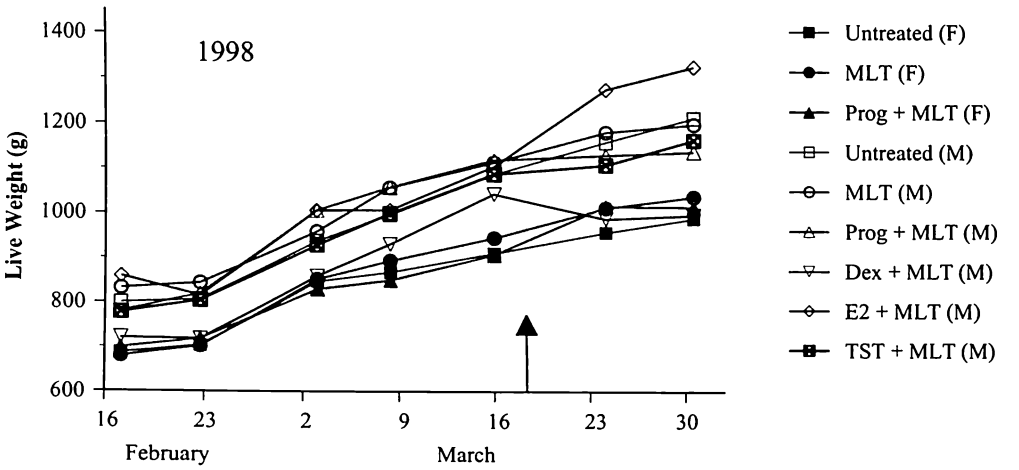


Fig. 4-2 Effect of the treatments on live weight gain in 1998 trial

4.3.2 Pre-treatment hair growth assessment

Results of pelt scoring: The pelt scores were very low when the ferrets were 11 weeks old. Scores increased quickly in the following weeks, despite differences among individuals (data not shown). The average pelt scores of the ferrets were similar when the experiments started in all the three 1997 and 1998 trials (Table 4-6).

Histological assessment of pretreatment hair growth on transverse sections: Skin biopsies were collected for histological assessments in transverse section for the ferrets used in 1998 trials. On average, 77 ± 3% (SEM) (15-100%) and 73 ± 2% (41-97%) of the original and derived follicles respectively were found to be in telogen when the ferrets were 84 days old (n=53, Table 4-7). Ten days later, when 10 of these ferrets were re-sampled, the resting original and derived follicles had increased to 91 ± 3% (64-100%) and 97 ± 1% (93-100%) respectively. It appeared that hair growth was progressing quickly towards completion of the juvenile coat. However, the hair growth was not well synchronised between animals. When the third assessment conducted another three days later (at 94 days of age) using different animals, the original and derived follicle activity were slightly lower: 88 ± 4% (67-100%) and 92 ± 2% (65-100%), respectively. A fourth assessment was

conducted six days later (after many ferrets with more advanced hair growth had been used in the first trial), and a lower percentage of telogen original follicles 76 ± 5% (32-100%) was found (Table 4-7). One ferret with some early proanagen follicles was recorded in the last two assessments. At the time, this was interpreted as an early sign of winter hair growth in these two ferrets. This assumption led to early commencement of the experiment. However, these two ferrets were siblings and both had low pelt scores (3.5 and 4). In retrospect, the proanagen follicles probably reflected the developing juvenile pelage, rather than a harbinger of the winter pelage.

Table 4-7 Average stage of follicle growth cycle in the pretreatment biopsies

Age (Days)	Ferret No.	Original follicles (%)			Derived follicles (%)		
		Anagen	Telogen	Proanagen	Anagen	Telogen	Proanagen
84	53	22.7	77.3	0	27.2	72.8	0
84	10[1]	16.8	83.2	0	20.6	79.4	0
91	10[1]	9.0	91.0	0	2.6	97.4	0
94	11[2]	9.9	87.5	2.6	7.8	92.2	0
100	10[3]	21.0	76.2	2.8	1.2	98.8	0

[1] the same ferrets (subgroup of the 53 ferrets initially sampled); [2, 3] different ferrets.

4.3.3 Plasma steroid concentrations after implantation

Oestradiol levels ranged from very low to undetectable before pellet implantation, averaging 14 ± 9 pg/ml (SEM) and 11 ± 10 pg/ml in 1997 and 1998. The plasma concentrations peaked at 1100 ± 182 pg/ml (1997) and 1115 ± 741 pg/ml (1998) after oestradiol administration (Fig. 4-3a and 4-3b). Judging from the 1998 data, when a smaller dose of the hormone was used, and additional plasma samples were collected from ferrets sacrificed before day 7, the maximal concentration was attained 2 days post implantation. High, though declining, levels were maintained until day 15.

Total testosterone was low or undetectable in the samples collected before pellet administration, averaging 173 ± 48 pg/ml and 400 ± 340 pg/ml for the two years, respectively. Although it was undetectable in some samples collected seven days after implantation, the concentrations averaged at 2400 ± 1215 pg/ml and 5464 ± 2941 pg/ml at this time (Figs. 4-3c and 4-3d). Total testosterone decreased to low or undetectable levels by day 14-15. As with oestradiol, testosterone was not

delivered constantly over a period of 21 days according to the manufacturer's specifications. Instead, the release appeared to be weighted towards the first few days after implantation. This accelerated release of the hormones was not unanticipated given that implant disintegration was observed in some slaughtered ferrets in the 1998 trials. The circulating concentration of progesterone could not be measured due to the interference of Saffan, the synthetic steroid anaesthetic used prior to blood collection.

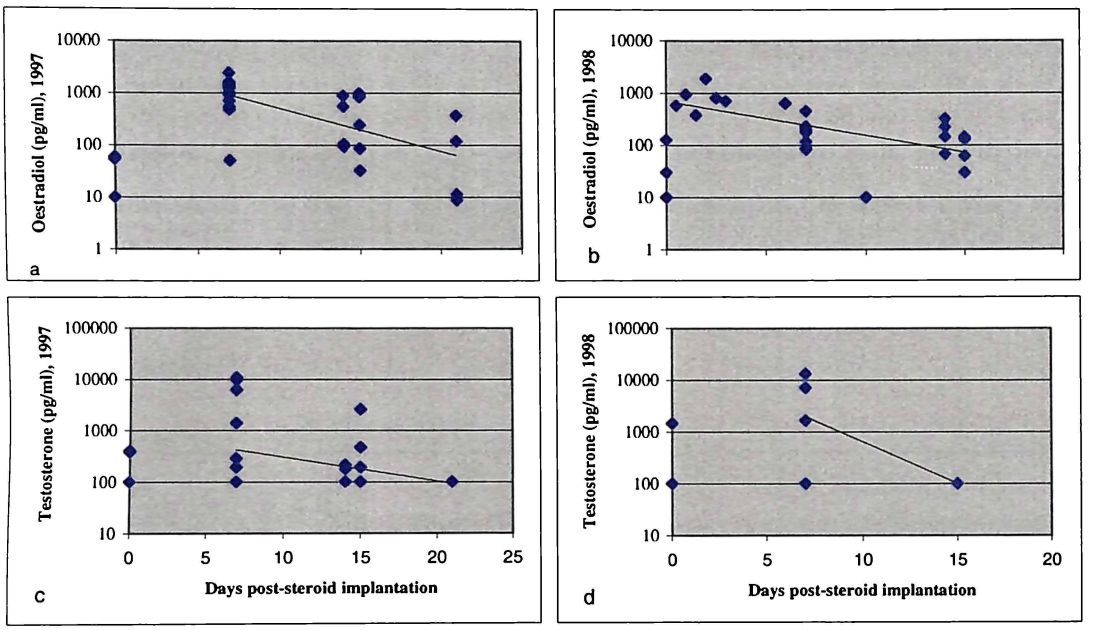


Fig. 4-3 Plasma oestradiol and testosterone concentrations after implantation at day 0

The dose of oestradiol was 15 and 5 mg for 1997 and 1998, while it was 15 mg in both years for testosterone. Diamond symbols may represent multiple data points.

4.3.5 Effects of melatonin and steroids on hair growth initiation

4.3.5.1 1997 trial

Melatonin: Among untreated ferrets, a majority of the original hair follicles were dormant on day 7, but hair follicle activity increased slowly over subsequent 8 observation days, due to spontaneous hair growth (Fig. 4-4a). In comparison, hair growth in ferrets treated with melatonin developed much faster. These ferrets had significantly fewer telogen follicles by days 11 (22 ± 9%) (SEM) ($P \leq 0.05$, Table 4-8) and 15 (6 ± 5%) ($P \leq 0.001$) (Fig. 4-4b), compared to 60 ± 11% and 51 ± 10% in the untreated group. A relatively constant proportion of mid-late proanagen follicles was present in both groups during the course of the experiment.

Testosterone: When testosterone (15 mg) was administered alone, it effectively inhibited spontaneous growth of original follicle. By day 15, more resting follicles (87 ± 8%) ($P \leq 0.01$) and no early proanagen follicles (0 ± 0%) ($P \leq 0.05$) were found (Fig. 4-4c) in comparison to untreated controls. Implantation of testosterone on the same day as melatonin had no effect on hair growth initiated by melatonin (Fig. 4-4d). When testosterone was implanted seven days before melatonin, there were more ($P \leq 0.001$) early proanagen follicles at day 7 (52 ± 8%), but fewer ($P \leq 0.05$) remained in this stage at day 15 (26 ± 12%), compared ferrets treated with melatonin alone (19 ± 8% and 46 ± 11% for the respective days) (Fig. 4-4e).

Oestradiol: Oestradiol (15 mg) was strongly inhibitory to hair growth in all circumstances. By day 15, all original hair follicles from the ferrets treated with oestradiol, either alone (100 ± 0%) or in combination with melatonin (100 ± 0%), were in telogen, in marked contrast to their respective controls ($P \leq 0.001$) (Fig. 4-4f, g and h).

Legends for Fig. 4-4

Left column: effects of steroids on spontaneous hair growth. a: untreated male control; c, f, i, l and o: results when each of the five steroids (testosterone, oestradiol, deoxycorticosterone, dexamethasone and progesterone) was administered on the same day (D0, time of melatonin administration in the othe other groups).

Central column: effects of steroids on hari growth when co-administered with melatonin. b: melatonin-treated male control (D0: time of melatonin and steroid administration); d, g, j, m and p: results for each of the five steroids.

Right column: effects of steroids on hair growth when administered 7 days prior to melatonin (d-7). e, h, k, n and q: results for each of the five steroids.

Yellow indicates percentages of follicles (Y axis) in proanagen IV to proanagen VI stages (PIV-PVI) at different times after melatonin administration (X axis).

Light blue indicates percentages of follicles in proanagen II to III stages (PII-PIII).

Blue indicates percentages of follicles in telogen.

TST, E2, DOC, DEX, Prog: abbreviations for testosterone, oestradiol, deoxycorticosterone, dexamethasone and progesterone, respectively.

M: males; F: females

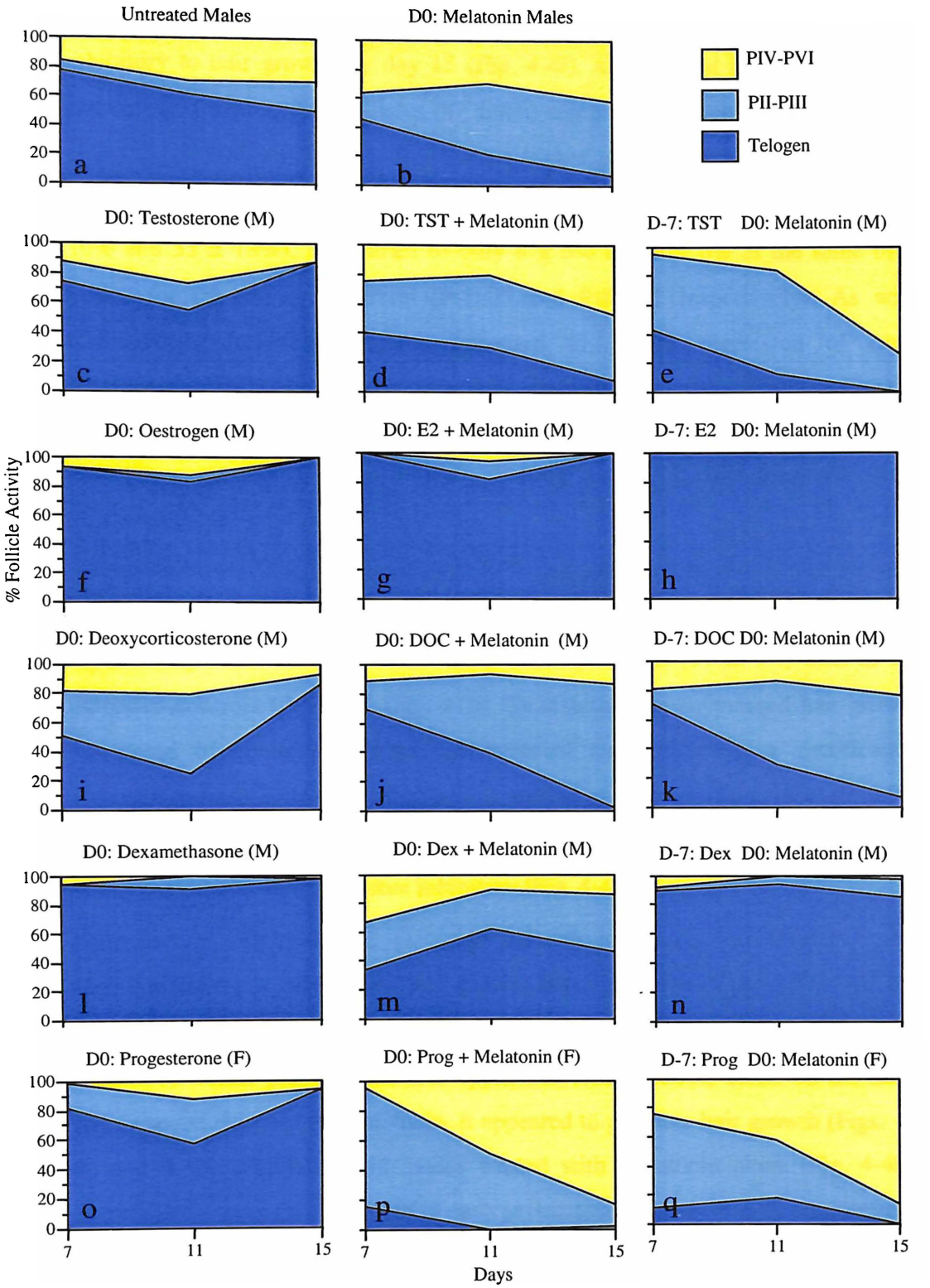


Fig. 4-4 Effects of steroids on hair growth in the 1997 trial

Mean percentages of follicles (Y axis) in mid to late proanagen (yellow), early proanagen (light blue) and telogen (blue) 7, 11 and 15 days after melatonin administration (X axis). See page 73 for legends.

Deoxycorticosterone: When deoxycorticosterone (25 mg) was given alone, it was inhibitory to hair growth by day 15 (Fig. 4-4i), as indicated by a much higher percentage of resting hair follicles (87 ± 9%) compared with the untreated control group (51 ± 10%) ($P \leq 0.001$). However, a higher percentage of follicles were in early proanagen on day 7 and 11 after deoxycorticosterone administration (31 ± 10% and 55 ± 13%), in contrast to only 8 ± 5% and 9 ± 6% at the same time points for the untreated ferrets ($P \leq 0.05$ and $P \leq 0.001$ respectively). As with testosterone, deoxycorticosterone appeared to induce regression of early proanagen follicles between day 11 and 15. Deoxycorticosterone implanted on the same day, or seven days ahead of melatonin did not affect hair growth. As in the controls, most hair follicles entered growing phase 11 days after melatonin treatment (Figs. 4-4j and k).

Dexamethasone: When dexamethasone (15 mg) was applied alone, it effectively arrested hair growth during the observation period. By day 15, there were significantly more resting follicles in the treated ferrets (97 ± 4%) than in the untreated controls ($P \leq 0.001$) (Fig. 4-4l). Dexamethasone suppressed hair growth stimulated by melatonin. When administered simultaneously, a significantly higher proportion of resting follicles was present on days 11 (62 ± 16%) ($P \leq 0.05$) and 15 (46 ± 12%) ($P \leq 0.01$) (Fig. 4-4m). Dexamethasone implanted seven days before melatonin was even more inhibitory (Fig. 4-4n).

Progesterone: Progesterone (15 mg) administered alone to female ferrets appeared to inhibit spontaneous hair growth as resting follicles peaked at 94 ± 6% by day 15 (Fig. 4-4o), compared to untreated males (51 ± 10%) (Fig. 4-4a) ($P \leq 0.001$). When progesterone was applied to female ferrets either on the same day or seven days before melatonin, it appeared to promote hair growth (Figs. 4-4p and 4-4q), compared with males treated with melatonin alone (Fig. 4-4b). Significantly more follicles were in early proanagen seven days after progesterone administration in both groups (81 ± 9% and 64 ± 10%) compared with melatonin alone (19 ± 8%) ($P \leq 0.001$). When it was given seven days before melatonin, more follicles (86 ± 14%) ($P \leq 0.01$) were found to be in mid-late proanagen on day 15 than in male melatonin controls (47 ± 11%).

Table 4-8 Significance of treatment differences on follicle growth stages in the 1997 trial

Comparison of treatments	Groups	Telogen			Early proanagen			Mid-late proanagen		
		day 7	day 11	day 15	day 7	day 11	day 15	day 7	day 11	day 15
untreated vs MLT	3 vs 4	0.113	0.047[1]	0.001	0.232	0.001	0.004	0.276	0.981	0.188
TST vs untreated	5A vs 3	0.817	0.598	0.006	0.609	0.383	0.029	0.452	0.905	0.254
(TST + MLT)=D0 vs MLT	5B vs 4	0.765	0.657	0.928	0.098	0.934	0.922	0.293	0.510	0.985
(TST=D-7) + MLT vs MLT	5C vs 4	0.086	0.614	0.612	0.001	0.056	0.023	0.007	0.349	0.057
E2 vs untreated	6A vs 3	0.232	0.222	0.000	0.636	0.690	0.029	0.235	0.195	0.042
(E2 + MLT)=D0 vs MLT	6B vs 4	0.000	0.002	0.000	0.050	0.003	0.000	0.002	0.093	0.001
(E2=D-7) + MLT vs MLT	6C vs 4	0.000	0.000	0.000	0.050	0.000	0.000	0.002	0.043	0.001
DOC vs untreated	7A vs 3	0.248	0.065	0.007	0.019	0.000	0.144	0.595	0.454	0.114
(DOC + MLT)=D0 vs MLT	7B vs 4	0.090	0.360	0.737	0.975	0.659	0.000	0.024	0.117	0.016
(DOC=D-7) + MLT vs MLT	7C vs 4	0.081	0.679	0.946	0.326	0.508	0.012	0.135	0.274	0.092
Dex vs untreated	8A vs 3	0.083	0.104	0.001	0.427	0.996	0.029	0.102	0.033	0.063
(Dex + MLT)=D0 vs MLT	8B vs 4	0.010	0.036	0.003	0.074	0.063	0.488	0.053	0.203	0.019
(Dex=D-7) + MLT vs MLT	8C vs 4	0.004	0.000	0.000	0.080	0.001	0.000	0.015	0.043	0.002
Prog vs untreated[2]	9A vs 3	0.382	0.891	0.001	0.330	0.083	0.029	0.047	0.206	0.100
(Prog + MLT)=D0 vs MLT[2]	9B vs 4	0.054	0.258	0.734	0.000	0.627	0.112	0.004	0.270	0.187
(Prog=D-7) + MLT vs MLT[2]	9C vs 4	0.026	0.872	0.612	0.000	0.393	0.001	0.291	0.357	0.007

[1] Colored entries represent significant (blue, P≤0.05) or extremely significant (red, P≤0.01) differences.

[2] Comparisons between female groups and male controls.

4.3.5.2 1998 trial

Controls: In both untreated male and female controls, hair follicles generally remained dormant throughout the experiment (Figs. 4-5a and b). Although female controls appeared to have fewer resting follicles (65 ± 15%) than untreated male controls (95 ± 6%) on day 15, the difference between the sexes was not significant ($P \geq 0.05$) (Table 4-9). The lower percentage of resting follicles was attributable to two females with more active hair growth. Melatonin clearly stimulated original follicle growth in both male and female ferrets. In females, significantly fewer telogen follicles (11 ± 11%) ($P \leq 0.01$), but more mid-late proanagen follicles (66 ± 15%) ($P \leq 0.01$) were seen 15 days after melatonin treatment, compared with untreated female controls (Figs 4-5b and d). When males and females treated with melatonin were compared (Figs. 4-5c and d), females entered the growing phase earlier than males, indicated by fewer follicles (23 ± 15%) remaining in early proanagen 15 days after melatonin implantation compared to 68 ± 16% among males ($P \leq 0.05$).

Progesterone: Progesterone (15 mg) was applied simultaneously with melatonin to both male and female ferrets. The effects on the two sexes were strikingly different. In females, while it appeared to accelerate hair growth initiation (Fig. 4-5f), compared with females treated with melatonin (Fig. 4-5d), the effect was not significant over all time points (Table 4-9). In contrast, progesterone strongly inhibited melatonin initiated hair growth in males. This was indicated by the small change in hair follicle activity (Fig. 4-5e), while hair growth in melatonin-treated male ferrets progressed rapidly (Fig. 4-5c). By day 15, significantly more telogen follicles were present in the male ferrets implanted with both progesterone and melatonin (71 ± 15%) compared with the male melatonin group (2 ± 4%) ($P \leq 0.001$).

Testosterone: Administration of 15 mg testosterone simultaneously with melatonin in males inhibited hair growth. By day 15, significantly more original follicles were in the telogen phase (44 ± 19%) compared with melatonin controls ($P \leq 0.05$) (Fig. 4-5g).

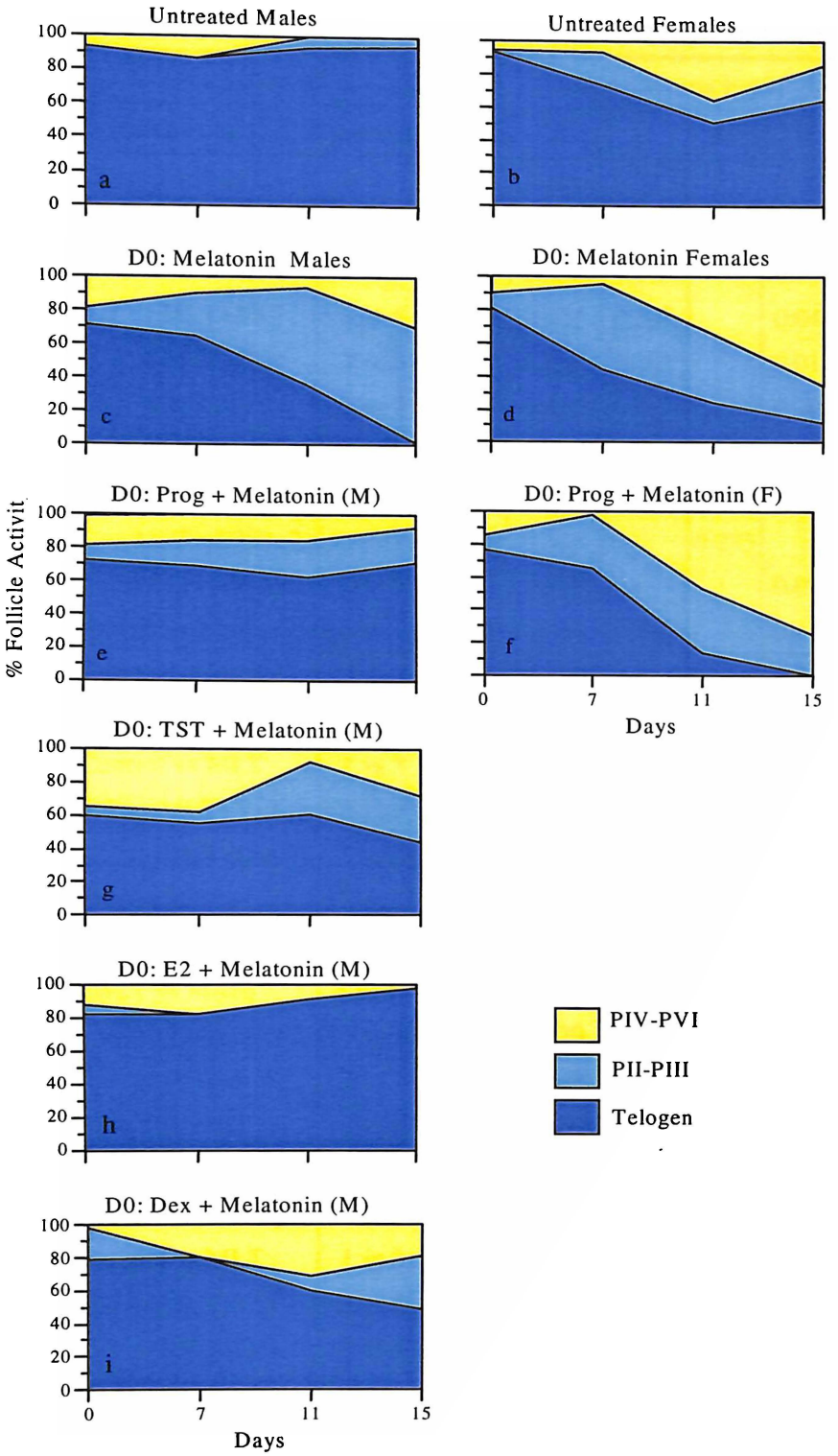


Fig. 4-5 Effects of melatonin and steroid administration on hair growth in the 1998 trial

Left column: untreated and melatonin-treated male controls and males co-administered with steroids and melatonin. Right column: untreated females or females treated with melatonin or melatonin and progesterone. Other legend information see Fig. 4-4.

Table 4-9 Significance of treatment differences on follicle growth in the 1998 trial

	Sex	Comparisons of		P value for each sampling day						
		treatments	groups	0	7	11	14	15	18	21
Telogen	F	Control vs MLT	2 vs 4	0.483	0.292	0.233		0.007		
	F	MLT vs MLT+Prog	4 vs 9	0.817	0.431	0.575		0.295		
	M	Control vs MLT	1 vs 3	0.291	0.353	0.005		0.000		
	M	MLT vs MLT+TST	3 vs 5	0.667	0.741	0.309		0.044		
	M	MLT vs MLT+Dex	3 vs 6	0.548	0.547	0.405		0.011		
	M	MLT vs MLT+E2	3 vs 7	0.636	0.489	0.007		0.000		
	M	MLT vs MLT+Prog	3 vs 8	0.960	0.885	0.275		0.000		
	M	MLT vs Mlt16	3 vs 17	0.493	0.418					
	M	MLT vs MLT+(E2 D+7)	16 vs 17	0.802	0.869		0.275		0.000	0.000
	F vs M	Control vs control	1 vs 2	0.971	0.561	0.035		0.073		
	F vs M	MLT vs MLT	3 vs 4	0.636	0.431	0.596		0.409		
PII-III	F	Control vs MLT	2 vs 4	0.336	0.134	0.137		0.932		
	F	MLT vs MLT+Prog	4 vs 9	0.907	0.377	0.889		0.938		
	M	Control vs MLT	1 vs 3	0.150	0.064	0.007		0.001		
	M	MLT vs MLT+TST	3 vs 5	0.586	0.238	0.230		0.104		
	M	MLT vs MLT+Dex.	3 vs 6	0.755	0.064	0.019		0.129		
	M	MLT vs MLT+E2	3 vs 7	0.556	0.064	0.001		0.000		
	M	MLT vs MLT+Prog	3 vs 8	0.915	0.581	0.095		0.035		
	M	MLT vs Mlt16	3 vs 17	0.150	0.064					
	M	MLT vs MLT+(E2 D+7)	16 vs 17	0.321	0.229		0.001		0.006	0.075
	F vs M	Control vs control	1 vs 2	0.571	0.138	0.626		0.297		
	F vs M	MLT vs MLT	3 vs 4	0.792	0.184	0.474		0.040		
PIV-VI	F	Control vs MLT	2 vs 4	0.631	0.879	0.941		0.007		
	F	MLT vs MLT+Prog	4 vs 9	0.776	0.680	0.516		0.653		
	M	Control vs MLT	1 vs 3	0.448	0.833	0.384		0.038		
	M	MLT vs MLT+TST	3 vs 5	0.390	0.142	0.904		0.940		
	M	MLT vs MLT+Dex	3 vs 6	0.184	0.546	0.237		0.538		
	M	MLT vs MLT+E2	3 vs 7	0.726	0.622	0.788		0.078		
	M	MLT vs MLT+Prog	3 vs 8	0.993	0.688	0.531		0.164		
	M	MLT vs Mlt16	3 vs 17	0.782	0.747					
	M	MLT vs MLT+(E2 D+7)	16 vs 17	0.470	0.443		0.278		0.094	0.010
	F vs M	Control vs control	1 vs 2	0.842	0.619	0.017		0.162		
	F vs M	MLT vs MLT	3 vs 4	0.579	0.629	0.101		0.082		

Oestradiol: Oestradiol (5 mg) given simultaneously almost completely inhibited melatonin-induced follicle growth by day 15 (97 ± 6%) (Fig. 4-5h). Given seven days after melatonin, when hair follicle growth was underway in the control group (Fig. 4-6a), a similar strong inhibition was observed (Fig. 4-6b). Significantly fewer early proanagen follicles were seen 14 days after melatonin administration (seven days post-oestradiol implantation) ($P \leq 0.001$) and more telogen follicles were present at days 18 and 21 (89 ± 10% and 95 ± 5% respectively) ($P \leq 0.001$).

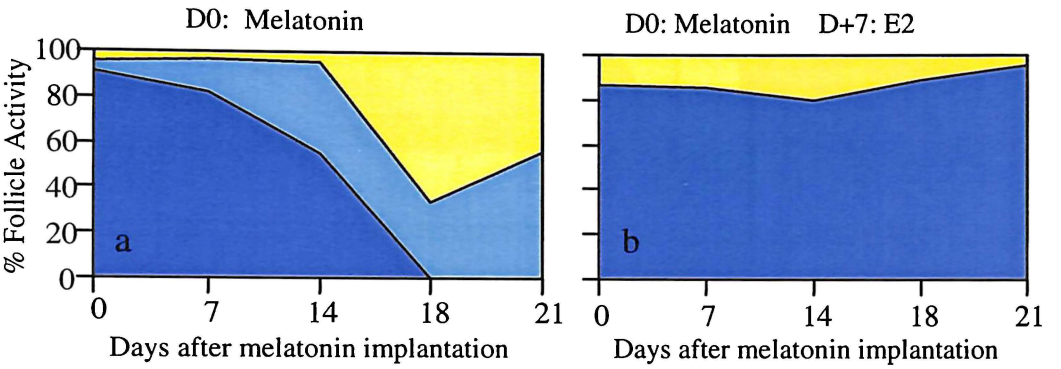


Fig. 4-6 Effects of oestradiol on hair growth when administered after melatonin

Melatonin administered on day 0 induced hair growth initiation (a) and oestradiol given 7 days after melatonin suppressed hair growth initiation (b). Legends see Fig. 4-4.

Dexamethasone: Dexamethasone (15 mg) partially inhibited follicle growth initiated by melatonin in male ferrets. Fifteen days after the treatment, the percentages of telogen follicles (48 ± 17%) were significantly higher than in the melatonin treated control group (2 ± 4%) ($P \leq 0.05$) (Fig. 4-5i).

4.3.5.3 Hair follicle growth status for skin samples collected for analysis of gene expression

All ferrets in the 1996 trial, those in Group 2 of the 1997 trial (Table 4-2) and in Groups 10-15 of the 1998 trial (Table 4-4) were sacrificed at various time points for collection of large piece of flank skin samples for differential display, Northern blot analysis and other gene expression studies (Chapters 5 and 7). The hair follicle growth status of these samples was also assessed by histology.

1996 trial: In 1996, 24 ferrets were implanted with melatonin and sacrificed over 14 days. When the experiment commenced, 26 ± 20% of the original follicles

were already in early proanagen, which indicated that spontaneous hair growth had occurred, at least in some animals. The rapid progression of hair follicle development within the first four days again suggested spontaneous hair growth in these ferrets. By day 14, all original follicles entered mid-late proanagen (Fig. 4-7a).

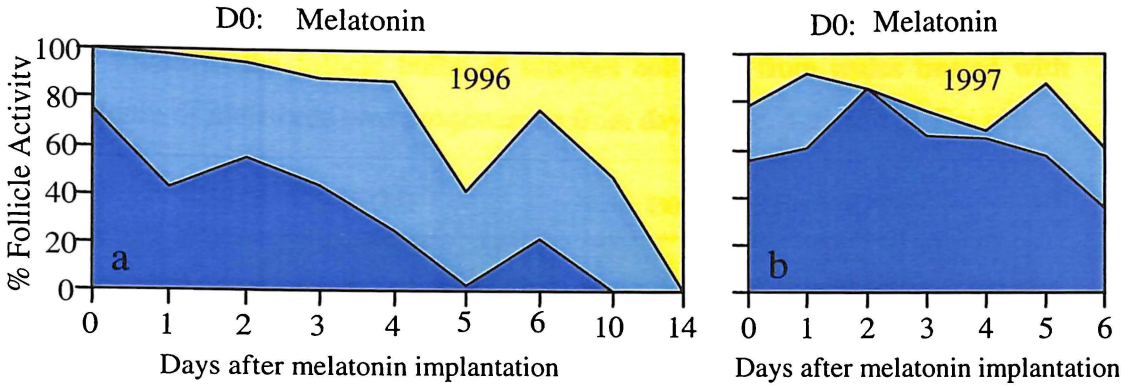


Fig. 4-7 Effects of melatonin on hair growth in the 1996 and 1997 trials

Hair growth proceeded rapidly in 1996 trial (a), but the response was slower and hair growth was less synchronised in 1997 trial (b). Y axis represents mean percentages of 2 or 3 ferrets (1998) or 2 ferrets (1997) over the observation period. Legends see Fig. 4-4.

1997 trial: When untreated ferrets were sacrificed on day 0, 56 ± 35% of the original hair follicles were in telogen. The percentages of original follicles in all three categories remained relatively stable over the five days after melatonin administration with an exception at day 2 (Fig. 4-7b). The percentage of resting follicles decreased to 35 ± 5% on day 6.

1998 trial: Hair growth initiation in males could not be identified by histology in longitudinal skin sections over the 10 days among males treated with melatonin or melatonin plus oestradiol or progesterone (Fig. 4-8a, b and c). The fluctuations in the percentage of growing follicles were likely have originated from differences in the growth status of each individual ferret, rather than associated with the treatments. In a female ferret treated with both melatonin and progesterone, all follicles had entered early proanagen on day 10 (Fig. 4-8d).

PCNA detection of hair growth initiation: In order to detect the earliest signs of hair growth, skin samples from 29 animals slaughtered in 1998 were assessed for

the presence of immunodetectable PCNA (proliferating cell nuclear antigen). Five to 14 original follicles were examined from each skin sample. Hair follicles from three of four male controls (day 0) were quiescent, with no PCNA detected in the hair bulb, dermal papilla or lower root sheath. Similarly, almost all follicles from samples taken before day 6 after the respective treatments were also negative for PCNA, confirming their resting status (Fig. 4-9). However, proliferating cells were found in the follicle bulbs of samples collected from males treated with melatonin or melatonin plus progesterone from day 6 (Fig. 4-9a and c). For male

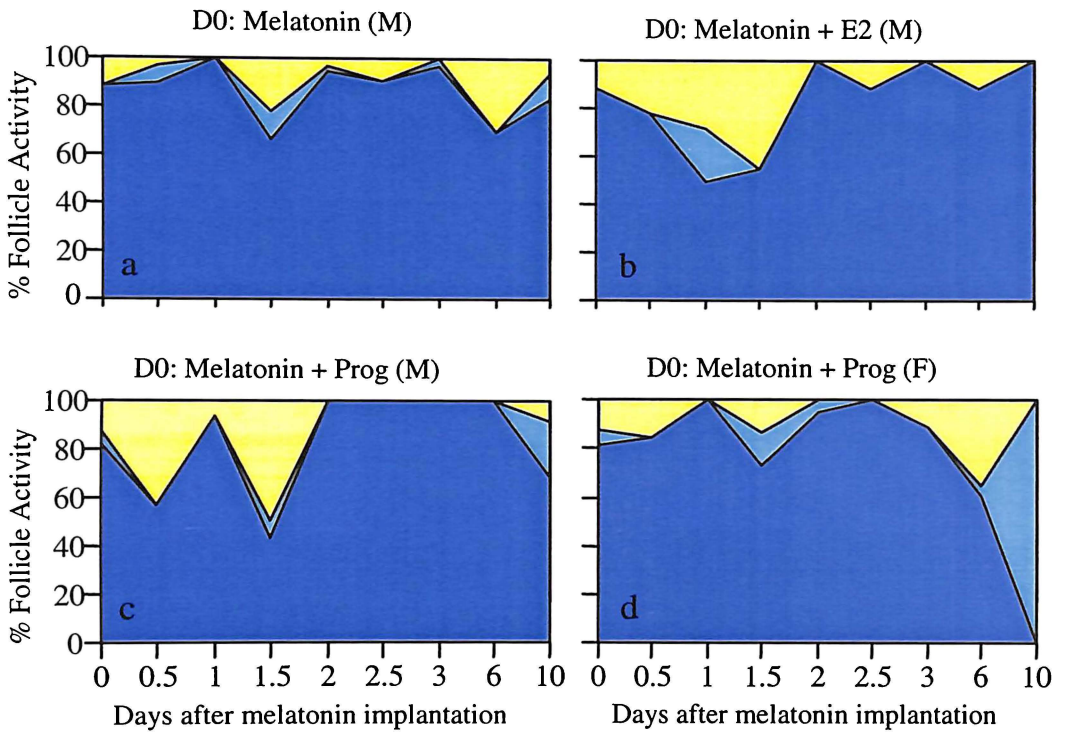


Fig. 4-8 Hair follicle activity in slaughtered ferrets in the 1998 trial

Percentages of hair follicle stages for male ferrets treated with melatonin (a) and melatonin with oestradiol (b) or progesterone (c); females administered with melatonin and progesterone (d). No hair growth initiation was observed for the three treatments in males, but all hair follicles was in early-mid proanagen 10 days after co-administration of melatonin and progesterone in females. Legends see Fig. 4.4.

ferrets treated with melatonin, approximately 8% of the follicles were in proanagen I at day 6 and this increased to nearly 61% by day 10 when follicles in proanagen II were also detected (Fig. 4-9a and Fig. 4-10), corresponding well to the previous histological results (Fig. 4-8a). Similarly, a male ferret treated with

melatonin and progesterone showed a high proportion of follicles in proanagen I and reduced number of telogen follicles on day 10 (Fig. 4-9c). In contrast, none of the samples from the ferrets treated with melatonin and oestradiol was PCNA-positive (Fig. 4-9b).

The follicles of four female controls slaughtered at day 0 were quiescent (Fig. 4-9d). Compared with males treated with melatonin only (Fig. 4-9a) or melatonin plus progesterone (Fig. 4-9c), hair growth initiation in the female ferret treated with melatonin and progesterone was more advanced (Fig. 4-9d). Proanagen I follicles comprised 30% of the total at day 6 and all follicles were in proanagen III or further advanced by day 10.

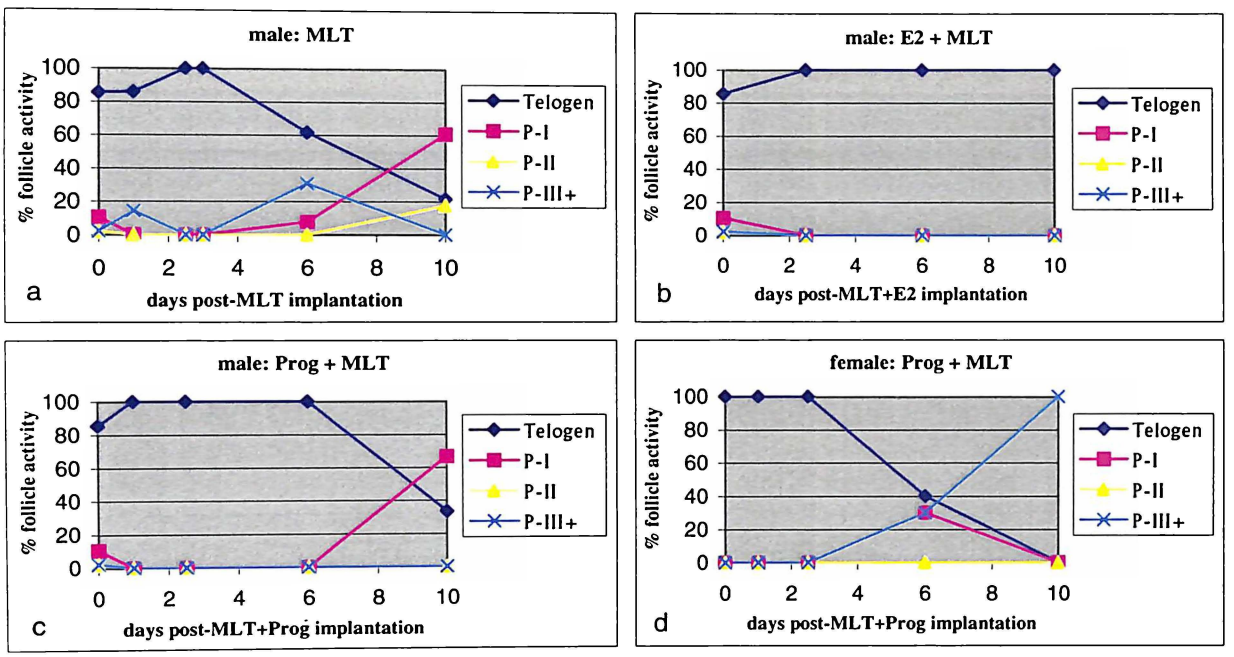


Fig. 4-9 Hair growth initiation detected by anti-PCNA immunocytochemistry

Ten days after manipulation, hair follicles were mainly in proanagen I for male ferrets treated with melatonin alone (a) and melatonin plus progesterone (c), while hair follicles were arrested in telogen after administration of melatonin and oestradiol (b). Hair follicles were all in proanagen III or advanced stages in females treated with melatonin and progesterone (d), Percentages at day 0 represent the means of four ferrets. Data for the subsequent points were derived from a single animal except for males treated with melatonin (n=2).

4.4 Discussion

4.4.1 Assessment of the melatonin-induced hair growth model

Exogenous melatonin stimulated hair growth in all four trials. However, the time required for hair follicles to enter proanagen was variable. In 1996, the majority of original hair follicles entered early proanagen four days after melatonin implantation. This was significantly earlier than previously reported. Six to 12 days were required to identify structural changes in goat hair follicles (Nixon *et al.*, 1992). In ferrets, proanagen II was the dominant stage at days 8 and 10 after melatonin administration (Nixon *et al.*, 1992; Nixon *et al.*, 1996). The faster hair growth initiation observed in this trial was likely caused by spontaneous hair growth initiation occurring before the experiment started, indicated by the presence of early proanagen follicles at day 0.

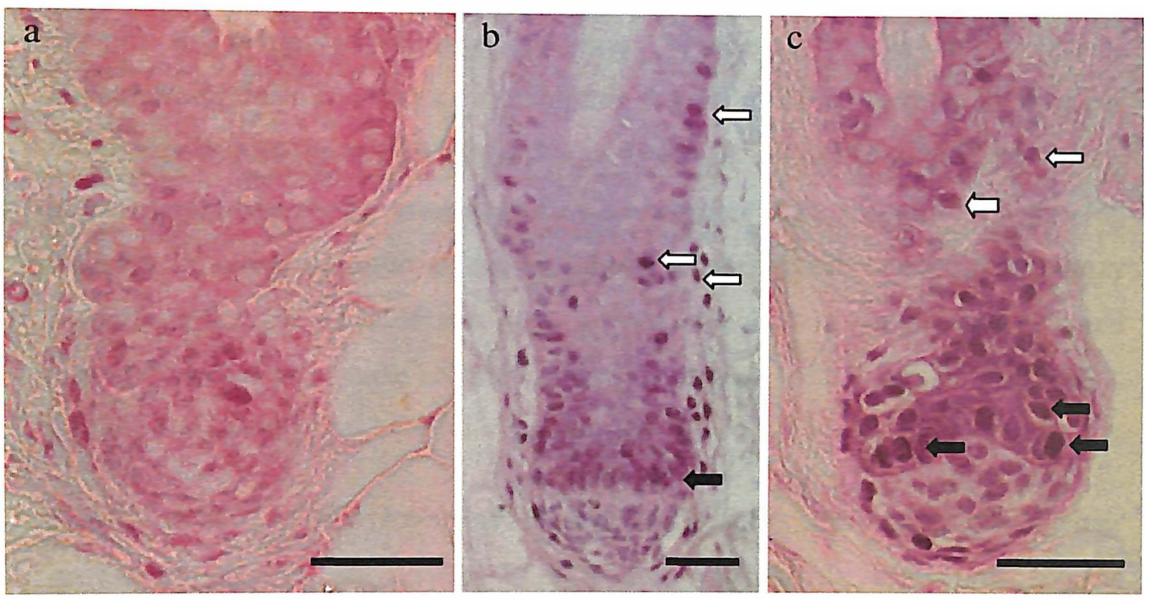


Fig. 4-10 Localisation of PCNA staining in histologically resting follicles

a: bulb end of a telogen follicle from a untreated ferret; b (proanagen I) and c (proanagen II): follicles bulbs were positively stained with anti-PCNA monoclonal antibody (PC10). Both samples were from ferrets sacrificed 10 days post-melatonin implantation. Black and white arrows point to the staining of matrix cells and outer root sheath cells, respectively. Black bars represent 50 µm. Counterstained with 2% eosin.

In the trial conducted in 1997, significant differences in the percentage of early proanagen follicles between melatonin treated and control animals were not seen until 11 days after treatment. In the slaughtered animals, no significant hair growth initiation could be seen during the six-day observation period, although

telogen follicles appeared to decrease by day six. Therefore, the hair growth progression initiated in this trial was comparable to that previously reported (Nixon *et al.*, 1992; Nixon *et al.*, 1996).

Similarly, the number of early proanagen follicles in the male ferrets used in the second trial in 1998 showed significant difference from the control group 11 days after melatonin implantation. However, the hair growth of ferrets used in the first 1998 trial was slow to commence. Apart from the female ferret treated with melatonin and progesterone, hair growth could not be clearly identified by histology during the 10 days following the treatment. However, growth initiation was confirmed by PCNA immunocytochemistry in samples collected 6 and 10 days after treatment. Again, this result was similar to that previously reported (Nixon *et al.*, 1992).

Overall, the comparatively high percentage of anagen follicles at the time of melatonin implantation was a disappointing feature of all the ferret trials. Most of the skin collected for mRNA extraction in 1997 was not useful in detecting cycle-related genes due to the existence of an undesirably high proportion of growing follicles in many animals at day 0. This less well-synchronised growth may partially reflect the more diverse genetic stock, which were not selected for rapid and uniform fur growth, in contrast to the ferrets used earlier (Nixon *et al.*, 1992; Nixon *et al.*, 1995).

For ferrets born in mid-November, it takes 12-14 weeks to complete juvenile hair growth (Nixon *et al.*, 1995). This is followed shortly afterwards by the development of the first winter pelage (Blomstedt, 1995; Nixon *et al.*, 1995). Hence the timing of melatonin administration is crucial to obtain effective and synchronised hair growth initiation as suggested previously (Pearson *et al.*, 1989). Apart from the genetic variations, a number of other factors different between these experiments are also likely to have contributed to the variable timing of the juvenile pelage completion and the response to melatonin (Table 4-6).

Long photoperiod generally halts ferret hair growth (Harvey and MacFarlane, 1958). However, at the age of 125 days, 1996 ferrets showed spontaneous winter fur growth despite of long day light treatment, suggesting that the long day light

treatment was applied too late, or the light intensity was not adequate. In contrast, ferrets between 98 and 109 days of age had not completed their juvenile pelage development under possibly a stronger long day light treatment in 1997, and earlier and even stronger light treatment in 1998. The response was the slowest when the ferrets were 98 days of age in the first trial in 1998. Ferrets in this trial were weaned much earlier and also had lower body weight. Thus, delayed juvenile hair growth completion observed in these trials may be related to a later birth date, the earlier weaning or the longer and stronger daylight treatment. As the ferrets used in 1997 trial responded to melatonin normally, despite of the presence of some growing juvenile hair follicles, the younger age and lower body weight may be implicated in the slow response of hair follicles to melatonin in the 1998 first trial.

This experience suggests that ferrets should be born as early as possible in the spring, and weaned as late as possible and that strong long day photoperiod should be applied at approximately 100 days of age when most hair follicles have already finished the juvenile pelage development. The appropriate ferret age for commencement of melatonin treatment in late February should be between 110 to 120 days.

4.4.2 Post-implantation steroid concentrations and their effects on ferret growth

The base line oestradiol concentration (14 pg/ml) measured in prepubertal ferrets in this study is comparable with the normal oestradiol levels (20 pg/ml) previously reported in the male ferret (Bernard *et al.*, 1983; Wagner and Dorn, 1994). Five or 15 mg of 17β-oestradiol in a subcutaneous pellet increased plasma concentrations to approximately 1000 pg/ml, comparable to the approximately 3 ng/ml reported previously after 15 mg of oestradiol given as an subcutaneous implant (Ryan, 1984). Oestradiol was maintained at nearly 10 times above base line during most of the observation period in both years.

Ferret testosterone levels are variable according to the physiological status of the animal. Reported values range from 700 pg/ml at 40 days of age (Erskine and Baum, 1982) and 2876 pg/ml in quiescent mature males, to 8 ng/ml in mature breeding ferrets (Rieger and Murphy, 1977). In this study, the average testosterone levels obtained for the ferrets before treatment were approximately

300 pg/ml. Administration of testosterone increased the circulating levels to 2.4 and 5.5 ng/ml in 1997 and 1998 respectively, approximately 100 times higher than baseline, but still within the physiological range in adult male ferrets.

The high maximal concentrations achieved soon after administration and the subsequent decline for both the total oestradiol and testosterone demonstrates that the hormones were not delivered uniformly over 21 days period as anticipated. This abrupt release profile is likely to have characterised all the administered steroids.

Only oestradiol and dexamethasone treatments had any effect on live weight. In 1997, when a 15 mg oestradiol implant was used, no effect on weight gain was observed. Ferrets are susceptible to prolonged, high dosages of oestrogen resulting in body weight loss and haematological disturbance (Bernard *et al.*, 1983; Hart, 1987). Although an effect on weight was not observed, six ferrets died towards the end of the experiment demonstrating that the dosage was indeed toxic. To avoid toxicity, a lower dose of 5 mg of oestradiol was administered in 1998. In this year, an increased weight gain was observed, reflecting the growth-promoting actions of oestrogenic compounds (McMartin *et al.*, 1978). The death of ferrets after the 1998 experiment indicated that the lower dose was still toxic. The reduction in live weight gain induced by dexamethasone is consistent with the catabolic actions of this steroid and losses in live weight observed in other species (Yang *et al.*, 1994).

4.4.3 Steroid effects and possible mechanisms of the actions

Testosterone: Testosterone showed different effects when administered in different circumstances. It effectively inhibited hair growth by day 15 when implanted alone, although the inhibition was not seen before day 11. This inhibitory effect of testosterone on hair growth is in line with that suggested by bilateral adrenalectomy in female mink (Rose and Sterner, 1992; Rose, 1995) and DHT administration in female mice (Chanda *et al.*, 2000). When given seven days prior to melatonin, this hormone showed modest stimulatory effect, indicated by more early proanagen follicles at day 7, but fewer at day 15. Injection of high dose of testosterone has also been reported to increased wool production (Slen and Connel, 1958).

When administered simultaneously with melatonin, testosterone had no effect in 1997, but was somewhat inhibitory in 1998. The cause of the differences observed for simultaneous administration in the two years is not known. There were important differences between the ferrets used in these two years: a later birth and a stronger artificial long day light treatment. Although a slightly higher total circulating testosterone concentration was seen in the 1998 ferrets, the results were not adequately accurate to associate it with the inhibitory effect, due to large variations between animals. Interestingly, the maximal testosterone concentration detected was close to the plasma concentration in adult men and male ferrets and that required to inhibit hair growth *in vitro* (Kondo *et al.*, 1990; Williams and Kealey, 1993).

The various effects of androgens on hair growth appear to be associated with the concentrations of androgen receptor and the activity of 5α-reductase in dermal papilla cells (Itami *et al.*, 1991a; Eicheler *et al.*, 1998). As androgen receptor binds DHT with a higher affinity, the inhibitory effect of androgens on scalp hair growth are likely to be enhanced when testosterone is converted into DHT (Keller *et al.*, 1996). Cultured dermal papilla cells have been shown to produce extracellular matrix components and mitogenic factors to regulate follicle growth in response to androgen (Randall *et al.*, 1991; Randall *et al.*, 1992a; Randall *et al.*, 1994), although the nature of the secreted factors remains unknown. The presence of aromatase and oestrogen receptors in the hair follicle, especially in the dermal papilla also allows for the possibility that androgens may undergo conversion and function through the oestradiol pathway (Schweikert *et al.*, 1974; Schweikert and Wilson, 1974; Schweikert and Wilson, 1981; Oh and Smart, 1996; Birch *et al.*, 1997).

Oestradiol: The profound suppressive effect of oestradiol on spontaneous and melatonin initiated ferret hair growth is in general agreement with results previously reported in ferrets and other species (Johnson, 1958b; Slen and Connel, 1958; Slen and Connel, 1960; Hale and Ebling, 1975; Wagner and Dorn, 1994; Oh and Smart, 1996; Chanda *et al.*, 2000). High levels of exogenous oestradiol suppressed hair growth regardless of growth stage, and the time oestradiol was applied relative to melatonin. Oestradiol caused regression of growing follicles

within 14 days, blocked spontaneous hair growth and hair growth initiated by exogenous melatonin, resulting in a lengthened telogen and a shortened anagen phase. This is in agreement with the observation in spayed rats, although different from the response reported in guinea-pigs. The subcutaneous administration of 10 mg of oestradiol to spayed rats shortened the anagen period and elongated telogen (Hale and Ebling, 1975). However, while 1 mg of oestradiol was found to reduce hair length and lengthen the resting phase, it did not affect the duration of the active phase of type II hairs in guinea-pigs over the first 48 days after birth (Jackson and Ebling, 1972). The difference in response could be due to the lower dose of oestradiol used in the guinea-pigs (1 mg) compared to higher doses in the ferret (5 or 15 mg) and the rat (10mg). The confirmation that oestradiol acts on both the growing and resting hair follicles is pertinent to both the controlling mechanisms of the hair cycle and possible clinical applications for the treatment of hirsutism.

The expression of oestrogen receptors-α in the dermal papilla has found to be hair cycle-dependent, with the highest levels of expression associated with telogen follicles (Oh and Smart, 1996; Chanda *et al.*, 2000). Application of an oestrogen receptor antagonist results in hair growth (Oh and Smart, 1996; Smart *et al.*, 1999; Chanda *et al.*, 2000). Hence, an oestrogen receptor-α pathway within the dermal papilla may be important to the induction of telogen, as well as the regulation of the telogen-anagen transition as originally proposed (Oh and Smart, 1996).

Deoxycorticosterone: DOC was found in this study to have little effect on hair growth initiated by melatonin, while it appeared to stimulate spontaneous hair growth within 11 days of treatment. However, by day 15, the hair follicles appeared to have regressed. The apparent inhibitory effect of DOC on later stages of proanagen was relatively weak, as it was counteracted by the stimulatory effect of melatonin. Previously, deoxycorticosterone has shown no effect (Mohn, 1958) or an inhibitory effect (Ralli and Graef, 1945; Baker, 1951) on hair growth induced by plucking, or associated with spontaneous replacement in either intact or adrenalectomised rats. No effect (Rose and Sterner, 1992) or stimulatory effects of DOC (Rose, 1995) in adrenalectomised mink have also been reported. DOC has also been found to delay the proliferation of the hair bud and suppress

deposition of melanin (Ralli and Graef, 1945). Therefore, the effect of DOC on hair growth, particularly its transient effects observed in ferret, merits further investigation.

Dexamethasone: Dexamethasone has been shown to inhibit both spontaneous hair growth and hair growth initiated by depilation in mice (Stenn *et al.*, 1993; Paus *et al.*, 1994a). The results obtained in the ferret studies confirmed an inhibitory action on both spontaneous and melatonin-initiated hair growth, supporting that blockage of an essential step required for hair growth has induced by dexamethasone as previously proposed (Stenn *et al.*, 1993; Paus *et al.*, 1994a). The lower level of inhibition seen after simultaneous administration of dexamethasone and melatonin, compared with dexamethasone alone or seven days earlier, indicates that the inhibitory effects of dexamethasone were attenuated by high circulating levels of exogenous melatonin.

Glucocorticoids have been found to inhibit prolactin secretion both *in vivo* and *in vitro* (Leung *et al.*, 1980; Nalda *et al.*, 1997; Spangler and Delidow, 1998). However, this effect is not responsible for hair growth inhibition of dexamethasone, as decreased prolactin level caused by melatonin in autumn is central to the stimulation of hair growth (Rougeot *et al.*, 1984; Rose *et al.*, 1987; Nixon *et al.*, 1992; Nixon *et al.*, 1995). Instead, the effects of dexamethasone on cell adhesion and cell-cell interactions, as seen in prolactin-producing pituitary tumour cells (Spangler and Delidow, 1998), might be linked to the hair growth inhibition (Chapter 8).

When dexamethasone was administered to ferrets with growing hair follicles, it induced the regression of these follicles, as also reported in mice (Paus *et al.*, 1994a) and sheep (Chapman *et al.*, 1982). While the mechanism is not understood, apoptosis is a feature of hair follicle regression (Lindner *et al.*, 1997; Matsuo *et al.*, 1998; Soma *et al.*, 1998) and dexamethasone is known to induce or prevent cell apoptosis, depending on the biological system or physiological states under investigation (Oikarinen *et al.*, 1998; Ramdas and Harmon, 1998; Ramdas *et al.*, 1999; Riccardi *et al.*, 1999).

Progesterone: No statistical difference in spontaneous winter hair growth between males and females were seen, as in a previous report (Nixon *et al.*, 1995). However, upon stimulation by exogenous melatonin, female hair growth progressed slightly more rapidly.

Progesterone was found to effectively inhibit spontaneous and melatonin-induced hair growth in male ferrets. As in the case of testosterone, the percentages of growing follicles at day 7 and day 11 were similar to those of untreated male controls, suggesting that progesterone inhibition operated in the later stages of proanagen. A stimulatory effect of progesterone on hair growth in female ferrets (Martinet *et al.*, 1983) and mink (Harvey and MacFarlane, 1958) has been suggested and daily topical application of progesterone stimulated hair growth in female mice (Chanda *et al.*, 2000). While progesterone appeared to accelerate female hair growth initiated by melatonin in the 1997 trial, no female controls were available to support this conclusion. In 1998, progesterone had no significant effect on melatonin-initiated hair growth in females when proper controls were available. Similarly, progesterone did not show inhibitory effects on hair growth initiated by plucking in female mice (Stenn *et al.*, 1993), but synthetic progesterone analogues with very low glucocorticoid activity showed marked hair inhibitory effects on hair growth in castrated mice (Houssay *et al.*, 1978). These results may suggest that the inhibitory effects of progesterone was overcome by the strong stimulatory effects of plucking or melatonin administration. Furthermore, sexual dimorphic effects of 17α-oestradiol have also been observed in mice. It does not affect hair growth in females (Oh and Smart, 1996), but blocks hair growth in males (Smart *et al.*, 1999). The existence of sex-dependent concentrations of progesterone receptors or their cofactors in skin, or different responses to high levels of exogenous progesterone, might be responsible for the sexual dimorphic effects (Smart *et al.*, 1999).

Inhibition of growing follicles was a common feature of the administered steroids. In some cases, the intervention appeared to occur at mid-late proanagen, most obviously seen with DOC, but also observed in ferrets treated with testosterone and progesterone. The presence of a high percentage of proanagen I hair follicles in a single male at day 10 after co-administration of progesterone and melatonin

was notable (Fig. 4-9c). This result contrasted strongly with the general inhibitory effect of melatonin and progesterone administration in male ferrets and supports differential effects of steroids on proanagen stages. Confirmation of this growth stage-specific effect of steroid hormones and studies to elucidate the mechanisms will aid in developing a better understanding of the biochemical pathways underlying proanagen progression.

Conclusion

Exogenous melatonin implanted in prepubertal ferrets in autumn initiated and synchronised the growth of the first winter pelage in trials over the three successive years. Ferret model was found to be difficult to apply because of unavailability of appropriate animals and some not fully understood factors. However, the animal trials have generated some novel information on steroid effects on hair growth and suitable samples for studying gene expression underlying hair follicle growth initiation.

As indicated by the plasma levels of oestradiol and testosterone, the release of steroids from the subcutaneous implants occurred relatively abruptly, though high levels were maintained for a week or more. The administration of steroids alone or steroids plus melatonin revealed that these steroids had various effects on spontaneous and melatonin-initiated hair growth. The inhibitory effects of oestradiol, testosterone and dexamethasone on spontaneous hair growth in prepubertal male ferrets observed in this study are in general agreement with results reported in other species. The effects of the steroids on melatonin-initiated hair growth have not been studied previously. The profound inhibitory effect of oestradiol on spontaneous as well as melatonin-initiated hair growth indicates that this steroid is a useful tool in deciphering hair growth control mechanisms. A similar effect of dexamethasone was found in relation to spontaneous hair growth and melatonin-induced hair growth when it was administered seven days earlier. A lesser effect after co-administration suggests an attenuation of inhibitory effect by melatonin, or that the blocking mechanism takes time to develop. Site-dependent stimulatory effects of testosterone on hair growth have been reported in males of many species. In ferrets, testosterone was inhibitory to spontaneous hair growth, inhibitory or non-effective towards melatonin-initiated hair growth when co-administered with melatonin, but

stimulatory when given seven days ahead of melatonin. The different effects of testosterone and deoxycorticosterone to spontaneous and, melatonin-initiated hair growth when co-administered, imply that their effects can be overcome by exogenous melatonin. Furthermore, the sexually dimorphic responses to progesterone on melatonin-initiated hair growth are potentially useful in understanding the developmental regulation of hair growth depending on gender.

Skin samples were taken after melatonin, or melatonin plus steroid administration for the purpose of identifying genes associated with hair growth initiation or inhibition. Samples collected from the ferrets slaughtered in 1996 trial covered all the growth stages and were well synchronised. On the other hand, samples obtained from the animals sacrificed after melatonin, or melatonin and oestradiol or progesterone in 1998 were in the very early stages of follicle growth initiation. Using these samples, the search for genes associated with hair growth is the subject of the remaining chapters in this thesis.

CHAPTER 5 OPTIMISATION OF THE DIFFERENTIAL DISPLAY PROTOCOL AND GENE EXPRESSION PATTERNS ASSOCIATED WITH HAIR GROWTH INITIATION

5.0 Abstract

An optimised differential display RT-PCR protocol was developed for ferret skin total RNA. Approximately 6% of the total expressed genes were screened using 18 primer sets. This survey provided a preliminary appreciation of gene expression patterns underlying hair follicle growth initiation or inhibition associated with melatonin and steroid administration.

When a set of RNA samples derived from skin containing telogen to anagen follicles was analysed, the expression of a large number of genes was altered. Approximately half were expressed only in flank skin, but not in footpad and liver, and hence likely to be specifically associated with hair follicle cycling. Other genes expressed in flank skin and footpad skin or liver, they were also likely to be involved in hair growth initiation, but with wider biological functions.

Most genes were up-regulated in response to melatonin, many with changes coinciding with hair follicle cell proliferation, differentiation and morphogenesis. Alterations in gene expression were also frequent prior to morphological changes in telogen follicles after melatonin treatment, and these are thus more likely to represent genes controlling hair cycle progression. The expression of many genes was affected by steroid administration, but more were down-regulated compared with melatonin administration alone. Comparison of expression patterns induced by the stimulatory and inhibitory hair growth treatments could provide leads to key candidate genes for further studies.

5.1 Introduction

The complex process of hair follicle growth initiation comprises cell proliferation, differentiation and structural reorganisation. As in hair follicle development, this process is believed to involve considerable cross-talk between cell populations in the hair follicle and surrounding integumental tissues (Hardy, 1992; Stenn *et al.*, 1996; Stenn and Paus, 1999). In mustelids, inactive follicles commence growth in autumn, leading to the formation of winter pelage with increased follicle density (Kondo and

Nishiumi, 1988; Blomstedt, 1995; Nixon *et al.*, 1995), accompanied by significant changes in the surrounding skin, (Hansen *et al.*, 1984; Valtonen and Blomstedt, 1988; Kondo *et al.*, 1996). This cycle phase transition can be stimulated and synchronised by exogenous melatonin (Valtonen and Blomstedt, 1988; Nixon *et al.*, 1992; Nixon *et al.*, 1995) via the suppression of pituitary prolactin secretion (Rose *et al.*, 1984; Rose *et al.*, 1985; Nixon *et al.*, 1993b; Nixon *et al.*, 1995). A reduction in prolactin receptor binding is thought to promote hair growth through a complicated, but poorly understood, signalling pathway (Brydon *et al.*, 1999; Witt-Enderby and Li, 2000). Steroids have also shown marked effects on hair growth (Section 2.4). In this study, the administration of melatonin and steroids to ferrets demonstrated distinct influences of these hormones on winter hair growth (Chapter 4). It was anticipated that a comparison of gene expression responses to these treatments could aid in an understanding of the molecular mechanisms underlying the control of hair growth. As the interactions between follicular cells and surrounding skin are important to the hair growth cycle (Hardy, 1992; Stenn and Paus, 1999; Widelitz and Chuong, 1999), whole skin RNA was used in the differential display analysis.

5.2 Materials and methods

5.2.1 Skin samples

Five sets of ferret skin samples were produced for identifying genes involved in hair growth initiation and inhibition (Chapter 4). The 1996 trials generated samples with hair follicles representing a complete transition from telogen to anagen after melatonin treatment. Four sets of flank skin samples were obtained in the 1998 trial from male ferrets treated with melatonin, melatonin plus oestradiol or progesterone and females treated with melatonin plus progesterone. These samples generally contained hair follicles in telogen or early proanagen. Flank skin, footpad skin and liver samples were also collected from two ferrets in mid-winter, when all hair follicles were in telogen.

5.2.2 Methods

The methods, including those for RNA extraction, differential display RT-PCR and electrophoresis are described in Chapter 3.

5.3 Results

5.3.1 Optimisation of differential display for ferret skin total RNA

5.3.1.1 Reverse transcription

The factors investigated were the treatment of the RNA sample with DNase I, the volume of the RT reaction, the amount of total RNA used, the amount of reverse transcriptase and the method of addition and RNase H treatment after RT.

DNase I treatment: Treatment with DNase I (0.3 U/µl, final concentration) made no difference to the band patterns, but often resulted in slightly heavier background in the differential display gel. However, in order to eliminate potential DNA contamination, all samples were treated with DNase I before RT.

Addition of reverse transcriptase: Conventionally, reverse transcriptase is added individually to each reaction before incubation. However, no difference could be identified in the differential display gel when the enzyme was included in the reaction mix cocktail.

Amount of Superscript II and reaction volume: The same differential display band patterns were obtained, when the full amount, or 1/4, 1/10 or 1/16 of the recommended quantity of Superscript II were used, as long as the incubation period was lengthened to one and half an hours, instead of 50 minutes recommended. However, as the bands became fainter with decreasing amounts of the enzyme (Fig. 5-1a), especially with shorter incubation times, the recommended quantity of the enzyme was normally used. When the concentrations of all the components except total RNA were kept the same, a 50 µl RT reaction with 1.5 µg RNA performed as well as a 20 µl reaction with 0.75 µg of RNA.

RNase H treatment: Treatment with RNase H (2 units) after first strand cDNA synthesis improved the results of differential display by producing denser bands, although the background was slightly heavier (Fig. 5-1a).

5.3.1.2 PCR

The annealing temperature, cycle number, the quantity of template, primer, dNTPs, the effect of TMAC (tetramethylammonium chloride) and the simultaneous use of two sets of PCR primers were tested.

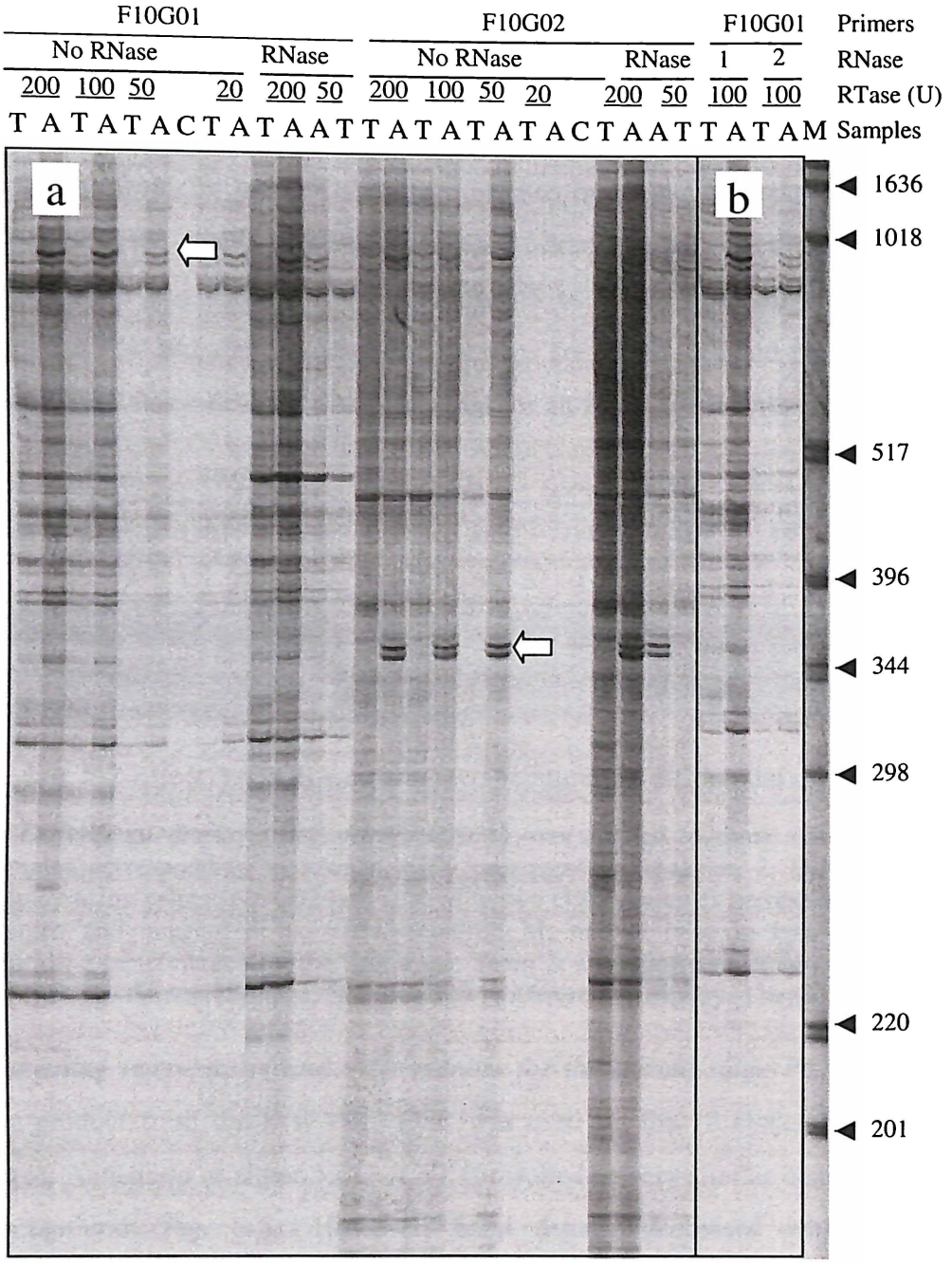


Fig. 5-1 The effects of reverse transcriptase, RNase H and primers

T: telogen sample; A: anagen sample; C: no cDNA control; M: marker; 1: one oligo dT primer + one arbitrary primer; 2: one oligo dT primer + two arbitrary primers. RTase: Superscript II reverse transcriptase; U: units; RNase: treated with or without RNase H; Solid arrows on the right point to marker sizes (bp); Hollow arrows point to differentially displayed bands. Panel a: effects of various amounts of RTase and RNase H treatment; and panel b: effects of one or two arbitrary primers used in the PCR reaction.

Annealing temperature for the first stage PCR: To determine the optimal annealing temperature for ferret skin total RNA, five cycles were conducted at 39°C, 41°C, 43°C, 45°C, 47°C and 50°C. As the annealing temperature was increased from 39 to 43°C, slightly weaker bands were produced (Fig. 5-2). However, the band density decreased dramatically when the annealing temperature was further increased from 45 to 50°C, especially for the bands between 150 and 300 bp (data not shown). To achieve a good balance of band density and resolution, 41°C was adopted for all first stage reactions.

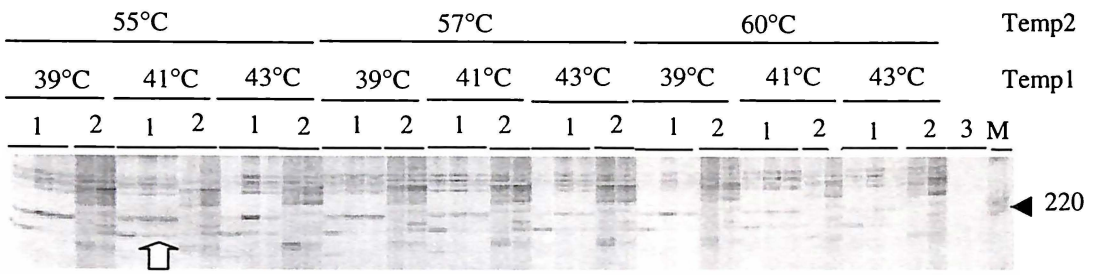


Fig. 5-2 Effects of PCR annealing temperatures on differential display

1: Differential display using primer F09F11 was applied to three skin cDNA samples corresponding to telogen, early proanagen and anagen. 2: Differential display using primer F09G02 was applied to two cDNA samples corresponding to telogen and anagen; 3: no cDNA control; M: marker (size in bp); Temp1: annealing temperatures for the first stage; Temp 2: annealing temperatures for the 18 cycles in the second stage; Hollow arrow: differentially displayed band.

Annealing temperature and cycle number for the second stage PCR: Four μl of the product from the first stage PCR was used for the 18 cycles in the second stage. Annealing at either 55°C or 57°C resulted in very similar band density and background (Fig. 5-2). However, band density decreased when annealing temperatures were further raised to 60°C or 63°C. Compared with 18 cycles, 20 cycles produced a heavier background and a reduced band contrast. When the cycle number was reduced to 14, no bands were visible. Thus, all second stage PCR reactions were conducted for 18 cycles with an annealing temperature of 55°C.

Concentrations of dNTPs: When 250, 25 and 10 μM dNTPs were compared, the results were similar, though 250 μM dNTPs produced slightly stronger bands for most reactions (data not shown). For differential display conducted with samples

from the 1996 trial, 125 μM or 200 μM dNTPs were used. All the reactions for the 1998 samples were conducted with 25 μM dNTPs.

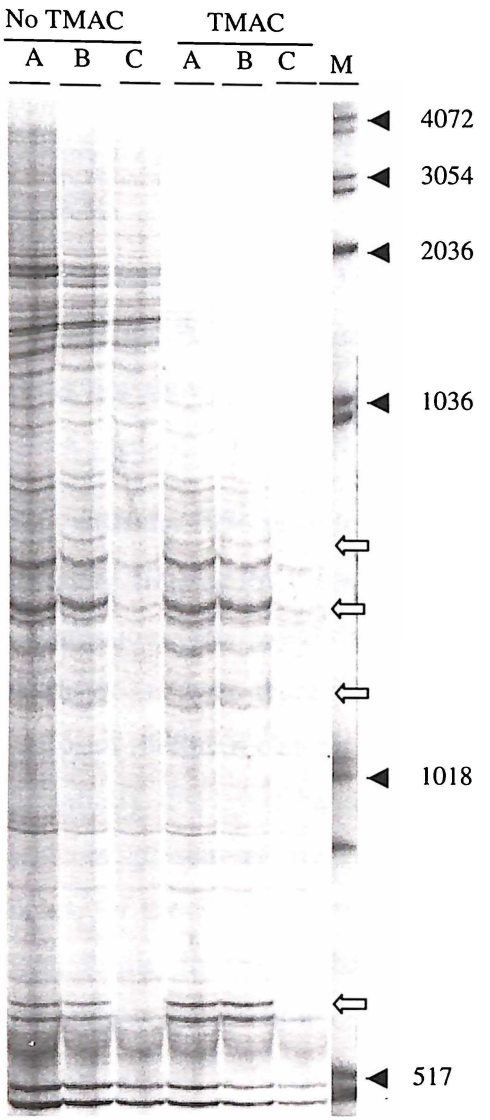


Fig. 5-3 Effects of TMAC on differential display

A, B and C: samples with anagen, proanagen II and telogen follicles; M: marker (bp); Hollow arrows point to differentially displayed bands.

Two sets of primers per reaction: Using two sets of primers simultaneously (the same oligo dT primer and two different arbitrary primers), without changing the other conditions, reduced the number of band displayed, although the main bands remained (Fig. 5-1b). Because of this reduced sensitivity, all PCR reactions were conducted with one primer set.

The effect of TMAC: A low concentration of TMAC has been shown to increase the specificity and yield of PCR products in some reactions (Chevet et al., 1995).

When 60 mM TMAC (final concentration) was added to both stages of PCR, it suppressed the reactions almost completely. When the TMAC concentration was lowered to 50 mM and used only in the later 18 cycles, a similar band pattern and density were seen in most regions compared with reactions without TMAC. However, the amplification of the DNAs around 1 kb was reduced, and those larger than 1.6 kb were totally inhibited (Fig. 5-3). Thus, no TMAC was used in differential display PCR.

The amount of cDNA template: When 0.1, 1.0 and 2.0 µl of first strand cDNAs were used as templates for PCR, comparable band densities and band patterns were generated (data not shown). However, to avoid possible selection against low abundance cDNA species, 1 µl of cDNA was used.

5.3.2 Differential display with the 1996 samples

Patterns generated using selected RNA samples: To obtain a general picture of the changes in gene expression, differential display analysis was conducted with 18 sets of primers with two or three representative cDNA samples containing hair follicles either in telogen (before melatonin treatment), proanagen II or anagen. A total of 230 differentially displayed bands (differentials) were detected. More bands were present, or present at higher density, in samples that contained growing hair follicles. Of the 55 differentials generated with three samples, 13% had the highest band density in telogen, 20% in proanagen II and 67% in anagen (Table 5-1). Some bands were present in only one or two samples. More than half of the bands were not present in telogen samples, a third were not in samples containing mainly proanagen II follicles, while approximately 10% were not in anagen samples (Table 5-1). Fourteen bands were present in only the anagen sample, 4 only in the proanagen II sample and only 1 in the telogen sample.

Patterns generated using additional skin samples: When the 18 sets of primers were used with samples covering all the main growth stages of hair follicle growth initiation (Fig. 5-4), approximately 1400 cDNA bands were screened. Of these, the density of 100 bands (6.9%) changed at least at one time-point between melatonin treatment and anagen (Table 5-2). Many of these changes were in the same direction, as the band density of the corresponding genes appeared to be further increased or decreased in the same direction. Approximately half the genes

were up-regulated continually, 14% were down-regulated throughout, while the residual differentials changed in different directions at different times.

Table 5-1 Differential display results with three representative samples

Bands	Telogen	Proanagen II	Anagen
Highest density (%)	7 (13)	11 (20)	37 (67)
No. absent from (%)	28 (51)	18 (33)	8 (15)
No. present in (%)	1 (5)	4 (21)	14 (74)

Table 5-2 General information of gene expression for follicle growth initiation

Primer sets	Mean bands/ primer set	No. of differentials	% of band changed	No. of changes	Mean changes/band
18	80	100	6.9	190	1.9

When these data were grouped according to time after melatonin treatment and hair follicle growth stage, the changes in gene expression appeared to be more common at some stages than at others. Many changes were seen in the first three days (especially the first day) after melatonin treatment, although most of the hair follicles still appeared to be in telogen. However, even more changes occurred after hair follicles entered proanagen II and beyond (Table 5-3). Overall, approximately two thirds of the changes were increases in band density. Up-regulation was particularly prominent in the first two days after melatonin treatment and when hair follicles entered into proanagen IV and V.

Table 5-3 Differential display patterns during hair growth initiation

Growth stages[1]	T	T	T	P-II	P-IIIa	P-IV	P-V & VI	Total
Days PMI[2]	1	2	3	1 & 3	3 & 6	10	5 & 14	
Up-regulation	19	7	5	16	17	26	36	126
Down-regulation	4	3	5	14	18	7	13	64
Subtotal	23	10	10	30	35	33	49	190
% up	83	70	50	53	49	79	74	66.3
% down	17	30	50	47	51	21	26	33.7

[1] Determined by Sacpic staining and microscopy. [2] Days post-melatonin implantation.

Gene expression in skin samples from autumn and winter: In mid-winter, ferret hair follicles were dormant. Flank skin RNA samples isolated from ferrets not treated with melatonin were used in some reactions to compare skin gene expression between autumn and winter, when the hair follicles were in telogen. Surprisingly, among the 61 bands compared, approximately one-third (22) differed in density. Of these, 15 bands were up-regulated in the autumn skin.

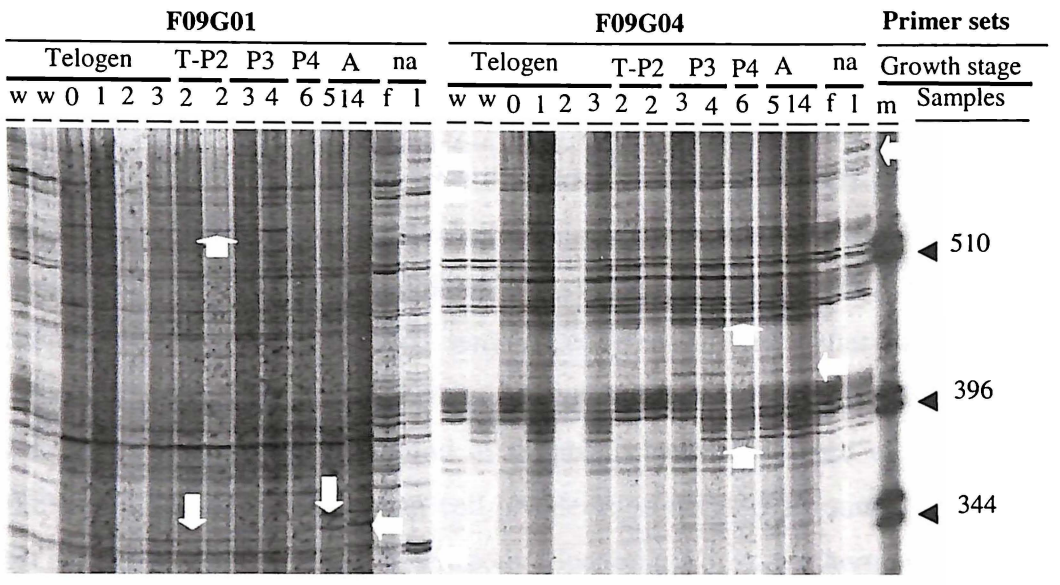


Fig. 5-4 A typical arrangement of the 1996 samples for differential display

A number of differentials (white arrows) are seen with primer sets F09G01 and F09G04. Black solid arrows on the right point to marker sizes (bp). Growth stages: telogen, telogen to proanagen II (T-P2), proanagen III (P3), proanagen IV (P4) and anagen (A). na: not applicable. w: samples from mid-winter; numbers indicate days post-melatonin treatment (PMI); f and l indicate footpad skin and liver, respectively. M: size marker.

Comparison between flank skin, footpad and liver samples: In an attempt to associate the differentials with hair follicles, RNA from either footpad skin or liver, derived from untreated ferrets in mid-winter, were included in differential display reactions for five sets of primers. Half of the 26 differentially displayed bands detected across the growth initiation stages were expressed in flank skin only (Table 5-4). The rest of the bands were also present in liver, while approximately 40% were expressed in both flank skin and footpad. Nearly a third of these (8 bands) were detected in all the three tissues.

Table 5-4 Differential display patterns: flank skin vs footpad skin and liver

	Flank only	Flank & liver	Flank & footpad	Flank, foot-pad & liver	Total
No.	13	13	10	8	26
%	50.0	50.0	38.5	30.1	100

Fourteen bands were detected in liver only, and 10 bands in footpad skin only, out of the approximately 400 bands present.

5.3.3 Differential display with the 1998 samples

Gene expression patterns post-melatonin treatment: When samples collected in the 1998 trial were analysed with the same 18 sets of primers (Fig. 5-5), the densities of 108 bands were found to change between 12 hours and 10 days after melatonin treatment, with a total of 203 changes in band density. The changes were most frequent in the first sample collected 12 hours after the treatment, which accounted for nearly 30% of the total changes (Table 5-5, Fig. 5-6 and Section 6.3.9). A second period of frequent changes occurred at 6 days after melatonin administration, when cell proliferation became prominent as detected by the anti-PCNA monoclonal antibody (Section 4.3.5.3). Furthermore, the ratios of bands that increased or decreased in band density varied at each time point. Down-regulation occurred most often at 12 hours after melatonin treatment, while many bands increased in density at 6 days after melatonin treatment, coinciding with increased cell proliferation in hair (Section 4.3.5.3).

Table 5-5 Number[1] and percentages of band density changes after melatonin treatment

Day (s) PMI	0.5 day	1 day	2.5 days	6 days	10 days	Total
Follicle stages[2]	Telogen	Telogen	Telogen	T, PI-PIII	PI, PII & T	
No. (%) down	31 (37)	11 (13)	18 (21)	9 (11)	15 (18)	84 (100)
No. (%) up	24 (20)	22 (18)	17 (14)	42 (35)	14 (12)	119 (100)
No. changes	55	33	35	51	29	203
% total change	27	16	17	25	14	100

[1] Some bands changed in density more than once during the 10 days.

[2] T: telogen; PI: proanagen I; PII: proanagen II; PIII: proanagen III.

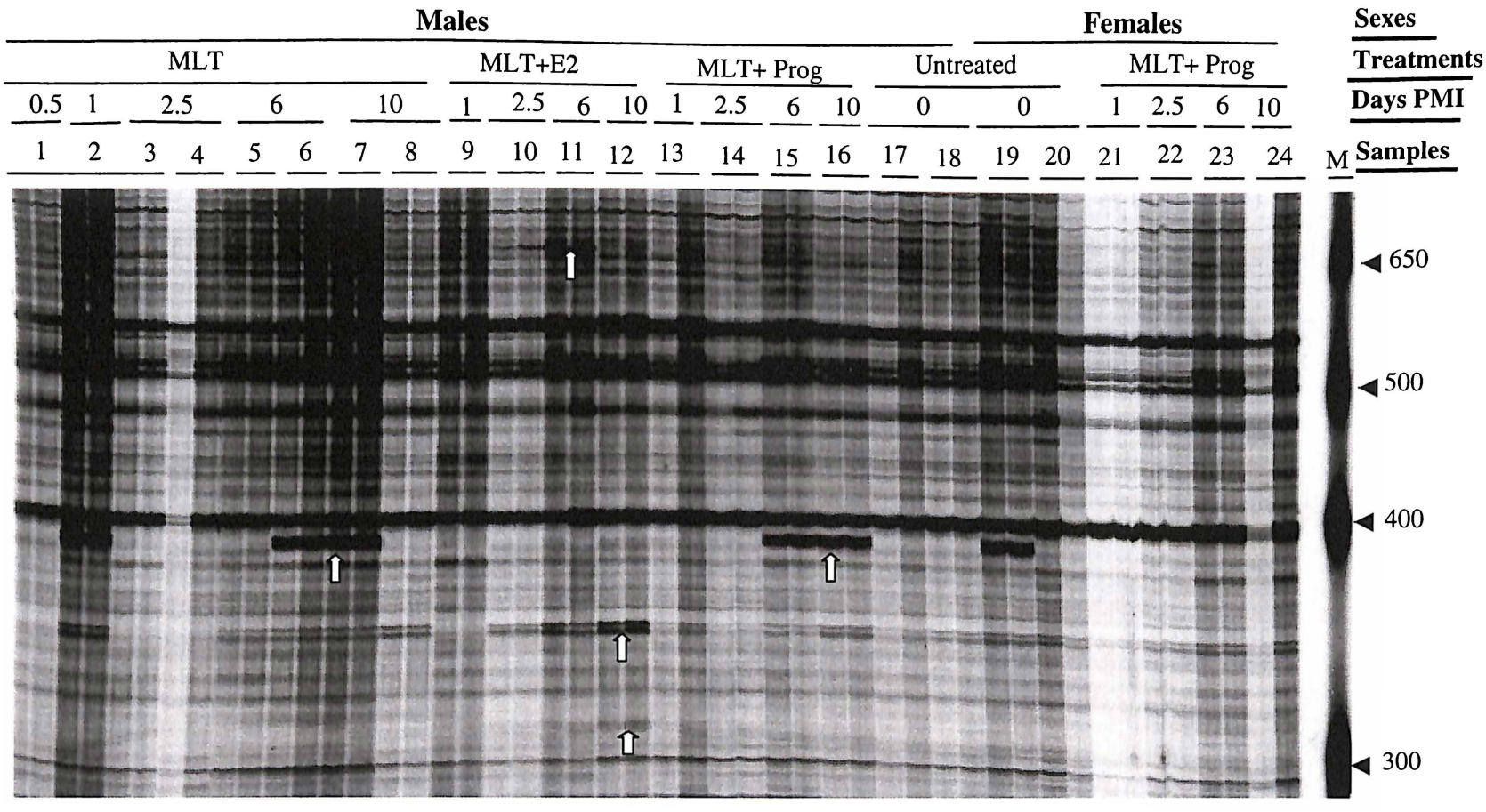


Fig. 5-5 A typical differential display gel with samples from the 1998 trials

There were two cDNA samples for each time point except days 0.5 and 1 for ferrets treated with melatonin alone. All reactions were conducted in duplicate. MLT: melatonin; E2: oestradiol; Prog: progesterone; PMI: post-melatonin implantation; Samples: each number stands for different samples; Solid arrows on the right point to marker sizes (bp); Hollow arrow: differentially displayed band. One lane for each of samples 4 and 24 and two lanes for sample 21 are of lower density, possibly resulting from pipetting errors.

Therefore, most of the changes in gene expression identified in the 1998 skin samples were likely to have arisen from genes involved in cell signalling or proliferation, rather than genes encoding the structural components of the hair follicle as was more likely in the 1996 samples (Chapter 6).

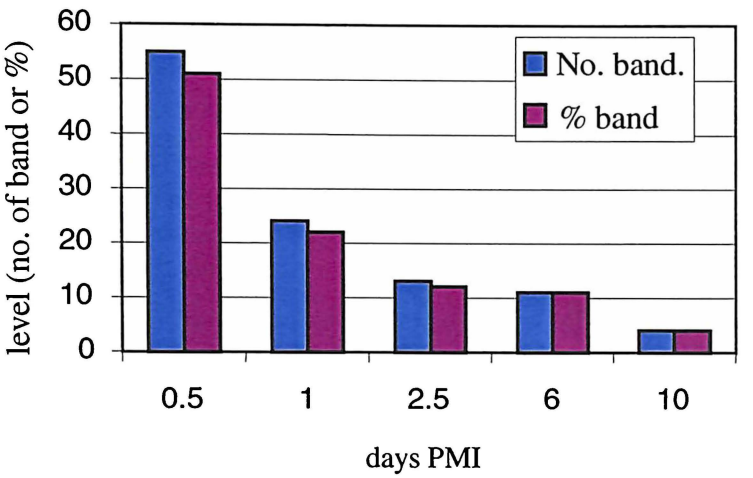


Fig. 5-6 Initial changes in band density post-melatonin implantation

No. of bands indicates the number that showed an initial difference in density at each time point; % band represents bands that changed in density as a percentage of total changes

Effects of oestradiol and progesterone on gene expression patterns: The effects of simultaneous administration of oestradiol or progesterone with melatonin on gene expression were interpreted with reference to the overall effects of melatonin implanted alone. Their effects were classified as inhibitory, stimulatory, no effect and no reaction conducted (No rex). After receiving melatonin, 44% of the differential display bands decreased in overall density, while 49% increased in density (Table 5-6).

The overall effects of oestradiol and progesterone in female ferrets were very similar. Compared with the bands derived from melatonin administration alone, 43% and 47% of the band densities were inhibited after melatonin with oestradiol or progesterone in male ferrets (Table 5-6). In addition, 20-30% of the genes altered by exogenous melatonin were not responsive to either oestradiol or progesterone, suggesting their expression was regulated by melatonin, but not further modified by these steroids. No difference in progesterone effects on gene

expression was seen between the sexes, although the percentage of genes whose expression was not influenced by progesterone was reduced.

Table 5-6 Effects of melatonin, oestradiol and progesterone on gene expression

	Inhibitory %[1]	Stimulatory %[2]	No effect %	No rex%
MLT[3]	44	49	na	na
MLT + E2[4]	43	27	30	0
MLT + Prog[4]	47	21	28	4
MLT + Prog (f)[4]	50	23	21	6

[1] percentage of bands that decreased in density, or similar band density occurred later in samples derived from the ferrets treated with melatonin plus oestradiol or melatonin plus progesterone, compared with the same band derived from ferrets treated with melatonin alone.

[2] percentage of band that increased in density, or similar band density appeared earlier in samples derived from the ferrets treated with melatonin plus oestradiol or melatonin plus progesterone, compared with the same band derived from ferrets treated with melatonin alone.

[3] increase or decrease of band density relative to day 0.

[4] band density alteration relative to melatonin pattern.

No rex: number of band could not be compared as no reaction conducted for the treatment.

f: females.

na: not applicable.

5.4 Discussion

5.4.1 Differential display optimisation

Differential display is a widely used method for the detection of altered gene expression. Various improvements have been introduced to increase its reliability and to reduce the incidence of false positives (Section 2.5.2), including longer oligo dT primers with one anchor base (Liang *et al.*, 1994) and a two-stage PCR (Ayala *et al.*, 1995; Linskens *et al.*, 1995). Some factors affecting the differential display RT-PCR were further optimised in developing the protocol used in this study.

Reverse transcription was conducted according to the manufacturer's instruction, except that reverse transcriptase was added into the reaction cocktail, instead of to each reaction tube. This change enhanced the efficiency and decreased pipetting errors. The dNTP concentration used with this protocol was 500 μM, much higher

than the 7.5 μM previously used (Linskens *et al.*, 1995). Although a reduced amount of reverse transcriptase is feasible (Rufaut, 1997), a lengthened incubation period was required. No clear advantage was found by treating with RNase H after reverse transcription, although it was maintained as a routine step.

For cDNA amplification, different temperatures for the first and second PCR stages were tested with ferret skin total RNA. Annealing at 43°C or higher temperatures for the first four cycles reduced the density of some bands, even to the point of elimination. Annealing at 57°C or higher temperatures for the second stage showed similar effects, although less pronounced. Therefore, the annealing temperature used for the second stage of amplification was 55°C, which is lower than the temperature used previously (Ayala *et al.*, 1995; Linskens *et al.*, 1995). This annealing temperature is adequately stringent. It is not only much higher than the annealing temperature of 41°C used in the original differential display protocol (Liang and Pardee, 1992), but is around the temperature required for the amplification of specific genes. Higher annealing temperatures can decrease the sensitivity of differential display, as the resultant band density and band number displayed on the gel were reduced, as seen in this study.

Increasing the number of amplification cycles from 18 to 20 in the second stage of PCR resulted in a higher background and reduced contrast of band to background, indicating the very high amplification efficiency of the protocol. A lower cycle number compared to the original protocol is also likely to be beneficial by increasing the percentage of bands still in their exponential phase by the end of the PCR amplification. However, 14 cycles in the second stage generated significantly fewer bands. With four PCR cycles annealing at 41°C and 18 cycles annealing at 55°C, the presence of between 25 to 250 μM dNTPs had little effect on the band pattern and density.

TMAC increases the stability of TA base pairing (Melchior and Von Hippel, 1973) and a low concentration of TMAC has been shown to enhance amplification yield and diminish non-specific priming (Chevet *et al.*, 1995). The optimal concentration for PCR was determined to be 60 mM. However, this concentration severely inhibited band production when used in both the first and

second stages of PCR. Addition of 50 mM TMAC only to the second stage did not affect the band pattern and density for the small and medium size bands, but reduced the density of bands around 1 kb and inhibited the synthesis of larger bands. The adverse effects of TMAC observed in this study were possibly due to the presence of multiple templates in differential display, as compared to normal PCR reactions (Chevet *et al.*, 1995).

When these various factors were taken into account, the modified differential display RT-PCR protocol developed, based on the conditions used with two-base anchor primers (Ayala *et al.*, 1995; Linskens *et al.*, 1995), proved to be stable and efficient in the presence of ferret skin total RNA (Chapters 6 and 7).

5.4.2 Estimated percentage of expressed genes screened

Using two-base anchor primers, it has been estimated that 240-300 sets of primers is required in order to display all mRNA species at the 0.90-0.95 confidence levels (Bauer *et al.*, 1993; Linskens *et al.*, 1995). For one base anchor primers, it is estimated that 240 primer sets are required to detect all mRNA at the confidence level of 0.96 (GenHunter Corporation, 1998). Considering 18 primer sets were used in this study, this would imply that approximately 7.5% of the ferret skin transcriptome was screened.

This estimate can be calculated by an alternative method using the results obtained in this study. Generally, 65 to 85 distinct bands were displayed on a denaturing acrylamide gel and could be compared. An additional 20-30 higher molecular weight bands near the top of the gel could be further separated by reloading the RT-PCR products into a reduced density gel and running for a longer distance. Assuming, for simplicity, 100 bands are displayed on each gel for each primer set, the 18 sets of primers produced 100 x 18 = 1800 bands. Although there is controversy regarding the number of genes expressed per cell, 15,000 distinct mRNAs per cell has been proposed (Albert *et al.*, 1989; Velculescu *et al.*, 1997). As skin and hair follicles contain many different types of cell, the total number of transcripts in skin will be greater (Adams *et al.*, 1995; Velculescu *et al.*, 1999). Assuming 25,000 in total, the number of cDNA displayed by the 18 sets of primers corresponds to approximately 7.2% of the total number of expressed genes. This may well be an overestimate considering that

approximately one fifth of the ESTs derived from differentially displayed bands are redundant (Chapter 6), or that the number of expressed genes in skin is larger than assumed. Therefore, perhaps only 6% or less of the ferret skin transcriptome was screened using 18 sets of primers.

5.4.3 Differential display patterns for hair growth initiation

Two sets of samples were used for studying gene expression patterns associated with ferret hair growth. The 1996 sample set spanned hair follicles over all stages of proanagen. The 1998 samples contained only the very early growth initiation stages which were likely to be largely absent from the former set (Chapter 4).

The expression of a relatively large number of genes was found to be altered during hair growth initiation. Two different experimental formats were used with the 1996 samples: a small number of selected samples and a more complete set of skin samples. When only two or three samples were screened without duplication, 230 differentials were recorded. In comparison, when more samples were arranged according to follicle growth stages, effectively acting as internal controls to each other, 100 differentially displayed bands with 190 changes in band densities were detected. This indicates that the use of a series of RNA samples containing progressively developed hair follicles excluded some false positives. When differential display reactions were conducted in duplicate as with the 1998 samples, false positives should have been further decreased. One hundred and eight differentially displayed bands were detected after melatonin administration and a total of 120 differentials were found across the four different treatments, indicating vigorous changes in gene expression after hormone administration linked to follicle responses.

In contrast to the differentials detected in the 1996 samples, the 1998 differentials were more likely to be involved in the early events of hair growth, including cell signalling, proliferation and regulation. The close association of the initial changes in band density with melatonin administration, the progressive decline with time and the subsequent sequence homology searches of the ESTs generated from these differentials (Chapters 6 and 8) also support this hypothesis. As most ESTs derived from the differentials were confirmed to be associated with hair growth and the treatment protocols (Sections 7.3.1 and 7.3.4), the total number of

skin genes whose expression is altered during proanagen is likely to be large, possibly a thousand or more. Hence, the intra- and inter-cell interactions involved in the cell signalling, proliferation, differentiation and morphogenesis of hair follicles and the control of these processes is likely be very complicated. Over 100 genes linked to hair cycling, have already been identified by other investigators, is further evidence of this complexity (Stenn *et al.*, 1994; Stenn *et al.*, 1996).

The finding that a majority of genes were up-regulated in growing follicles is in line with the knowledge that many structural genes are expressed only in growing follicles (Langbein *et al.*, 1999). The gradual increase in the band density for many genes and the large number of changes that coincide with follicle cell proliferation and structural re-organisation support this contention. As the growth process commenced, the expression of some genes were shut down or gradually decreased, suggesting these genes are expressed only or predominantly in resting follicles. Some of these may encode hair growth inhibitors (Paus, 1996; Stenn and Paus, 1999). Transiently expressed genes may represent regulatory factors for the control of specific cycle stages.

However, as the density patterns of some differentials were highly variable, these results need to be treated with caution. In many cases, differential display patterns were confirmed by Northern hybridisation, but for others the expression profiles differed (Chapter 7). Some of these observations may also reflect the suboptimal synchronisation of follicle growth (Section 4.3.5.3). The differentials for hair keratin *fHa8* were an example (Chapters 6 and 7). In the 1996 samples, bands corresponding to this transcript gradually increased in density as follicles entered late proanagen. It first appeared anomalous that only one of the two replicate 1998 samples taken at the same time point generated a prominent band. In fact, all samples that produced this band contained a percentage of anagen follicles to which the strength of the band was proportional. This further demonstrates that synchronisation of hair growth is crucial for high specificity and efficiency of differential display in identifying cycle-specific genes. On the other hand, it also indicates that differential display is sensitive in detecting the variations in hair follicle gene expression. Finally, even pipetting errors can cause variations in band densities (Fig. 5-5). Hence, rigorous technique is required.

Although the extent of variations in gene expression between autumn and winter skin samples was not anticipated, these could be attributable to season, age and, most importantly, to spontaneous hair growth which had already commenced in the autumn samples (Section 4.3.5.3).

Inclusion of footpad and liver RNA as controls in differential display proved to be informative. Half of the total number of differentials were found solely in flank skin which contained hair follicles at different growth stages. Although the corresponding genes may also be expressed in tissues other than liver and footpad skin, most are likely to represent genes specifically associated with hair growth. Shared differentials are likely to represent genes participating in hair growth and in related biological processes in other tissues.

5.4.4 Differential display patterns modified by oestradiol or progesterone

As melatonin and steroids are directly or indirectly involved in many physiological processes (Chapter 2), it was not surprising that many of the genes which responded to melatonin were also modified by oestradiol and progesterone. The interactions were complicated, as steroid effects could be stimulatory, inhibitory or absent. Although the molecular interactions of melatonin and steroids are not the focus of this study, the inclusion of samples from ferrets treated solely with steroids would enable the identification of specific steroid-responsive genes.

The samples collected in 1998 were derived from four treatments, two of which (melatonin in males and melatonin and progesterone in females) stimulated hair growth, while the other two (oestradiol and progesterone in males) inhibited hair growth. Genes for which expression was regulated in the same direction in treatments giving the same effects, but differently for the treatments producing opposite effects, could be important candidate genes for hair growth regulation (Section 8-10).

5.5 Conclusion

The differential display protocol used in this study combined the advantages of a longer, one base-anchored oligo dT primer and two-stage PCR. It was optimised for ferret skin total mRNA to give repeatable results.

With 18 sets of primers, an estimated 6% of the transcriptome in skin was screened. The inclusion of non-hair follicle samples as a control for the 1996 samples suggested that not more than half of the total differentially displayed bands were likely to be associated with hair growth cycle. The 1996 skin samples appeared particularly suitable for identification of genes associated with hair follicle structural formation. In contrast, the 1998 samples, most of which were derived from early proanagen without morphological changes, were more likely to reveal genes linked to early proanagen events. The large number of differentials detected in this study suggests that the expression of many genes is altered during hair follicle growth initiated by melatonin and influenced by steroids. These transcripts are likely to include the primary target genes of melatonin, oestradiol and progesterone, as well as the subsequent gene expression cascades initiated by these hormones.

The gene expression events following simultaneous administration of melatonin and steroids are likely to be complicated. Overall, more genes appeared to be down-regulated by the steroids, in contrast to up-regulation by melatonin. Although confirmation of the differential display expression patterns detected is required by using other methods (Chapter 7), differentials were identified for further studies. The determination of the sequence identities of these differentials was the next step and is described in the following chapter.

CHAPTER 6 THE CLONING, IDENTIFICATION AND BIOINFORMATIC ANALYSIS OF DIFFERENTIALLY DISPLAYED SEQUENCES

6.0 Abstract

Differentially displayed cDNA bands derived from ferret skin were amplified and cloned. One hundred and thirty-three of 146 isolated differentials were successfully reamplified. From these, 321 clones were generated. At least one clone derived from each differential was sequenced and almost all were bounded by the primer sets used. Sequences derived from clones generated from the same differential were frequently, but not always, identical. The size of the derived sequences ranged from 154 to 1167 bp, with the majority between 200 and 500 bp.

When the redundant sequences were excluded, 112 different ferret ESTs were identified. Most of these were associated with the early stages of hair follicle growth in flank skin prior to morphological changes. Forty-two were identified by nucleotide sequence homology (BLASTn, expectancy value ≤e-10). Of these, 19 were also identified by amino acid sequence homology (BLASTx, expectancy value ≤e-5), indicating a significant portion of the sequences were located within the mRNA coding region. Of those not identified in the GenBank non-redundant database, over a third showed homology to entries in the DBest database, suggesting that they were transcripts for novel genes.

Approximately half of the identified ferret ESTs were derived from the 3' ends of their respective mRNAs, judging by sequence alignments, the presence of a poly-adenylation signal and a poly-adenylation site. Binding of anchor primers to adenine-rich internal sequences, rather than 3' ends, had occurred to generate the remaining identified transcripts. This phenomenon frequently resulted in isolation of more ESTs derived from the coding region, but sometimes caused redundancy. However, as cDNAs larger than 1kb are not displayed efficiently on screening gels, the resultant shorter fragments, due to internal binding of anchor primers, allowed the detection and cloning of more differentially expressed transcripts, especially as a limited number of arbitrary primers were used. However, redundant sequences arising from a variety of causes comprised approximately a quarter of the 150 sequences generated, reducing the efficiency of differential display in screening the target transcriptome.

6.1 Introduction

The differential display patterns observed during hair growth initiation gave a preliminary indication of the overall genetic response to endocrine signals controlling hair growth (Chapter 5). However, the EST sequences and the identities of the genes from which they arise are required in order to develop an understanding of the biochemical systems involved. For transcripts which are associated with known genes by virtue of sequence comparisons, their functions in hair follicles and skin can sometimes be inferred from knowledge of their roles in other tissues. Cloning of the differentials also enables detailed investigation of expression profiles across the hair growth cycle and localisation of expression sites. Bioinformatic analysis of the generated sequences also assist in understanding some aspects of the results. The determination of the location within the mRNA from which the differential is derived and the degree and causes of EST redundancy enable an assessment of differential display as a method for gene discovery.

6.2 Material and methods

6.2.1 Cloning and sequencing

After a differentially displayed band (differential) was identified, it was isolated from the acrylamide gel and the cDNA was extracted. It was then reamplified by PCR using the same set of primers from which it was derived. The reamplified DNA was cloned and sequenced (Section 3.11).

6.2.2 Bioinformatic analyses

Multiple sequences from the same differential, together with the vector flanking sequences and primer sequences, were aligned using DNA Star (DNA Star Inc., Madison, WI, USA). The 150 EST consensus sequences were retrieved, and corrected for ambiguous bases by reference to the sequence electrophenograms. Text sequence files were established for subsequent bioinformatic analyses by removing approximately 10 bases from each end of the ESTs to eliminate of primer sequence and regions of lower fidelity. These were uploaded into an internet-based bioinformatics analysis programme (Bionavigator, eBioinformatics Inc., Sunnyvale, CA, USA) (WWW.bionavigator.com) and transformed to sense sequences by reverse complementation, if necessary. Redundant sequences among

the 150 ESTs were detected using the OVERLAP programme within BioNavigator.

The consensus sequences were used to search the non-redundant GenBank database for DNA and protein sequence homologies (BLASTn and BLASTx). The unidentified ferret ESTs were subjected to further BLASTn searches against the GenBank EST database, DBest. Given the cross-species comparisons involved and theoretically that the gene fragments were derived from the 3' end of the mRNA, a threshold expectancy (E) value of e-10 was adopted for BLASTn and e-5 for BLASTx searches.

Using the results from the BLASTn searches, the locations of the ESTs within their homologues were identified using the Clustal W multiple alignment programme in Bionavigator. The 3' location of the query sequences was further confirmed by searches for the upstream polyadenylation signal AAUAAA or the most common variant sequence AUUAAA (Wahle and Keller, 1992; Chen *et al.*, 1995). A 3' end location for an EST was assigned when the polyadenylation signal was within 50 bases of the terminal poly-adenine sequence.

6.3 Results

6.3.1 Reamplification of isolated differentials

1996 differentials: Seventy-five isolated differentials were extracted from acrylamide gels. Of these, 56 were amplified successfully with the original primer sets, using 40 cycles of PCR with annealing at 55°C. A singular band, or a dominant band of the expected size, was obtained for most differentials, estimating from cDNA bands on agarose gel. A repeated reamplification was carried out for almost half of these differentials, generating identical or very similar results.

1998 differentials: DNA was eluted from 119 differential display bands from the 1998 skin samples. DNA fragments of the expected sizes were reamplified for 109 bands with 35 PCR cycles. A majority of the reactions (63%) generated just one band or a dominant band of the expected size. These cDNA bands were used for direct cloning. Thirty-four reactions (30%) generated multiple bands, including one of the expected sizes. The targeted bands were gel-purified before

cloning. Nine differentials produced bands which were smaller than expected and one did not generate any DNA, even after repeated attempts.

Table 6-1 Reamplification and cloning of differentials derived from the1998 samples

Poly T Primer Set	ABR Primer	No. of Bands Eluted	No. of Bands Reamplified	No. of Bands Cloned	Total No. of Clones
F08	G01	8	8	8	35
	G02	8	8	8	18
	G03	9	9	9	22
	G04	3	3	3	8
	F11	8	6	8	13
	F12	8	8	8	20
Subtotal		44	42	44	116
F09	G01	5	5	4	6
	G02	7	7	6	17
	G03	6	6	6	16
	G04	11	11	11	30
	F11	8	7	5	16
	F12	6	6	6	14
Subtotal		43	42	38	99
F10	G01	2	2	1	3
	G02	6	6	5	10
	G03	5	5	5	13
	G04	6	6	5	11
	F11	10	10	10	20
	F12	4	4	4	12
Subtotal		33	33	30	69
Total		120	119[1]	110	283

[1] One sample was lost.

6.3.2 Cloning

1996 differentials: Twenty-three differentials out of 26 attempted were cloned into a pGEM-T vector as indicated by the presence of inserts of the expected size after excision with appropriate restriction enzymes. A total of 38 clones were obtained.

1998 differentials: Of 119 differentials amplified successfully, 110 (92%) were cloned into pGEM T vector, with a total of 283 clones being produced (Table 6-1). Seventy-one of the differentially displayed bands were cloned without gel-purification, while 39 were cloned after gel-purification to select the band of expected size for ligation. Of these 110 bands, 86 (78%) generated inserts of approximately the same sizes as estimated from the acrylamide gels, while eight (7%) produced inserts of the same sizes as those generated by reamplification, but different from the sizes of the original differentials. Eight (7%) were of unexpected sizes, either smaller (6) or larger (2). Nine others (8%) were unable to be cloned.

6.3.3 Sequencing and size distribution of the cloned differentials

At least one clone from each isolated differentially displayed band was sequenced, generating a total of 187 sequences. Of these, 172 sequences were of good quality (92%), while 15 (8%) were readable, but of poor quality. Sequences of improved fidelity were usually obtained from these clones after resequencing with a higher concentration of plasmid DNA or using a different clone from the same differential. Two clones from the 1996 differentials did not produce readable sequence. The sizes of the cloned differentials ranged from 154 bp to 1167 bp, with the majority between 200-500 bp (Fig. 6-1).

6.3.4 Multiple sequences alignment

The sequences derived from the same differential were aligned to each other and against vector flanking sequences and the appropriate primers. For 33 differentials, which had two or three sequenced clones, 21 comprised multiple copies of the same sequence. Clones from each of the remaining 12 differentials contained distinct sequences, indicating that the same differentially displayed band was comprised of more than one cDNA species or, alternatively, that some contamination had occurred during isolation of the differentials. Most of the sequences from the same differentials differed only slightly in size (usually 0-4 bp). However, clones from three differentials were more variable in length. Three clones derived from one differential contained inserts of more than 900 bp, two of which were identical and a third which varied by 24 base pairs in length. When distinct ESTs from the same differentials were counted separately, the number of

sequences added up to 150, which includes redundant ESTs from different primer sets.

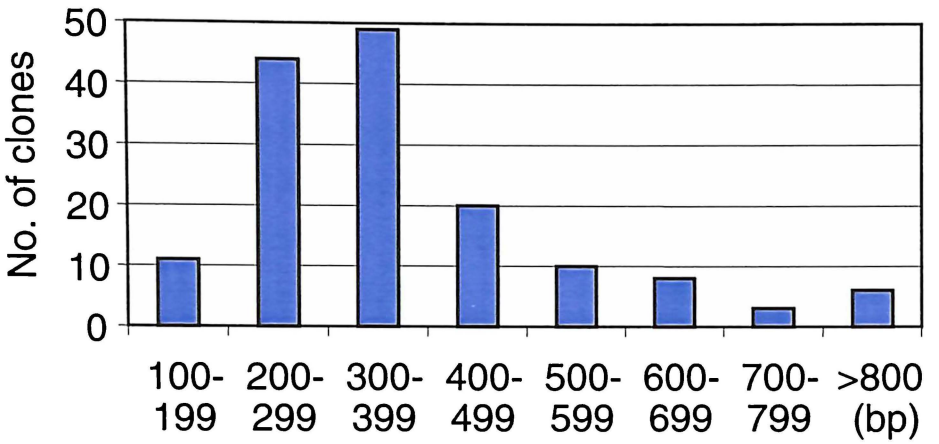


Fig. 6-1 Size distribution of 150 cloned ESTs

By aligning all the sequences with their corresponding primers, it was found that 174 sequences out of 187 (93%) were defined by the primer sets. However, four fragments were amplified solely by the oligo dT anchor primers and one by the same arbitrary primer. Another eight were likely to have been derived from the primer sets used, but the binding site for only one primer could be clearly located, due to the low fidelity sequences at the vector-insert junctions.

6.3.5 EST redundancy

When the 150 ESTs were subjected to OVERLAP to identify homologous sequences, some derived from independent differentials arose from the same mRNA, although sometimes these were different in length (Tables 6-2 and 6-8). Thirty-eight of the 150 ESTs were redundant. However, as five of these were identified in both the 1996 and 1998 skin samples using the same primer sets, the actual redundant ESTs totalled 33, comprising 22% of all ESTs. In most cases, the ESTs were repeated only once (Table 6-2). While a redundancy of four times or more was rare, it did occur when there were multiple adenine-rich segments in the templates. Sequences of different lengths derived from the same primer set usually had the same 5' sequence, but varied in length at the 3' ends, caused by binding of the anchor primer at different sites on the template. Such an example was 98F08G01-228-1.2 and its homologues (Table 6-8, No. 40). These 10

sequences were of four different lengths caused by annealing of the anchor primers to different adenine-rich locations (a minimum of 10 adenines out of 13 nucleotides). The sequence with five copies (Table 6-8, No. 16) had a mRNA template (ferret hair acidic keratin Ha8, Chapter 7) with a stretch of adenines 659 bases upstream to the 3' poly A tail (Appendix IA). For an EST (stearoyl-coA desaturase, SCD) with three copies, two (98F08G04-1002-2 and 98F10G04-1200-1) (Table 6-8, No. 68) were over 1 kb which was almost at the limit of differential display detection. These two sequences were derived from the 3' end of the mRNA. Another sequence aligned to the same transcript was less than half the size, truncated by the binding of the same anchor primer to an internal cDNA site.

Table 6-2 Redundancy of the ESTs

Copies	Two	Three	Five	Ten	Total
No. of sets	19	3	1	1	24
No. ESTs redundant	19	6	4	9	38

6.3.6 Results of sequence homology search

The 112 unique sequence tags obtained from the two years were subjected to BLASTn and BLASTx searches for nucleotide and amino acid homologies in the non-redundant GenBank database on the 16 and 21 September, 2000, respectively.

Table 6-3 Number and percentages of sequences identified by BLASTn

No. (%) identified	No. (%) not identified	Total
42 (37.5)	70 (62.5)	112

Table 6-4 The ESTs identified in GenBank by BLAST searches

Identity by BLASTn	BLASTn E value	BLASTx E value	Clones[1]
ATP citrate lyase (ACL)	1e-10	-	98F09G03-780-4
Basic transcription element binding protein 1 (BTEB1)	4e-46	-	98F09G03-243-2 98F10G03-238-1
CBF1-interacting corepressor (CIR)	6e-85	e-30	98F10F11-220-1.2
c-Myc single stranded DNA binding protein 1/2 (MSSP1/2)	2e-91	-	98F10F12-214-1

Collagen α2 type (I) (Colgn α2I)	3e-62	-	98F08G01-620-2
Cyclin D-interacting Myb-like protein (DMP1)	8e-59	3e-21	96F08F12-171-1
Cysteine-rich intestinal protein (CRIP)	e-43/2e-32	e-24	98F09G02-323-3
Cytokeratin type II (K5) (keratin K5)	3e-18	-	96F10F12-180-2
Decorin	0	e-117	98F08F11-673-1.8
Ezrin	3e-93	2e-53	98F10F11-278-6
Fibulin1(isoform D) (Fibulin 1D)	3e-12	-	98F08F11-488-p1
Glyceraldehyde-3-phosphate dehydrogenase (GAPDH)	e-158	7e-55	98F08F12-545-1.8 98F09F12-358-1
Glutamine synthetase (GS)	9e-18	-	98F09G04-370-3
Immunoglobulin superfamily 4 (IGSF4)	e-89	-	98F08G03-300-6 98F08G03-302-5.6
Guanylate kinase associated kinesin (GAKIN)	e-45	2e-15	98F08G03-160-2
Latexin or carboxypeptidase inhibitor (CPI)	e-143	2e-94	98F08F12-705-2.3
Leukocyte common antigen related protein (LAR)	e-101	-	98F09G04-301-1
Mannosyl-glycoprotein acetylglucosaminyltransferase (GnTI)	e-17	-	96F09G02-520-2
MAP/ERK kinase kinase 1(MEKK1)	8e-14	-	98F08G02-273-1
Matrix G1a protein (MGP)	e-28	-	98F08F11-227-1 98F09F11-880-4 98F10F11-355-1
Mitochondrial hinge protein (MHP)	2e-45	2e-15	98F08G02-341-2 98F09G02-344-3
Myosin heavy chain IIa (MyHC IIa)	0	3e-82	98F09F11-740-1
Pombe CDC5-related protein (PCDC5RP)	0	-	98F08G03-570-2
Proteasome subunit (HSPC)	e-51	2e-22	98F08G02-828-2 98F10G02-395-1
Ribosomal protein L11 (RPL11)	e-164	3e-50	98F08G03-480-2
Ribosomal protein L14 (RPL14)	e-128	4e-50	98F09G03-630-2
Ribosomal protein L4 (RPL4)	3e-34	-	96F10G02-205-1 98F08G02-229-1.2
Ribosomal protein L7 (RP L7)	e-128	3e-59	96F08F12-405-1 98F08F12-768-3

Signal recognition particle-72 (SRP72)	2e-95	-	98F08G04-390-7
Sox 9	2e-40	-	96F10G01-144
Stearoyl-coA desaturase (SCD)	4e-45	-	98F10G04-476-1 98F08G04-1002-1 98F10G04-1200-1
Sterol regulatory element binding protein 1 (SREBP1)	7e-26	-	98F08F11-198-1
SUMO-1-activating enzyme E1 C subunit (SAE1C)	2e-43	-	98F09G04-350-5
TATA box binding protein (TBP)	0	e-81	98F10G03-900-3
Ten integrin EGF-like repeat domains protein (TIED)	4e-83	2e-29	96F10G03-180-2.3.698F09G02-528-1.2
TI1	6e-34	-	98F10F12-255-1
Titin	0	e-157	96F10F11-284-1 98F09F11-880-8.14
Transmembrane protein 9 superfamily member 3 (TM9SF3)	3e-80	-	98F09G03-243-3
Tunicamycin-responsive protein (RTP) /N-myc-dependent regulation gene 1 (Ndr1)/ differentiation regulated gene 1 (Drg1)/TDD5	4e-28/ 2e-16	-	98F09F11-248-2
Ubiquitin-like protein 3 (UBL3)	2e-75	-	98F09G04-225-2 98F09G04-227-1
Xenobiotic/medium-chain fatty acid CoA ligase form XL-III (CoA ligase)	2e-28	6e-15	98F09G02-377-3

[1] The first two digits indicate the sample set from which the EST was derived. This is followed by the primer set used, then the approximate size of the differential. The last digit, or digits separated by period(s), indicates the clone number(s).

When the 112 unique ESTs were subjected to homology searches, 42 unique ESTs (including two different *titin* ESTs) (37.5%) were identified by BLASTn searches (Tables 6-3 and 6-4). Among these, BLASTx searches confirmed the identities of 19 sequences (45.2%), based on predicted amino acid sequences.

A few other clones showed more limited homology to specific genes with BLASTn E values >e-10. These include *epithelial V-like antigen precursor (EVA)* (6e-7, 98F08G01-480-2, Table 6-8, No. 47), *lysozyme* (6e-5, 98F09G02-228-5, Table 6-8, No. 89), *hair acidic keratin 8 (Ha8)* (3e-5, 96F10G02-295-1.2 Table 6-8, No. 16) and *differentiation gene 2 (DIF2)* (e-4, 96F10G02-380-3,

Table 6-8, No. 20). In this context, sequence tags from 5 distinct differentials contained the same piece of cDNA weakly homologous to the human hair acidic keratin Ha8. With extended sequence, the identity of these ESTs was subsequently confirmed to be the ferret Ha8 orthologue (fHa8) (Chapter 7).

6.3.7 The alignment of the ESTs with mRNA

When the 149 ESTs derived from one of the three anchor primers were examined, all had a poly-adenine site and only 4 (2.7%) did not match with the anchor base (Table 6-5). This demonstrated that the anchor base performed as anticipated and the oligo dT also annealed to a stretch of adenines. However, only approximately half of the ESTs generated were derived from the 3' end of a mRNA, indicated by the presence of both a polyadenylation signal and a poly-adenine site (Tables 6-6 and 6-8). The other ESTs were derived from internal adenine-rich sequences. Examples were ESTs 96F08F12-171-1(Table 6-8, No. 1) 98F08G03-570-2 (Table 6-8, No. 66) and 98F10F11-220-1.2 (Table 6-8, No. 119), which all showed sequence homology to the coding sequence at or close to the 5' end.

Table 6-5 The use of the anchor base in the oligo dT anchor primer

Anchor primer	F08	F09	F10
Anchor base	C	G	A
No. (%) with correct base	53 (96.4%)	44 (95.6%)	47 (100%)
No. (%) with incorrect base	2 (3.6%)	2 (4.4%)	0 (0%)

The alignments of the 43 identified gene tags (including *fHa8* and two different *titin* fragments) to their homologues showed that 25 (59%) of these gene tags were aligned to the 3' end (Table 6-8). In only two cases (Table 6-8, Nos. 61 and 64) the locations of the EST alignment was to the 3' end of their homologues, but a poly-adenylation signal was absent. This suggests that in these cases the ferret 3' UTR could be longer than their homologues. The other 17 identified gene tags were located in the other upstream regions of the mRNA, but none was located in the 5' UTR.

When all the unique ESTs were considered, 53 % were located to the 3' end of the mRNA (Table 6-6). The remainders were derived from other regions of their respective transcripts.

Table 6-6 Locations of the ESTs in their mRNAs for the 112 unique ESTs

Locations	3' end		otherwise		Total	
Status	Known	Unknown	Known[1]	Unknown	Known	Unknown
No. and (%)	25 (23.3)	34 (30.4)	17 (15.1)	36 (31.2)	42	70
Subtotal and (%)	59 (52.7)		53 (47.3)		112 (100)	

[1] *fHa8* included.

6.3.8 Unidentified ESTs

All 70 unique sequences which were not identified in the GenBank non-redundant database were further subjected to a search against the GenBank EST database (DBest) across all species. All those that showed homology to cDNA clones also had homology to DBest entries (Table 6-7). More than half of the sequence tags that showed homology to genomic DNA clones also had homologues in DBest. Approximately a quarter of the remainder also aligned to DBest sequences. When all the 70 unknown sequences were considered as a whole, more than a third (25) had homologues in DBest.

Table 6-7 Homology search results in DBest for unidentified ESTs

	To cDNA clone	To DNA clone	No or low homology	Total
No. from non-redundant database search	5	12	53	70
No. homologous to DBest entries	5	7	13	25
No. lacking DBest homology	0	5	40[1]	45

[1] Five of these which showed low homology to hHa8 were confirmed, with extended sequences, to be the ferret Ha8 orthologue (Chapter 7).

6.3.9 Association of gene expression with the hair follicle growth cycle

When the timing of the initial changes in expression detected by differential display were analysed, most of the cloned 1998 differentials showed an initial alteration of expression at the first sample (0.5 day) collected following melatonin administration. Subsequently, the number of ESTs decreased with the time after the treatment (Fig. 6-2). Among those which showed homology to known genes, a high proportion were extracellular matrix (ECM) or cell adhesion (CAM) and other structural molecules. The number of transcription and signalling molecules was also significant (Chapter 8).

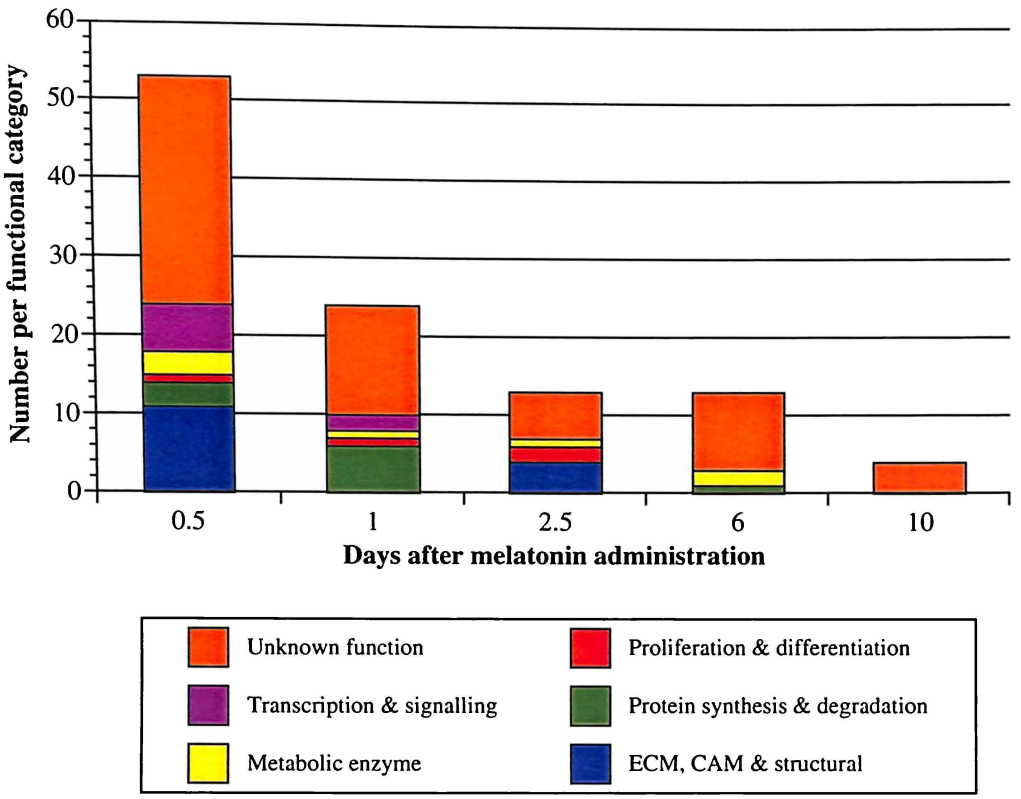


Fig. 6-2 Number and class of 1998 ESTs associated with melatonin administration

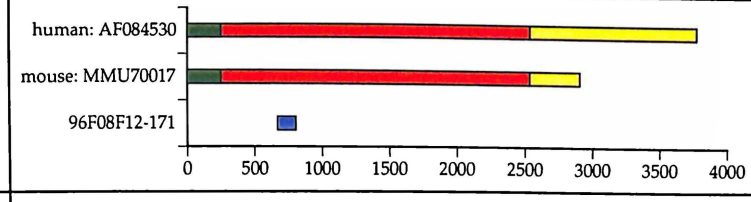
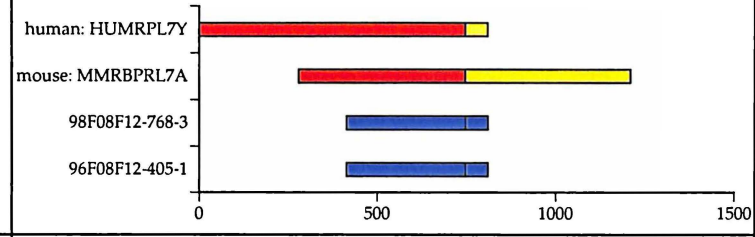
To further develop the relationship between the ferret ESTs and the hair growth cycle, all 150 ESTs generated (including those derived from ferrets treated with melatonin plus steroids) were assigned to different hair follicle growth stages, according to the time of their first alteration in expression, determined either by differential display or Northern blot hybridisation. Where available, the results from the latter technique superseded those from the former. The ferret ESTs were predominantly linked to proanagen II, or earlier stages when morphological changes in the hair follicle were not apparent by light microscopy (Fig. 6-3).

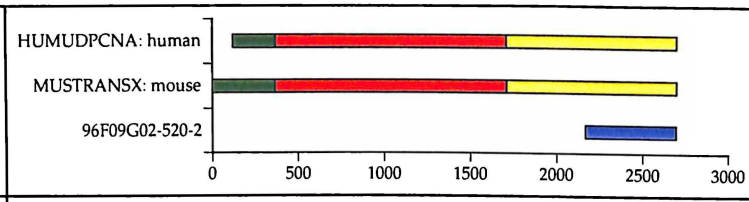
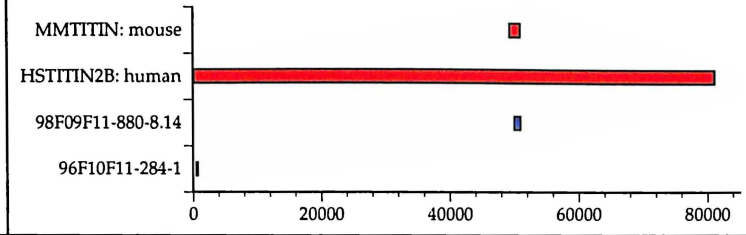
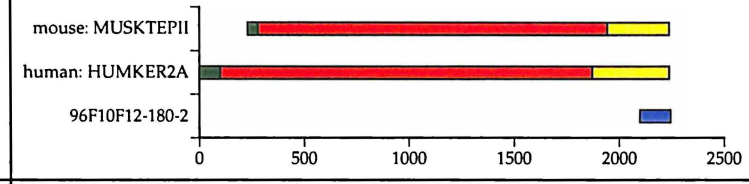
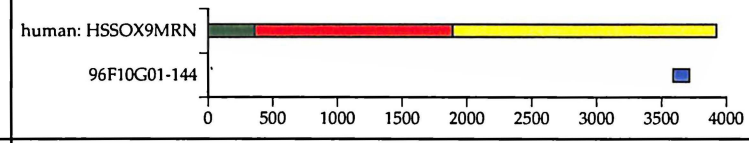
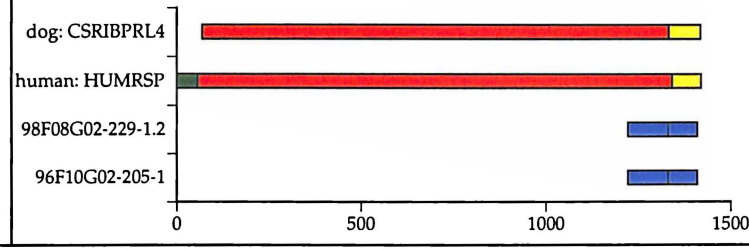
6.4 Discussion

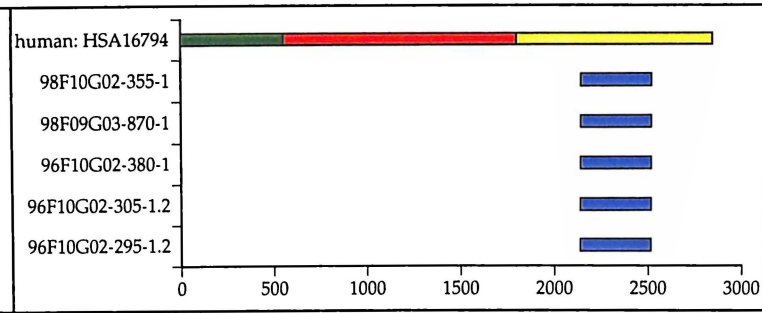
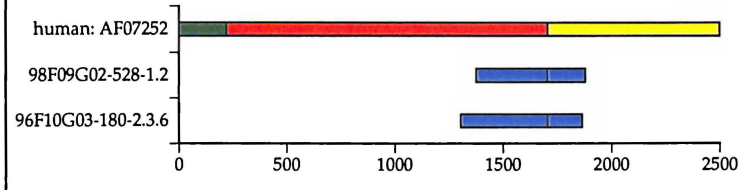
6.4.1 Amplification and cloning

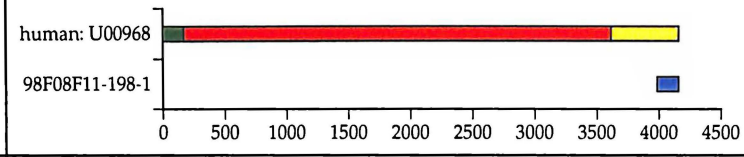
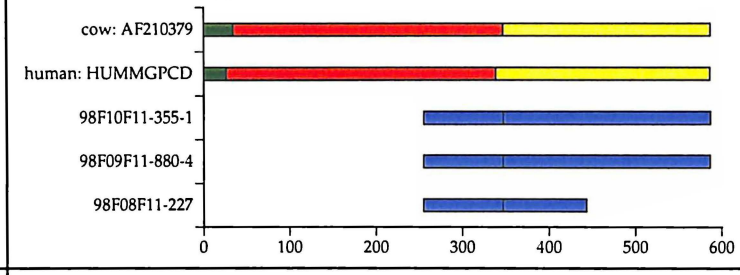
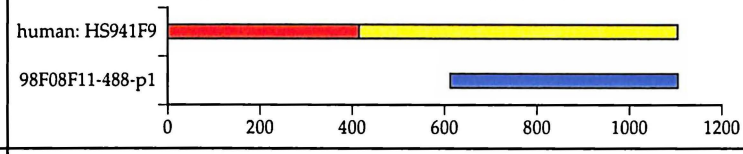
Most of the differentials isolated from the acrylamide gels were amplified successfully, indicated by the presence of a single band or dominant band of the estimated size. Compared with the higher cycle number, or even the second round of PCR required when using short primers (Liang and Pardee, 1992; Rufaut, 1997), sufficient DNA was produced from the isolated cDNA with 35-40 cycles.

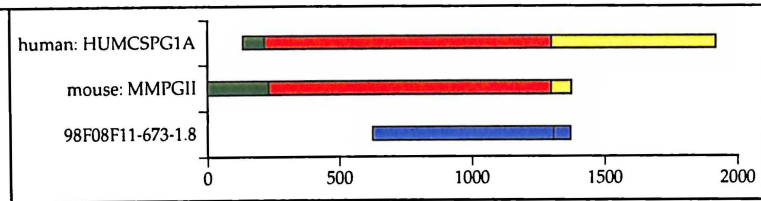
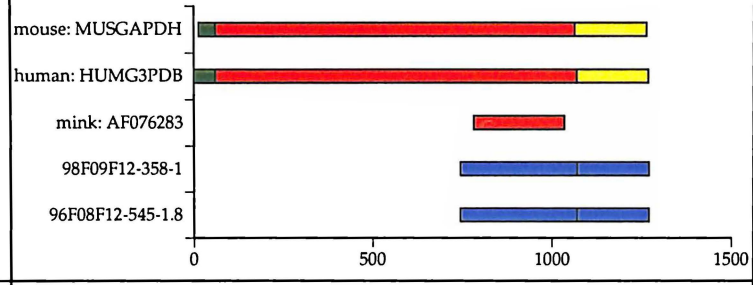
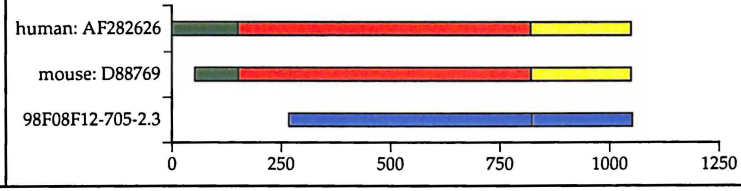
Table 6-8 Features of the ferret ESTs

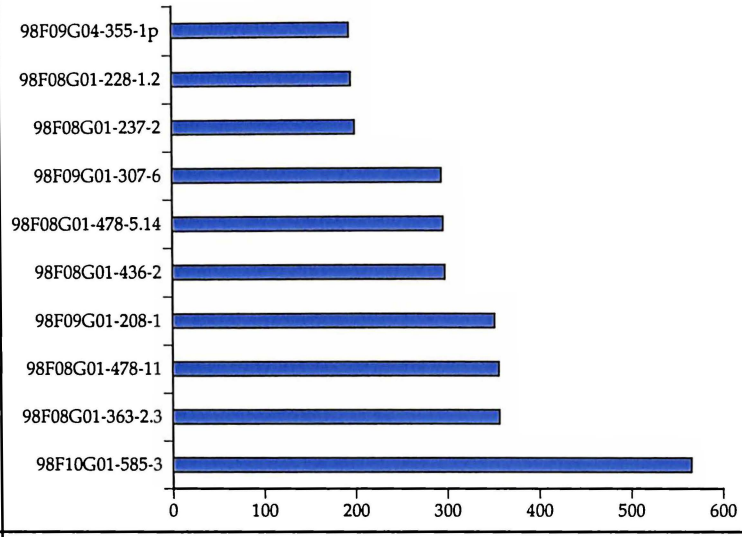
EST	Identity	Alignment diagram	Other features	No.
96F08F12-171-1	DMP1		A: 147 B: P-V* P-IIIb C: np D: Transcription regulators and signalling	1
96F08F12-359	unknown		A: 383 B: P-II C: 178	2
96F08F12-368-7 96F10F12-396-5	unknown		A: 374 B: P-II* P-IIIa C: np	3
96F08F12-405-1 98F08F12-768-3	RPL7		A: 403 B: P-II no change C: 49 D: Protein synthesis and degradation	4
96F08F12-470-1	unknown		A: 479 B: P-II* P-V C: 23	5
96F08G01-170-2.3 98F08G01-141-3	unknown		A: 144 B: P-V C: np E: homologous to a DNA clone	6
96F08G04-450-3	unknown		A: 466 B: T1 T3 C: 42 E: homologous to a DNA clone and a DBest entry	7
96F08G04-462-4	unknown		A: 480 B: P-IV C: 22 E: homologous to a DNA clone and a DBest entry	8

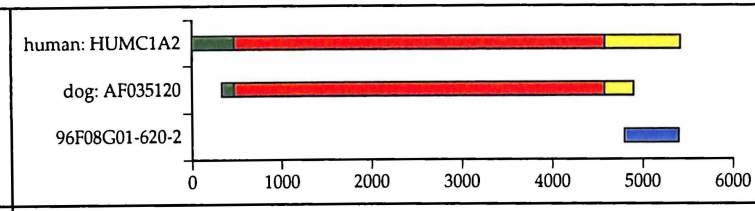
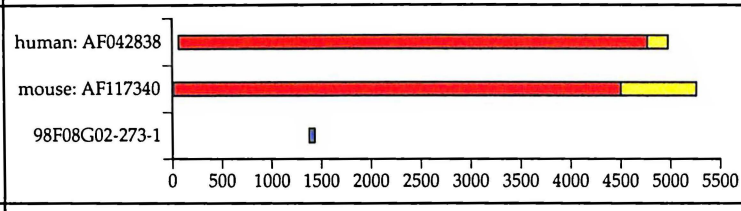
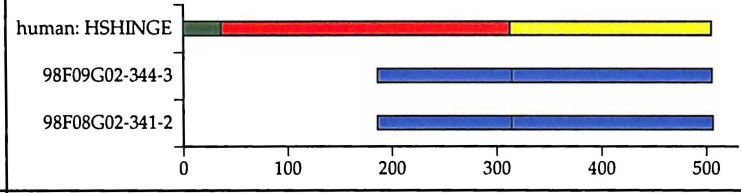
96F09G02-520-2	GnTI		A: 522 B: T1 T2 C: 18 D: Metabolic enzymes	9
96F09G04-710-3	unknown		A: 598 B: P-V nd C: 140 [a] E: homologous to a DNA clone	10
96F10F11-284-1 98F09F11-880-8.14	Titin		A: 251 B: 0.5 C: np D: ECM, CAM and other structural	11
96F10F12-180-2	Keratin K5		A: 151 B: P-II* P-IV C: 17 D: ECM, CAM and other structural	12
96F10F12-396-5	see 3		A: 377 B: P-II* P-IIIa C: np	13
96F10G01-144	Sox9		A: 126 B: T1 C: 46 [a] D: transcription regulators and signalling	14
96F10G02-205-1 98F08G02-229-1.2	RPL4		A: 204 B: P-IIIa C: 20 D: Protein synthesis and degradation	15

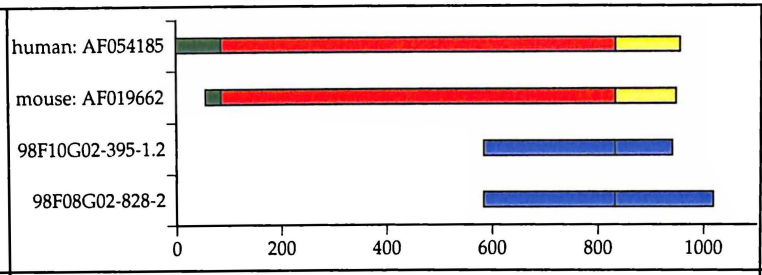
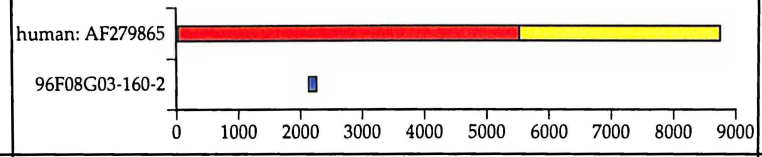
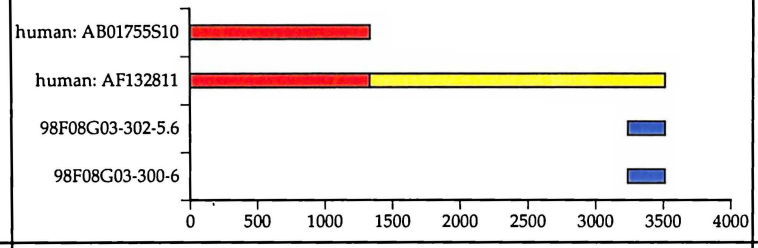
96F10G02-295-1.2 96F10G02-305-1.2 96F10G02-380-1 98F09G03-870-1 98F10G02-355-1	fHa8		A: 327 B: P-IIIa P-IIIb C: np D: ECM, CAM and other structural E: Identical sequences which were homologous to hHa8 with E value of e-5. Extended sequence confirmed the identity.	16
96F10G02-305-1.2	see 16		A: 327 B: P-IIIb C: np	17
96F10G02-380-1	see 16		A: 327 B: P-IIIa P-IIIb C: np	18
96F10G02-380-2	unknown		A: 323 B: P-IIIa C: np E: While 96F10G02-380-2 has good homologies to DNA clones AF140594 and EST AF221609, it also has some homology to the mink tyrosine aminotransferase gene (AF163863), but the matches are to intron sequences. It shares limited homology with 98F09G03-242-1 and 98F10F12-255-1. These sequences all have BLASTn matches to mink TI1 and mink tyrosine aminotransferase genes.	19
96F10G02-380-3	unknown		A: 410 B: P-IIIa P-IV C: 22 E: It showed some homology to differentiation gene 2 (DIF2), radiation early response gene (IEX1) and apoptosis inhibitor gene (IEX1L) and homology to a DBest entry*.	20
96F10G03-180-2.3.6 98F09G02-528-1.2	TIED		A: 570 B: P-V C: np D: ECM, CAM and other structural E: 96F10G03-180-2.3.6 and 98F09G02-528-1.2 are equally matched to another GenBank entry AB008375: osteoblast specific cysteine-rich protein. No information apart from the GenBank	21

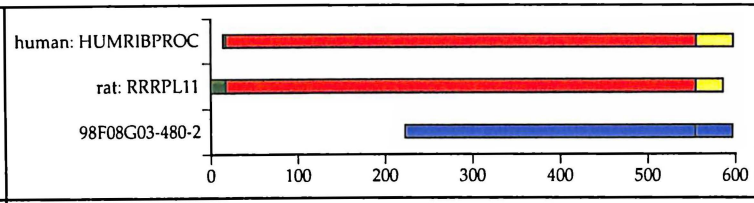
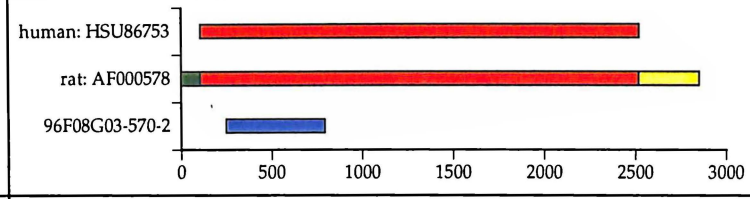
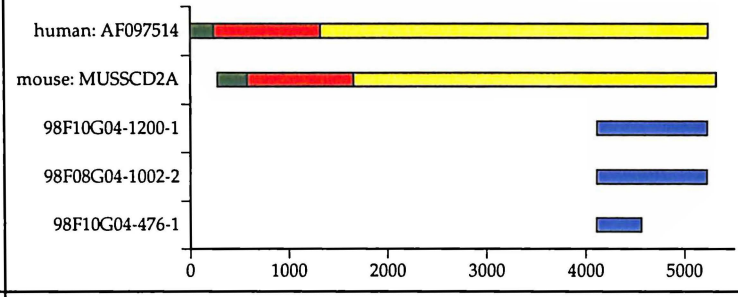
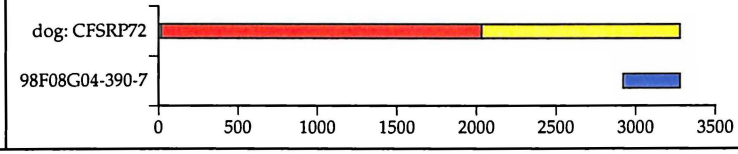
			entry is available on this sequence. AB008375 and AF07252 are closely related sequences but differ by 7 base substitutions, a single base insertion in AB008375 and a 69 base insertion in AF07252. Further 5' EST sequence information would be required to make a definitive distinction.	
96F10G03-346-1 98F10G03-378-1	unknown		A: 339 B: P-II P-IIIa C: np	22
96F10G03-430-1	unknown		A: 395 B: P-V* T-w C: 126	23
98F08F11-198-1	SREBP 1		A: 176 B: T0.5 nd C: 21 D: transcription regulators and signalling	24
98F08F11-227 98F09F11-880-4 98F10F11-355-1	MGP		A: 195 B: T2.5 C: np D: ECM, CAM and other structural	25
98F08F11-488-1.2	unknown		A: 467 B: T1 C: np	26
98F08F11-488-p1	Fibulin 1D		A: 469 B: T1 C: 39 D: ECM, CAM and other structural	27
98F08F11-520-2	unknown		A: 479 B: T1 C: 19 E: homologous to a DBest entry	28
98F08F11-521-5	unknown		A: 482 B: T2.5 C: np	29

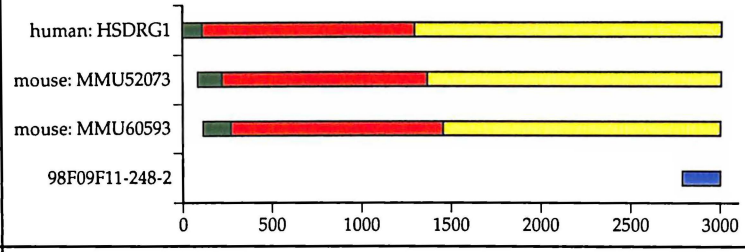
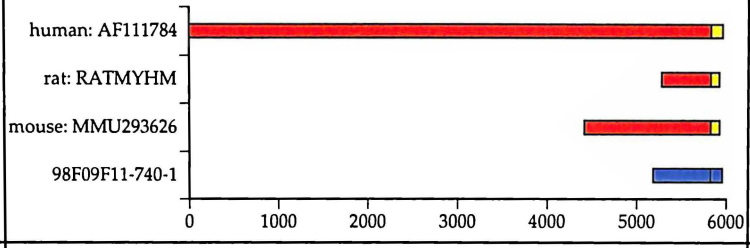
98F08F11-673-1.8	Decorin		A: 750 B: T1 C: np D: ECM, CAM and other structural	30
98F08F12-265-7	unknown		A: 242 B: T1 C: 22 D: homologous to a DBest entry	31
98F08F12-274-1 98F08F12-456-2 98F08G04-390-1	unknown		A: 257 B: PI C: np	32
98F08F12-358-1 98F09F12-358-2	unknown		A: 404 B: T2.5 C: 23	33
98F08F12-456-2	see 32		A: 257 B: PIIIa[b] C: np	34
98F08F12-545-1.8 98F09F12-358-1	GAPDH		A: 545 B: T2.5 C: 25 D: Metabolic enzymes	35
98F08F12-680-2.4	unknown		A: 762 B: T1 C: np E: homologous to a DNA clone	36
98F08F12-705-2.3	Latexin		A: 790 B: T2.5 C: 19 D: Proliferation and differentiation	37

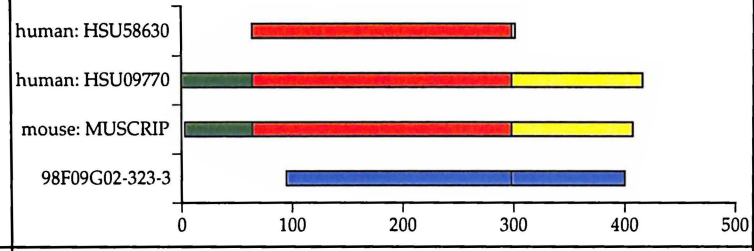
98F08F12-768-3	see 4		A: 402	B: T0.5 no change	C: 22	38
98F08G01-141-3	see 6		A: 141	B: T1	C: np	39
98F08G01-228-1.2 98F08G01-237-2 98F08G01-363-2.3 98F08G01-436-2 98F08G01-478-11 98F08G01-478-5.14 98F09G01-208-1 98F09G01-307-6 98F09G04-355-1p 98F10G01-585-3	unknown		A: 193	B: P-IIIa[b]	C: np	40
98F08G01-237-2	see 40		A: 198	B: T2.5[b]	C: np	41
98F08G01-237-6	see 40		A: 226	B: T2.5[b]	C: np	42
98F08G01-363-2.3	see 40		A: 355	B: T2.5[b]	C: np	43
98F08G01-436-2	see 40		A: 296	B: T2.5[b]	C: np	44
98F08G01-478-11	see 40		A: 355	B: T2.5[b]	C: np	45
98F08G01-478-5.14	see 40		A: 294	B: T2.5[b]	C: np	46
98F08G01-480-2 98F08G03-575-2	unknown		A: 469	B: T1[b] nd	C: 23 E: 98F08G01-2 and 98F08G03-575-2 have some homology to epithelial V-like antigen (AF03455) and are homologous to a DBest entry.	47

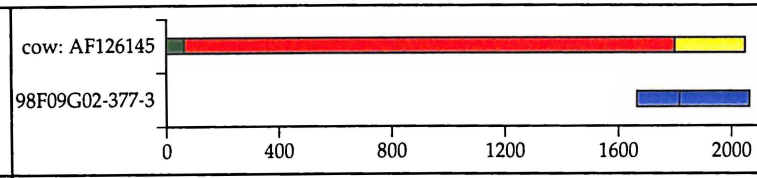
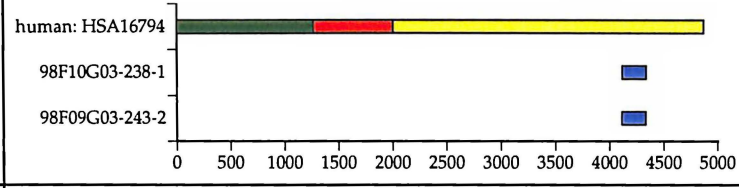
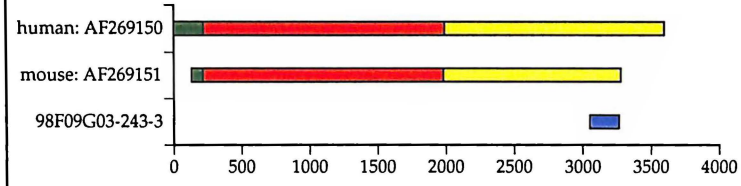
98F08G01-620-2	Colgn α2I		A: 612 B: T2.5 C: 26 D: ECM, CAM and other structural	48
98F08G02-229-1.2	see 15		A: 205 B: T0.5 P-IIIa C: 20	49
98F08G02-231-3.4	unknown		A: 207 B: T0.5 C: 38D: homologous to a DBest entry	50
98F08G02-272-1	unknown		A: 255 B: T2.5[b] C: np	51
98F08G02-273-1	MEKK1		A: 252 B: T0.5 nd C: np D: Transcription regulation and signalling	52
98F08G02-341-2 98F09G02-344-3	MHP		A: 323 B: T0.5 C: 22 D: ECM, CAM and other structural	53
98F08G02-492-1.2	unknown		A: 489 B: T0.5 C: np	54
98F08G02-600-2.3	unknown		A: 593 B: T1 C: 89 E: homologous to a DNA clone and a DBest entry	55

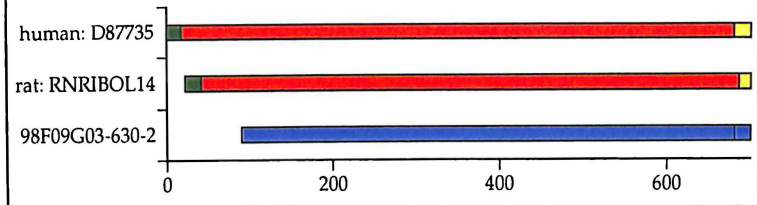
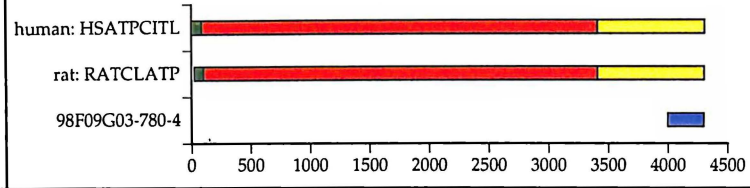
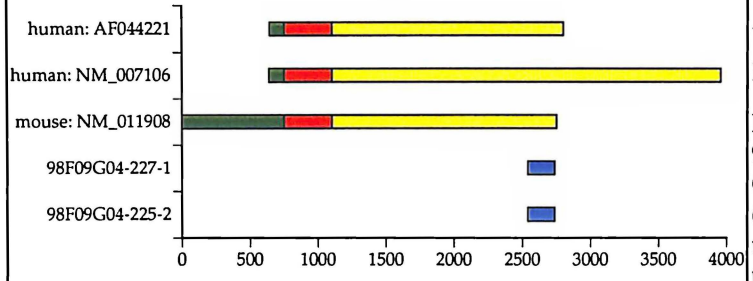
98F08G02-828-2 98F10G02-395-1.2	HSPC		A: 445 B: T1 C: 22 D: Protein synthesis and degradation	56
98F08G03-150-1	unknown		A: 119 B: T6 C: 19 E: homologous to cDNA clone and a DBest entry	57
98F08G03-160-2	GAKIN		A: 133 B: T2.5 C: np E: ECM, CAM and other structural	58
98F08G03-280-4	unknown		A: 269 B: T2.5 C: 219	59
98F08G03-300-2	unknown		A: 234 B: T0.5 C: 21	60
98F08G03-300-6 98F08G03-302-5.6	IGSF4		A: 284 B: T0.5 C: np D: ECM, CAM and other structural	61
98F08G03-302-5.6	see 61		A: 284 B: T0.5 C: np	62
98F08G03-480-1 98F08G03-525-2	unknown		A: 470 B: T1 C: 22	63

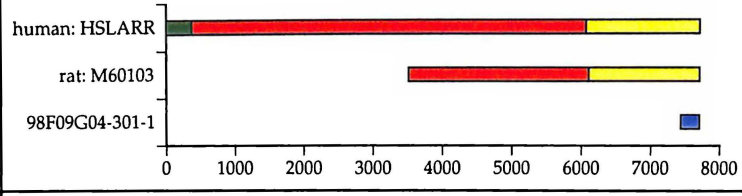
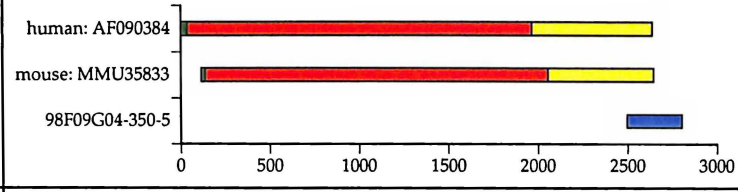
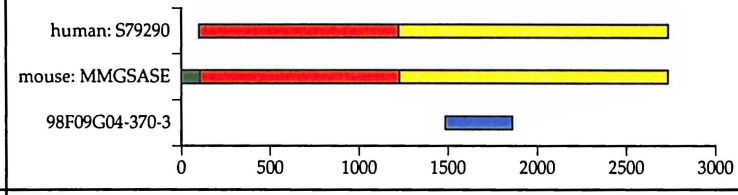
98F08G03-480-2	RPL11		A: 382 B: T1 C: np D: ECM, CAM and other structural	64
98F08G03-525-2	see 63		A: 466 B: T0.5 nd C: 22	65
98F08G03-570-2	PCDC5RP		A: 545 B: T1 nd C: np D: Transcription regulation and signalling	66
98F08G03-575-2	see 47		A: 468 B: T0.5 nd C: 23 E: homologous to a Dbest entry	67
98F08G04-1002-2 98F10G04-1200-1 98F10G04-476-1	SCD		A: 1118 B: T6 C: 20 D: Metabolic enzymes	68
98F08G04-270-2	unknown		A: 257 B: P-I C: 20 E: homologous to a DNA clone	69
98F08G04-390-1	see 32		A: 256 B: T6 C: np	70
98F08G04-390-7	SRP72		A: 365 B: T6 C: 26 D: Protein synthesis and degradation	71

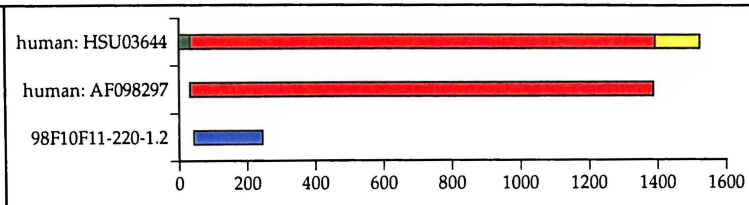
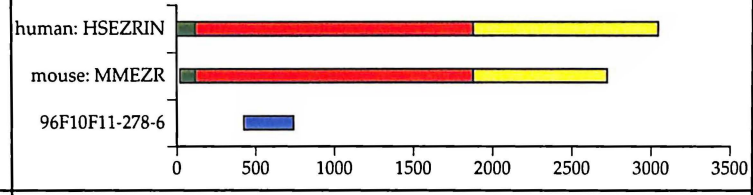
98F08G04-390-8	unknown		A: 374 B: T6 C: np	72
98F09F11-230-2	unknown		A: 193 B: T0.5 C: 21	73
98F09F11-248-2	RTP/Ndr1/ Drg1/TDD5		A: 223 B: T2.5 nd C: 18 D: proliferation and differentiation	74
98F09F11-391-1	unknown		A: 374 B: T0.5 C: 21 E: homologous a DBest entry	75
98F09F11-740-1	MyHC IIa		A: 780 B: T0.5 C: 29 D: ECM, CAM and other structural	76
98F09F11-880-4	see 25		A: 334 B: T0.5 T2.5 C: 25	77
98F09F11-880-8.14	see 11		A: 962 B: T0.5 P-II C: na	78
98F09F12-301-1	unknown		A: 284 B: P-IIIa[b] C: 26 E: homologous to a DNA clone	79
98F09F12-358-1	see 35		A: 541 B: T2.5 C: 23 D: homologous to a DBest entry	80
98F09F12-358-2	see 33		A: 322 B: T0.5 C: 23	81

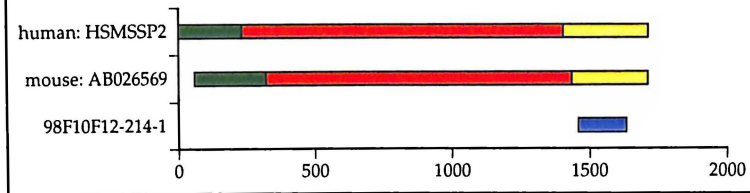
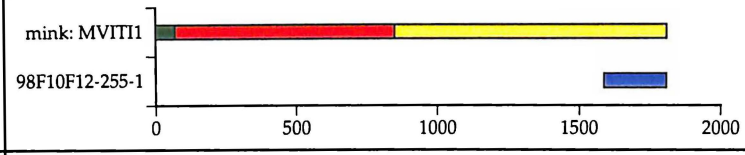
98F09F12-388-2	unknown		A: 362 B: T1 C: np E: homologous to a DBest entry	82
98F09F12-390-3	unknown		A: 363 B: T1 C: 82	83
98F09F12-655-2	unknown		A: 666 B: T2.5 C: 15	84
98F09G01-204-1	unknown		A: 187 B: T0.5 C: np	85
98F09G01-208-1	see 40		A: 350 B: T6[c] C: np	86
98F09G01-307-6	see 40		A: 292 B: T6 C: np	87
98F09G01-390-1	unknown		A: 361 B: T1 C: np	88
98F09G02-228-5	unknown		A: 195 B: T0.5 C: np E: homologous to a DNA clone	89
98F09G02-272-3	unknown		A: 251 B: T0.5 C: 29	90
98F09G02-323-3	CRIP		A: 290 B: T0.5 C: 16 D: Proliferation and differentiation	91
98F09G02-344-2	unknown		A: 316 B: T0.5 C: np	92
98F09G02-344-3	see 53		A: 319 B: T0.5 C: 20	93

98F09G02-377-3	CoA ligase		A: 374 B: T6 C: 20 D: Metabolic enzymes	94
98F09G02-528-1.2	see 21		A: 511 B: T2.5 C: np	95
98F09G03-242-1	unknown		A: 216 B: T0.5 C: 32	96
98F09G03-242-2	unknown		A: 216 B: T0.5 C: np D: homologous to a DBest entry	97
98F09G03-243-2 98F10G03-238-1	BTEB1		A: 221 B: T0.5 nd C: 16 D: Transcription regulation and signalling	98
98F09G03-243-3	TM9SF3		A: 221 B: T0.5 nd C: np D: ECM, CAM and other structural E: 98F09G03-243-3 aligns to four human GenBank entries (a cDNA clone, transmembrane protein TM9SF3, endomembrane protein emp70 precursor, and T84 colon carcinoma cell IL-1β regulated HSCC1) with the same score and e value. None of these entries are supported by published information and are likely to represent the same mRNA species on the basis of their multiple sequence alignment. The HSCC1 alignment is to the reversed and complemented GenBank entry of a partial mRNA sequence.	99
98F09G03-424-1	unknown		A: 408 B: T2.5 C: 16 E: homologous to a cDNA clone and a DBest entry	100

98F09G03-630-2	RPL14		A: 617 B: T1 C: 18 D: Protein synthesis and degradation	101
98F09G03-780-4	ACL		A: 304 B: T0.5 C: 25 D: Metabolic enzymes	102
98F09G03-870-1	see 16		A: 327 B: T6 P-IIIb C: np	103
98F09G04-205-1 98F09G04-207-2	unknown		A: 179 B: T1 [d] C: 23	104
98F09G04-207-2	see 104		A: 181 B: T1[d] C: 25	105
98F09G04-217-1.7	unknown		A: 189 B: T1 C: np	106
98F09G04-225-2 98F09G04-227-1	UBL3		A: 204 B: T1 C: np D: Protein synthesis and degradation E: The two identical clones 98F09G04-225-2 and 98F09G04-227-1 have significant homologies to GenBank entries for human and mouse HCG-1 (AF044221 and AF044222 respectively). However the HCG-1 sequences do not have any relationship to human chorionic gonadotrophin sequences. AF044222 is identical to mouse UBL3 (NM_011908) while AF044221 is very similar, but not identical, over their aligned section to human UBL3 (NM_007106).	107

98F09G04-227-1	see 107		A: 204	B: T1	C:	108
98F09G04-281-1	unknown		A: 250	B: T0.5	C: 15	109
98F09G04-301-1	LAR		A: 278	B: T0.5	C: 27 D: Transcription regulation and signalling	110
98F09G04-303	unknown		A: 281	B: T6	C: np	111
98F09G04-350-5	SAE1C		A: 318	B: T1	C: 17 D: Protein synthesis and degradation	112
98F09G04-355-1p	see 40		A: 192	B: T1	C: np	113
98F09G04-367-2	unknown		A: 331	B: T0.5	C: 17 E: homologous to a DNA clone and a DBest entry	114
98F09G04-370-2	unknown		A: 366	B: T0.5	C: 18	115
98F09G04-370-3	GS		A: 363	B: T0.5	C: np D: Metabolic enzymes	116
98F10F11-151-1	unknown		A: 137	B: T0.5	C: 22	117
98F10F11-187-2	unknown		A: 174	B: T0.5	C: np	118

98F10F11-220-1.2	CIR		A: 199 B: T1 nd C: 76 D: Transcription regulation and signalling E: 98F10F11-220-1.2 shows strong homology to two GenBank entries: recepin (HSU03644) and CBF1 interacting corepressor. These two mRNAs differ in their 1356/1353 bp CDS by only 15 bases and are likely to represent the same transcript. No additional published information is currently available on recepin function.	119
98F10F11-241-1.2 98F10F11-244-2	unknown		A: 217 B: T0.5 C: np	120
98F10F11-244-2	see 120		A: 215 B: T6 C: np	121
98F10F11-254-1	unknown		A: 233 B: T6 C: np	122
98F10F11-278-6	Ezrin		A: 330 B: T0.5 C: np D: ECM, CAM and other structural	123
98F10F11-341-2 98F10F11-376-1	unknown		A: 315 B: T2.5 C: np	124
98F10F11-355-1	see 25		A: 335 B: T0.5 T2.5 C: 22	125
98F10F11-376-1	see 124		A: 355 B: T0.5 C: np	126
98F10F12-204-1	unknown		A: 182 B: T0.5 C: 81	127

98F10F12-214-1	MSSP1/2		A: 193 B: T0.5 nd C: np D: Transcription regulation and signalling	128
98F10F12-255-1	TI1		A: 232 B: P-IIIa[b] C: 10, 14 D: Proliferation and differentiation	129
98F10F12-255-2	unknown		A: 233 B: P-IIIb C: 19 E: homologous to a cDNA clone and a DBest entry	130
98F10F12-264-1.2	unknown		A: 245 B: T0.5 C: 16, 20	131
98F10G01-585-3	see 40		A: 565 B: T0.5 C: np	132
98F10G02-308-1	unknown		A: 281 B: T0.5 C: 7, 11	133
98F10G02-315-1	unknown		A: 288 B: T0.5 C: 36 E: homologous to a DBest entry	134
98F10G02-355-1	see 16		A: 327 B: P-IIIb C: np	135
98F10G02-395-1.2	see 56		A: 376 B: T0.5 C: 20	136
98F10G02-616-3	unknown		A: 581 B: P-I C: 46[a] E: homologous to a DNA clone and a DBest entry	137
98F10G02-616-4	unknown		A: 580 B: P-I C: np	138
98F10G03-238-1	see 98		A: 221 B: T0.5 nd C: np	139
98F10G03-376-2	unknown		A: 339 B: T0.5 C: 30	140

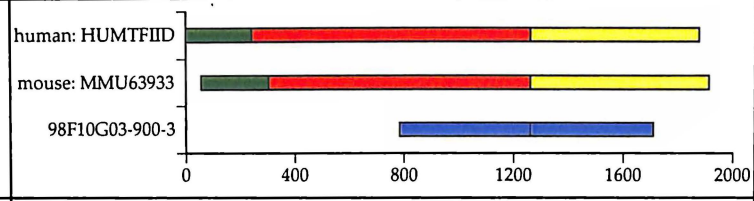
			E: homologous to a DNA clone and a DBest entry	
98F10G03-378-1	see 22		A: 344 B: T0.5 P-IIIa C: np	141
98F10G03-477-2	unknown		A: 449 B: T6 C: 17 E: homologous to a cDNA clone and a DBest entry	142
98F10G03-477-3	unknown		A: 452 B: T6 C: 20 D: homologous to a DBest entry	143
98F10G03-900-3	TBP		A: 919 B: T0.5 C: np D: Transcription regulation and signalling	144
98F10G03-900-5.7	unknown		A: 903 B: T0.5 C: 22 E: homologous to a DBest entry	145
98F10G04-1200-1	see 68		A: 1108 B: T6 C: 18	146
98F10G04-268-1	unknown		A: 220 B: T1 C: np	147
98F10G04-338-2	unknown		A: 300 B: T0.5 C: np	148
98F10G04-374-1	unknown		A: 344 B: T6 C: 29 E: homologous to a cDNA clone and a DBest entry	149
98F10G04-476-1	see 68		A: 431 B: T6 C: np	150

Table 6-8 Notes:

5' untranslated region; coding sequence; 3' untranslated region, gene tags generated in this study.

A: Length in bp.

B: Time after melatonin treatment or cycle phase at which the first alteration in expression was detected by differential display or Nothern blots. The results of Northern blots (Chapter 7) are underlined and supersede those from differential display (only result from Northern blots is provided if they are the same). A: anagen; P: proanagen; T: telogen. Number after T indicates days after melatonin administration. w indicates winter sample. nd: no signal detected in Northern blots. * Differential display using only three representative samples, the patterns were thus less reliable (See Chapter 7 for details).

C: Numbers indicate the position of the first adenine of the poly-adenylation motif AAUAAA (nucleotides away from the first adenine of the 3' poly A tail). na: not applicable as 98F09F11-880-8.14 was defined by the same arbitrary primer at both ends. np: not present.

D: Class of molecules (Table 8-1).

E: Notes.

[a] Derived from only the oligo dT anchor primer only.

[b] Differential derived from females treated with melatonin and progesterone only.

[c] Differential derived from males treated with melatonin and oestradiol or progesterone.

[d] Differential derived from males treated with melatonin and oestradiol only.

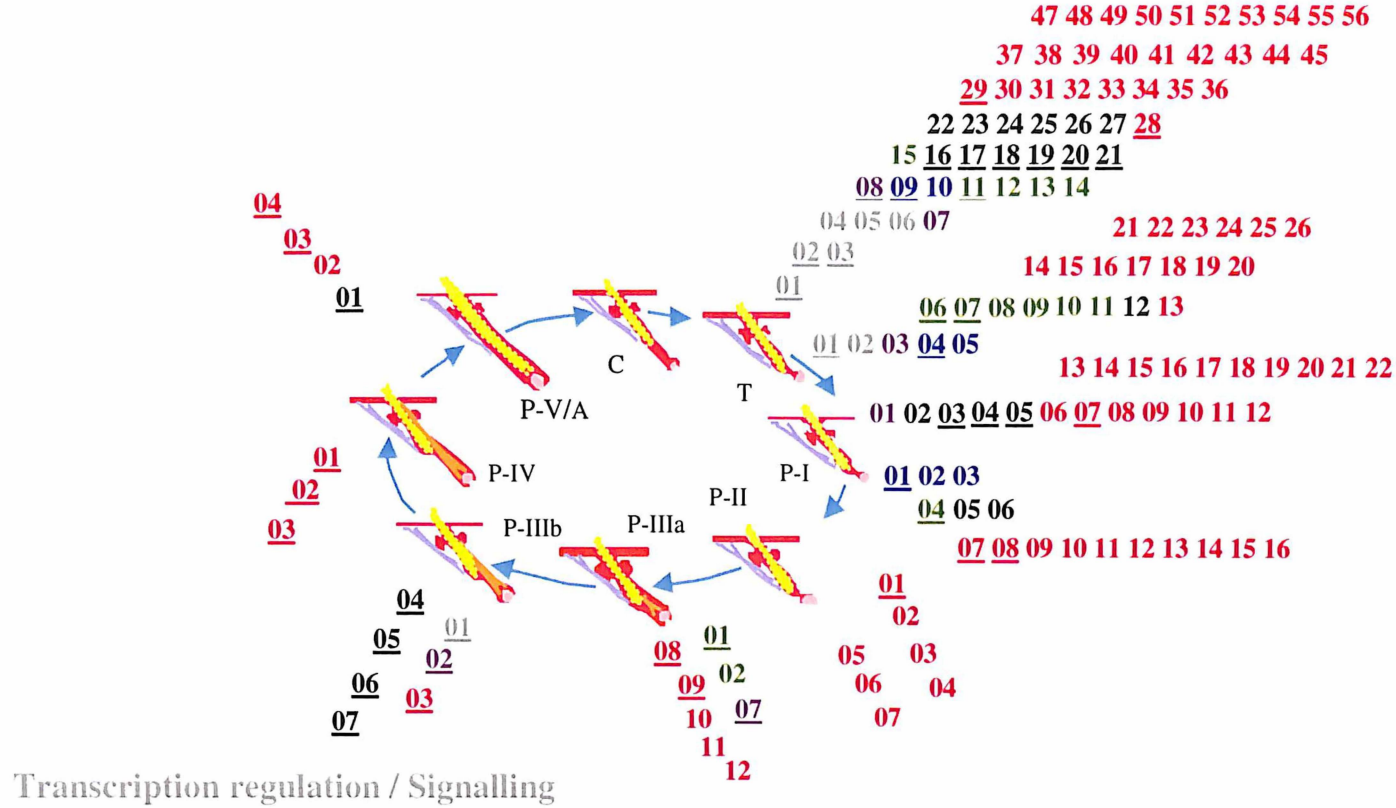


Fig. 6-3 The association of the expression alteration of 150 ESTs with the hair follicle growth cycle

Two digit numbers represent a single EST. The assignment of the ESTs was according to the first changes in expression detected either by differential display or Northern blots (underlined and superseding that from the former).

The majority of these amplified cDNAs were cloned. There were larger numbers of differentials and ESTs arising from anchor primers F08 (anchor base C) and F09 (anchor base G). This suggests a higher efficiency of annealing and is in agreement with the higher melting temperature (Tm) for G-C binding than T-A binding and similar to the results reported by others using two-base anchor primers (Linskens *et al.*, 1995).

Although most of the multiple clones derived from the same differential contained the same cDNA species, distinct inserts of the same or different lengths were found in some cases. This is a common outcome of differential display and believed to be caused by the co-migration of different cDNA species in the acrylamide gel (Bauer *et al.*, 1993; Callard *et al.*, 1994; Li *et al.*, 1994). These co-migrated cDNAs may, or may not, be differentially expressed, requiring confirmation of expression by independent methods.

6.4.2 Identities of the ESTs

Close to 40% of the ESTs were identified to known genes. The possible roles of these genes and the likely biological processes in which they are involved during hair follicle growth initiation are discussed in Chapter 8. In common with other studies, the majority of the sequence tags could not be identified by sequence homology searches (Linskens *et al.*, 1995; Uchiyama *et al.*, 1995; Too, 1997; Rufaut *et al.*, 1999a; De Vries *et al.*, 2000). These unidentified sequence tags are likely to represent novel transcripts and many have sequence similarity to DBest entries. The expression of several gene tags in this category was also confirmed to be related to the hair growth cycle by Northern blot and *in situ* hybridisation (Chapter 7).

One reason that some ferret ESTs could not be identified is low sequence conservation between species, especially when the ESTs are derived from the 3' UTR. An example was the five ESTs which showed limited homology (E≥e-5) to human Ha8. When more sequence information was obtained, it was found that the ferret and human orthologues were highly homologous in the coding region, but with reduced identity in both the 3' and 5' UTRs (Appendix I-A). It was noticeable that when homologous sequences were available for the mink, dog,

human, mouse and rat, the degree of homology usually declined in this order, reflecting the evolutionary divergence of the species.

6.4.3 The binding of primers and sequence redundancy

The binding sites of the anchor and arbitrary primers: Differential display divides a large number of transcripts into subcategories by the binding of three different single-base (or 12 different two-base) oligo dT anchor primers to the 3' polyA tail. The combination of the arbitrary primer and the anchor primer enables the transcripts to be systemically displayed (Liang and Pardee, 1992; Liang *et al.*, 1994). Although poly-adenine sequences were present in all the gene tags derived from the anchor primers, the lack of poly-adenylation signal for nearly half of the ferret ESTs suggests that the anchor primers did not anneal specifically to the 3' end of the templates. Binding of the anchor base to internal adenine-rich cDNA sequences was common in this study, as has been reported previously with two-base anchor primers (Guimaraes *et al.*, 1995; Frost and Guggenheim, 1999; Trenkle *et al.*, 1999).

As the most common match between the arbitrary primer and a template is seven out of eight bases at the 3' end of the primers (Linskens *et al.*, 1995), the high stringency of binding has been shown to result in very little redundancy (Trenkle *et al.*, 1999). Although the binding specificity of the arbitrary primers used in this study remain to be determined, it is likely to be similar. In the situation that there is only one binding site for the arbitrary primer, the shorter fragment derived from the internal binding of anchor primers is likely to be produced more efficiently in both the reverse transcription and PCR reactions than the longer fragment containing the polyA tail, assuming the same priming efficiency. On the other hand, as the sizes of the cDNA displayed typically range between 150 bp to 1 kb, cDNA fragments larger than 1 kb are much less likely to be represented on a acrylamide gel, even if they are produced. Hence, the effect of these multiple priming events of anchor primers is that the smaller fragments derived from the regions closer to the 5' end are selectively displayed, resulting in cloning of more differentials located inside the coding region. By aligning the gene tags to their homologues (mainly those from humans and mice), approximately half of ESTs were derived from regions other than the 3' end, many of which included coding sequence. If no internal binding of the anchor primer occurs, large cDNA species

from mRNA with only one arbitrary binding site far away from the 3' end, are likely to be missed. This can be particularly significant when the number of primer sets is limited, as in this study. An example is the identification of *titin*, which is encoded by a very large mRNA (over 80 kb). Two differentials, 96F10F11-284-1 and 98F10F11-880-8.14 (Table 6-6, No. 11), were detected and both located thousands of nucleotides away from the 3' end. cDNA synthesis commencing from the 3' end of the mRNA is very unlikely to proceed that far. Even then, it would be impossible for such a large cDNA to be displayed on a gel.

The redundant bands resulting from internal binding of the anchor primers can reduce the efficiency of differential display (Frost and Guggenheim, 1999). As this redundancy was found to be mainly caused by mispriming during reverse transcription (Frost and Guggenheim, 1999), raising the annealing temperature in the subsequent PCR reaction is not effective in reducing the internal binding. Higher annealing temperatures can also lead to a reduction in the number of bands displayed by each set of primers (Chapter 5). In this study, the annealing temperature for reverse transcription was 42 °C, which is commonly used and already much higher than the calculated melting temperature (Tm) for binding of 12 adenine and an anchor base (26-27°C depending on the anchor base).

Redundancy derived from electrophoresis: Another source of redundant sequences was derived from electrophoresis. A few differentials, which were adjacent to each other in the denatured acrylamide gel showed similar density patterns, represented identical cDNAs. These include 96F10G02-295-1.2 and 96F10G02-305-1.2 (Table 6-8, No. 16), 98F08G02-341-2 and 98F08G02-344-3 (Table 6-8, No. 53), 98F08G03-300-6 and 98F08G3-302-5.6 (Table 6-8, No. 61) 98F09G04-225-1 and 98F09G04-227-2 (Table 6-8, No. 107), and 98F10F11-241-1.2 and 98F10F11-244-2 (Table 6-8, No. 120). These identical doublets have been reported previously (Liang *et al.*, 1993; Too, 1997). They may arise from the addition of dATP by Taq DNA polymerase and the different electrophoretic mobility of the two cDNA strands in the denatured acrylamide gel. The introduction of non-denatured gel was considered to alleviate the problem (Bauer *et al.*, 1993).

6.5 Conclusion

Most of the isolated differentials were amplified and cloned. Of the 112 unique ESTs generated, 38% were identified to a specific known mRNA in other species and the remaining ESTs are likely to represent novel genes. Further studies to confirm an association of their expression patterns with the hair growth cycle and to elucidate their functions are likely to significantly advance our understanding the control of hair growth.

Analysis of the locations of the identified gene tags within their homologous mRNA revealed that the anchor primers annealed to complementary sequences at both the 3' end and to internal regions of the templates during reverse transcription and/or PCR amplification. Although binding to internal regions caused some redundancy, it also enabled the discovery of many differentially displayed bands that would not have been otherwise detected.

Hair follicle growth initiation involves biological processes such as cell proliferation, differentiation and the structural formation of a new hair. It was originally proposed that a focus on the early events associated with hair growth re-initiation would aid in understanding hair growth control. Most of the ESTs obtained in this study were derived from skin samples in which no obvious structural transformation had occurred. The identification and cloning of these differentials enabled further studies which are described in the following chapter.

CHAPTER 7 FURTHER STUDIES OF SELECTED CLONES

7.0 Abstract

Further studies described in this chapter generated new information on the association of selected ESTs with hair follicle growth. Thirty-five ESTs derived from differentially displayed bands were analysed by Northern blot analysis using total RNA derived from skin and some other tissues. Of these, 21 (60%) were shown to be differentially expressed over hair follicle growth initiation with or without steroid treatment, 2 (6%) did not show significant variation, and 12 (34%) did not produce any signal. The specific associations of these transcripts with hair growth were further confirmed by the general lack of expression, or low expression in tissues not containing hair follicles. Only one of 15 ESTs derived from the 1996 samples was not detectable by Northern blot hybridisation. The much higher proportion of the 1998 ESTs which were not detectable is likely to be due to low levels of expression during early proanagen. Localisation of some ESTs using DIG-labelled RNA probes also demonstrated a close association with hair follicle function. All nine clones, whose expression sites could be visualised, were localised to hair follicles, either in a particular compartment, or throughout the follicle. Most of these transcripts were also present in other parts of skin. Extended sequences were generated for two ESTs: *fHa8* and *fDMP1*, both of which were uniquely expressed in the keratinising cortex of mid-proanagen and anagen follicles. The mRNA of *fHa8* and hHa8 were similar in size and the predicted protein encoded by *fHa8* shared high homology to its human orthologue. However, *fHa8* has a distinct asymmetrical cortical distribution and a higher level of expression. The sequence obtained for *fDMP1* (760 bp) is highly homologous to its human and mouse equivalents at both the mRNA and protein levels. However, a unique expression pattern in keratinising cortex cells and a smaller size (0.9 kb) suggest an alternatively spliced isoform of *DMP1*, which might specifically mediate hair cortical cell growth and proliferation.

7.1 Introduction

A common problem of differential display is the incidence of false positive results (Bauer *et al.*, 1993; Liang *et al.*, 1995; Hsiang *et al.*, 1996). Although the use of longer oligo dT primers and two-stage PCR increases the specificity, some spurious

changes in band density still occur (Linskens *et al.*, 1995). Hence, although the differential display band patterns (Chapter 5) and the identities of the cloned differentially displayed fragments (Chapter 6) have built a preliminary picture of gene expression involved in follicle growth and the effects of steroids, their expression profiles still need to be established by other methods. Among the various methodologies which have been used for verification of differentially displayed bands, Northern blot hybridisation remains the most widely used. Northern blot hybridisation is straightforward (no requirement for amplification), providing not only the levels of expression, but also the number and size of transcripts (if more than one exist). The disadvantages are relatively low sensitivity and the resources required to check each EST separately.

Reverse RNA dot blots provide a capability to screen all the clones derived from the same set of primers simultaneously (Vogeli-Lange *et al.*, 1996). In this method, total RNA samples are reverse transcribed and then used to make probes by PCR using the same set of primers that generated the ESTs. When the amplified ESTs requiring confirmation are dotted on a membrane in excess to a particular probe, the intensity of the signal produced by hybridisation should indicate the expression levels of an EST in samples from different time points or treatments. As multiple probes are produced from a set of primers, all of the ESTs derived from the same primer set could theoretically be checked simultaneously. In addition, the sensitivity of the screen should also be enhanced by the amplification of probe. This can be important for detecting the genes expressed at low levels. Furthermore, a much smaller amount of RNA is needed compared with Northern blots.

Localisation of gene expression sites within the follicle not only verifies the association of the genes with hair follicles, but also indicates specific cycle stages in which they are expressed. RNA probes made from digoxigenin (DIG) labelled nucleotides have the advantage of long probe life and avoid the use of radioisotopes. The sensitivity and specificity of the method has been greatly improved by using Fab fragments of anti-DIG monoclonal antibodies (Miller *et al.*, 1993a).

As the ESTs generated are often quite short and come from the 3' end of mRNA, they may or may not contain coding sequence (Chapter 6). To better characterise ESTs representing novel genes, longer sequence is required. A cDNA library was

made from a pool of skin samples containing follicles at various growth stages (Chapter 3). Rapid Amplification of cDNA Ends (RACE) is a PCR based method which can be used to extend unknown sequences in both the 5' or 3' directions in order to obtain a complete cDNA sequence (Frohman *et al.*, 1988; Frohman, 1993). Additional sequence information generated for *fHa8* and *fDMP1* confirms that it is well suited for obtaining longer sequences for cloned differentially displayed bands, especially when the EST cloned is located at the 3' end of the transcripts. In such cases, only 5' RACE is required.

7.2 Material and methods

7.2.1 Methods for Northern blots and *in situ* hybridisation

To accelerate Northern blot screening, clones generated with the 1996 samples were first hybridised to a small blot containing only seven representative RNA samples and run for a short distance. Some detectable clones were then subjected to standard Northern blotting. As the number of ESTs obtained from the 1998 samples was much larger, reverse RNA dot blots were initially utilised, but changed to use Northern blot analysis. To confirm expression localisation and sensitivity of DIG-labelled RNA probes, ^{35}S-labeled RNA probes were used for three ESTs derived from the 1996 samples. See Sections 3.12 and 3.14 for protocol details.

7.2.2 Selection of clones for further studies

Most of the ESTs derived from differential display with the 1996 samples were subjected to Northern blot hybridisation. ESTs derived from the 1998 samples were selected for Northern blot analysis based on their sequence homologies and differential display patterns. Preference was given to ESTs with homologies to known genes and those which showed early changes in band density after the hormone treatments. Two ESTs, *fHa8* and *fDMP1*, were selected to obtain additional sequences on the basis of interesting Northern blot and *in situ* expression patterns.

7.2.3 Primers used in RACE

fHa8:

5' RACE	antisense primer 1	5' CCTTGGGTAATGGTTCTTGC 3'
	antisense nested primer1	5' GGGGCAATGGAACTAGATACTA 3'
	antisense primer 2	5' CTGCATAGAGAAAGTCAGCAAT 3'
	antisense nested primer 2	5' CCATACTGCCTGGATGTATAAT 3'
3' RACE	sense primer 1	5' AAGCCTGGTCCTTTGTCAGA 3'
	sense primer 2	5' CTGCATAGAGAAAGTCAGCAAT 3'
	nested sense primer 2	5' CCATACTGCCTGGATGTATAAT 3'

fDMP1:

5' RACE	antisense primer 1	5' GCTAGCATGGTTTACAACTAAAGAA 3'
	antisense primer 3	5' GATAGCGTTCAATATTGTTCATCAA 3'
	nested anti-sense primer 3	5' AGACCACATCCCCTGTTTCC 3'
3' RACE	sense primer 1	5' CTATCTAAAGGCACGCGGAA 3'
	sense primer 2	5' GAAGATAAGGATTCTCTGACTAA 3'
	sense primer 3	5' GAAGATAAGGATTCTCTGACTAACA 3'
	nested sense primer 3	5' GGATGTGGTCTAAGGAAGGA 3'

7.3 Results

7.3.1 Northern blot hybridisation

Northern blot analyses were conducted for 35 ESTs. Of these, 21 (60%) were shown to be differentially expressed (Table 7-1), 2 did not show variation across the hair growth cycle or treatments, and 12 (34%) did not provide any detectable signal. When the results from the two sample sets were compared, the major difference was that expression of more than half of the ESTs derived from the 1998 samples were undetectable, while only one out of 18 generated from the 1996 samples could not be detected (Table 7-1).

1996 ESTs: Differential gene expression for all 7 ESTs which showed homology to known genes was confirmed. *fHa8* and *fDMP1* were expressed only in samples with late proanagen or anagen follicles (Fig. 7-1). Three others, including *GnTI*, *titin* and *RPL4* (Fig. 7-1) were also differentially expressed. They were associated with all stages of hair follicle growth, but with different expression levels across hair growth cycle. *GnTI* expression was not present in footpad or mid-winter skin,

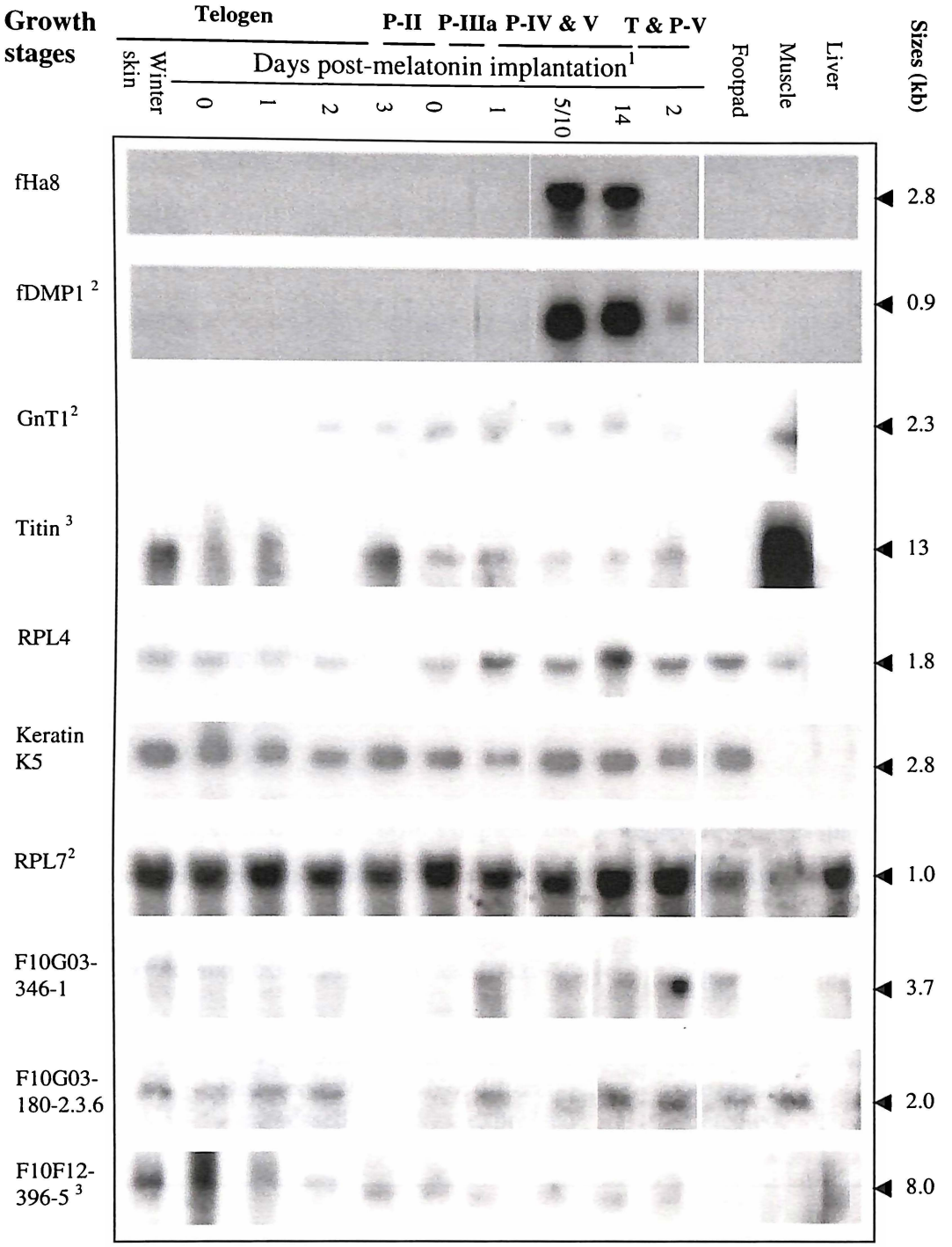


Fig. 7-1 Northern blot hybridisation for 1996 ESTs (I)

[1] Samples were arranged primarily according to follicle growth stages, then by days after melatonin treatment. Due to spontaneous hair growth, hair growth was not totally correlated with the time after the treatment (Chapter 4). Samples were selected to cover the whole proanagen period. P-II: proanagen II; P-IIIa: proanagen IIIa; P-IV: proanagen IV; P-V: proanagen V; T: telogen.

[2, 3] Results derived from the same blot have a superscript in common.

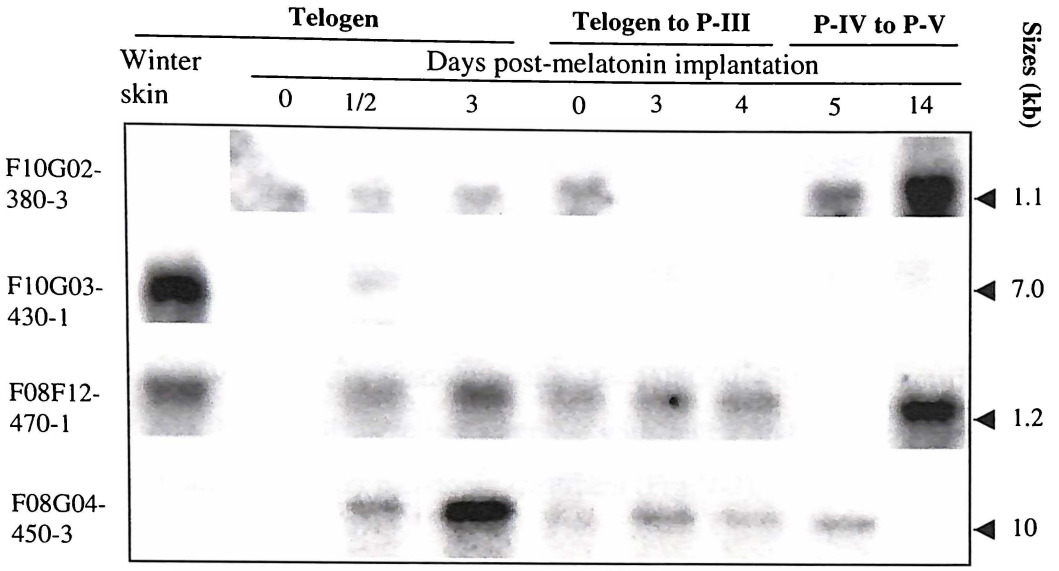


Fig. 7-2 Northern blot hybridisation for 1996 ESTs (II)

but was higher in samples with growing follicles. *Titin* is expressed at a higher level in skin samples with resting follicles, and its expression decreased as hair follicles entered proanagen. It expressed at a very high level in muscle, but not in footpad skin or liver.

RPL4 was expressed in all skin samples, but a higher level was particularly associated with anagen follicles. *Keratin K5* and *RPL7* were expressed relatively evenly across the follicle growth initiation, but *keratin K5* differed from *RPL7* in that it was not expressed in muscle and liver.

Table 7-1 Summary of Northern blot hybridisation for selected clones

	Differentially expressed	Not differentially expressed	No signal detected	Total
1996	12	2	1	15
1998	9	0	11	20
Total	21	2	12	35
%	60.0	5.7	34.2	100

For most of the ESTs which showed low, or no homology to known genes, their differential expression was also confirmed. The expression of F10G03-346-1 decreased during early proanagen and then increased in samples containing late proanagen follicles (Fig. 7-1). F10G03-180-2.3.6 showed higher expression in

samples containing proanagen V follicles. The expression of F10F12-396-5 was higher in samples without melatonin treatment and lower in samples with growing follicles (Fig. 7-1). The pattern was similar for its homologous EST, 96F08F12-368-7 (not shown). The expression level of F10G02-380-3 progressively increased, as follicles progressed through proanagen (Fig. 7-2). F10G03-430-1 was almost uniquely expressed in the winter skin sample (Fig. 7-2). Transcript F08F12-470-1 was expressed at higher level mainly in samples containing fully-grown follicles. In comparison, message for F08G04-450-3 was not detected in winter skin, but in all the other samples, with much higher level in the sample collected three days after melatonin treatment (Fig. 7-2).

1998 ESTs: Northern blot hybridisation was conducted for 20 ESTs, or 21 including *fHa8,* using different formats. With between 1-3 RNA samples from each treatment, differential gene expression was confirmed for 8 out of 14 ESTs examined (Fig. 7-3). One of these, *fHa8* was detected only in samples containing late proanagen and anagen follicles, which were collected 6 days after melatonin or melatonin plus progesterone treatments. For the other seven genes, melatonin administration either promoted or suppressed their day 0 expression. The expression of *colgn α2I* and *CoA ligase* were progressively enhanced between days 0.5 and 6 following melatonin treatment. However, *MGP* expression was increased at 0.5 and 2.5 days after the treatment and then fell at day 6. There was little change for *TIED* at day 0.5, but expression increased at days 2.5 and 6 after melatonin treatment. Expression of the unidentified EST F09G02-344-2 was raised to the same level at all the three time-points after melatonin administration. The expressions of *GS* and *UBL3* were decreased at 0.5 and 1 days after melatonin treatment, but returned to the day 0 level by day 6.

The effects of melatonin plus oestradiol or progesterone were interpreted by comparison with the effects of melatonin alone. The administration of melatonin plus oestradiol suppressed the expression of all seven genes. The expressions of *colgn α2I* and *TIED* were reduced at day 1 and totally suppressed at day 10 after the treatment. In contrast, the expression of *UBL3* was suppressed more markedly at day 1 than at day 10. For *CoA ligase*, the expression was totally suppressed at both days 1 and 10. The expression of *MGP* was almost completely suppressed at

day 10, with little change at day 1. The melatonin-increased expression level of the unidentified EST (F09G02-344-2) was not affected by oestradiol at day 1, but by day 10 it was reduced to the day 0 level. Similarly, the expression of *GS* was reduced at day 10.

Melatonin plus progesterone in males did not alter the expression level of *CoA ligase, UBL3* and F09G02-344-2 compared with day 6 levels of the same genes when only melatonin was administered. However, it promoted the expression of the other four transcripts. In females, the effects of progesterone and melatonin administration tended to be the same as that in males, although *colgn α2I, MGP, TIED* and *UBL3* expression levels at day 6 seemed to be somewhat reduced, by comparison.

Total RNA from four control tissues without hair follicles were included in the Northern blots. Transcripts *fHa8*, *CoA ligase* and *TIED* were solely expressed in samples with hair follicles, while *colgn α2I* was also expressed at very low level in footpad, but not in muscle, liver or adrenal gland. *MGP* and *UBL3* were expressed in footpad at approximately the same level as in flank skin. However, the expression of *UBL3* was not detected in other tissues examined, and *MGP* at a very low level. Interestingly, *GS* expressed at a much higher level in muscle than in flank skin and footpad, but not in liver and adrenal gland. In contrast, the unknown EST F09G02-344-2 was expressed in all tissues, with much higher levels in muscle. The expression of six other ESTs was not detectable by Northern blot hybridisation using the same RNA samples. These included genes coding for *TM9SH3, RTP/Ndr1/Drg1/TDD5* and an EST with some homology to *EVA*.

Five other ESTs with homology to signalling molecules or transcription regulators, namely *LAR, MEKK1, PCDC5RP, SREBP1/2* and *CIR* were examined by Northern blot hybridisation with samples that covered more time points after each treatment. However, expression was detectable only for *LAR*. A message of 7.1 kb was clearly detected in the sample collected 1 day after melatonin administration, but only weakly expressed in samples at other time points (results not shown).

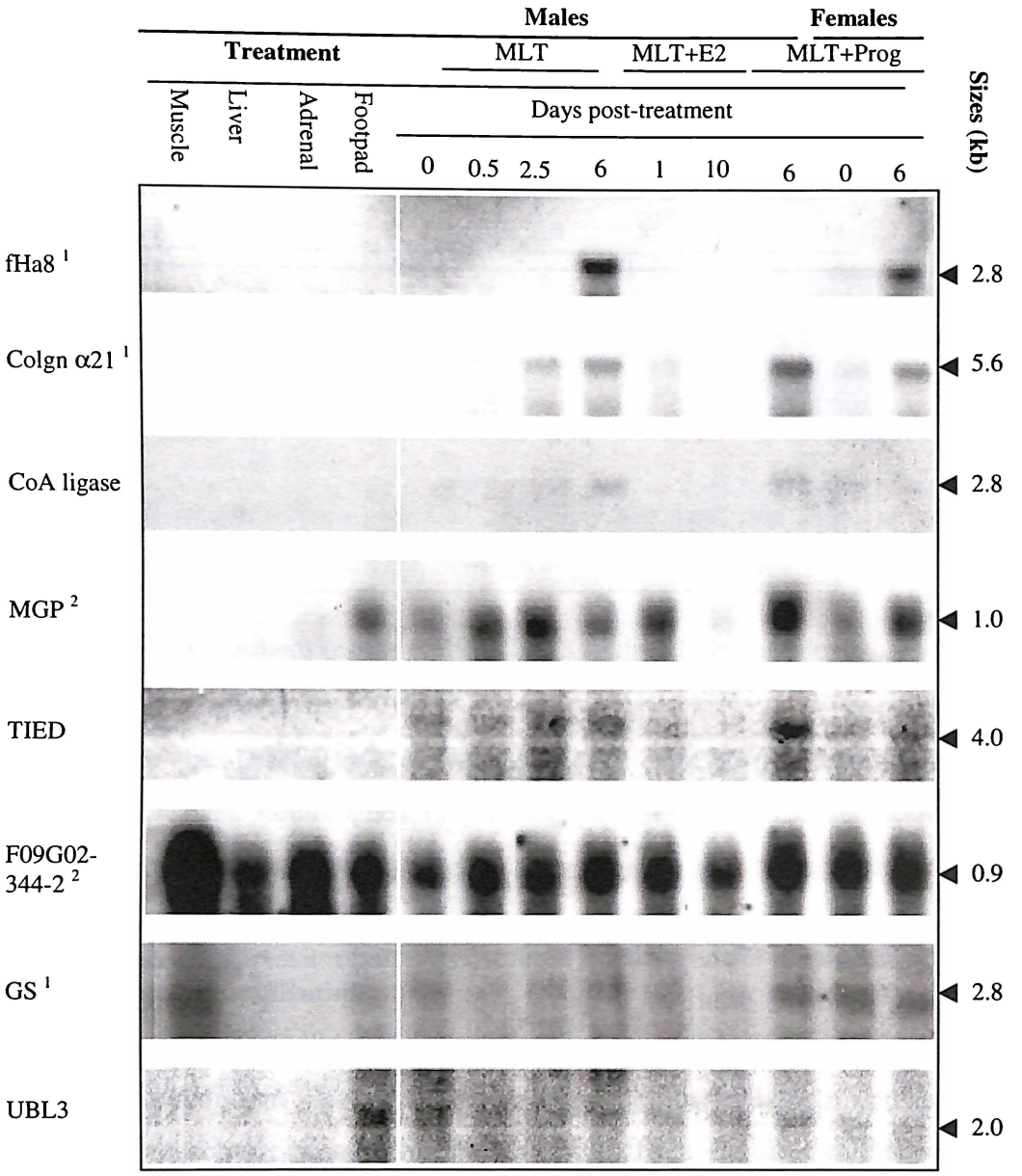


Fig. 7-3 Results of Northern blot for 1998 ESTs against 1998 RNA samples

[1, 2] Results derived from the same blot have superscript in common.

To establish the relationship between gene expression and all proanagen growth stages, three ESTs were subjected to Northern blot hybridisation using the 1996 RNA samples. The expression of *fHa8* commenced at a low level when follicles entered proanagen IIIb and increased markedly as follicle growth advanced. The expression was maximal when most follicles had entered anagen (Fig. 7-4). In contrast, the expression of an unidentified EST F10F12-255-2 was detected in samples from all growth stages, but was stronger in samples containing proanagen

follicles and maximal after follicles had entered anagen (Fig. 7-4). The expression of *MSSP1/2* was not detected using the same set of RNA samples.

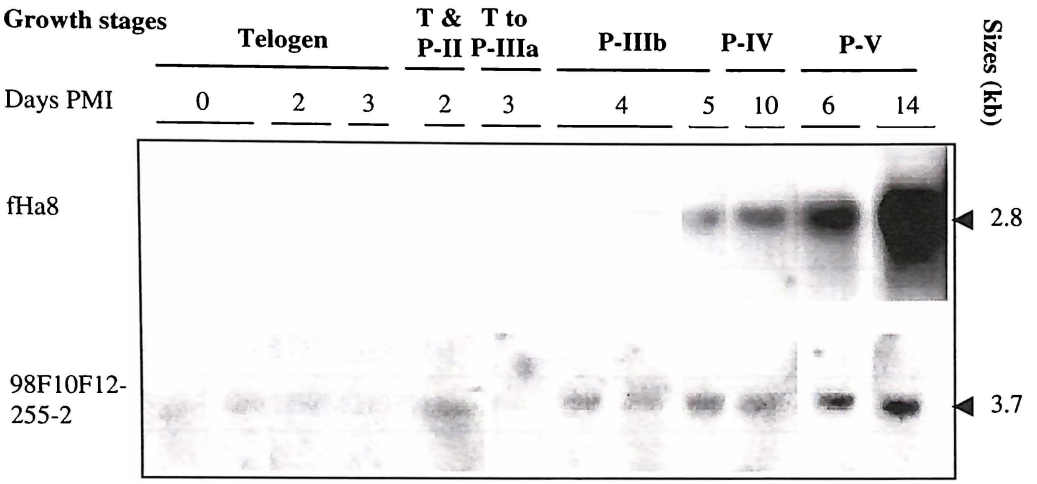


Fig. 7-4 Northern results of 1998 ESTs against 1996 samples

The sizes of the detected mRNA were generally close to that recorded in the literature for their homologues (Table 7-2). However, size differences were apparent for *DMP1* and *titin*.

7.3.2 Comparison of expression patterns detected by differential display and Northern blot hybridisation

The expression patterns of differential display were compared with the patterns generated by Northern blot hybridisation. Only the post-melatonin implantation patterns were compared, as insufficient samples from steroid treatments were included in Northern blot hybridisations to enable valid comparisons. The pattern was assessed as matched (Md), different (Dt) or reversed (Rd) (Tables 7-3 and 7-4). The distributions for the clones examined were 38%, 38% and 23% for matched, reversed and different, respectively (Table 7-5). However, the ratios for the same category varied significantly when the two years were compared separately. Only 4 out of 17 clones had matched patterns with samples from the 1996 trial, while the ratio was 6 out of 9 with 1998 samples.

Table 7-2 Comparison of the mRNA sizes detected with their homologues

Identity	Size in human[1] (kb)	Size in other species (kb)	Size in ferrets (kb)
CoA ligase		2.0 (AF126145, bovine)	2.8
Colgn α2I	5.4 (J03464)	4.6 (AB008683, bovine)	5.6
DMP1	3.8 (AF084530.1)	2.9 (U70017, mouse)	0.9
GS	2.7 (X59834)	2.8 (M91652, rat)	2.8
GnTI	2.6 (M55621)	2.5 (D16302, rat)	2.3
Ha8	2.8 (NM_006771)		2.8
Keratin K5	2.5 (M21389)		2.8
LAR	~8[2]		7.1
MGP	0.6 (NM_00090)	0.5 (X07363, bovine)	1.0
TIED/OSCP	2.4 (AB008375)/2.5 (AF072752)		4.0
RPL7	0.8 (NM_000971)	0.8 (NM_011291, mouse)	1.0
RPL4	1.4 (NM_000968)	1.3 (X99909, dog)	1.8
Titin	81.9 (NM_003319)		13
UBL3	2-4.5[3]		2.0

[1] Genbank entry numbers indicated in brackets.

[2] Variable between tissues (Longo *et al.*, 1993).

[3] Variable between tissues (Chadwick *et al.*, 1999).

Table 7-3 Comparison of expression patterns for 1996 ESTs

ESTs	Identity	Expression pattern in differential display	Expression pattern in Northern blot hybridisation	Classi-fication
F08F12-171-1[1]	DMP1	Only in samples with anagen follicles	Only in samples with anagen follicles	Md
F09G02-520-2	GnTI	Higher in telogen follicles, present in early proanagen follicles, but not in late proanagen follicles	Higher expression in samples with proanagen follicles	Rd
F10F12-180-2[1]	Keratin K5	Stronger in a telogen sample	Similar in all skin samples with hair follicles	Dt
F10G02-205-1[1]	RPL4	Strongest in samples with anagen follicles	Stronger in samples with anagen follicles	Md

F08F12-405-1[1]	RPL7	Only in samples with anagen and proanagen follicles	Similar in samples with follicles of different stages	Dt
F10F11-284-1	Titin	Mainly in samples with proanagen follicles	More strongly expressed in samples with telogen follicle	Rd
F10G02-295-1.2	fHa8	Present in P-II and P-IIIa samples and maximised when follicles entered anagen	Only in samples with late proanagen and anagen follicles	Md
F10G02-305-1.2	fHa8	Only in samples with P-IIIa and more advanced follicles	Only in samples with late proanagen and anagen follicles	Md
F10G02-380-1	fHa8	Only present in samples with telogen follicles	Only in samples with late proanagen and anagen follicles	Rd
F10G02-380-2	Not known	Only present in samples with telogen follicles	No difference across hair growth stages	Dt
F10G02-380-3	Not known	Only present in samples with telogen follicles	Gradually increased from telogen to anagen follicles	Rd
F10G03-430-1[1]	Not known	Stronger in the samples with anagen follicles	Strongest in winter sample, weaker in telogen day 1 PMI sample and even weaker for other samples	Dt
F10G03-346-1	Not known	Slightly stronger in telogen follicles	Higher expression in samples with P-IIIa or more advanced follicles	Rd
F10F12-396-5[1]	Not known	Present in early proanagen follicles	High expression in winter skin, but no difference across hair growth stages	Rd
F08F12-368-7[1]	Not known	Present in proanagen and anagen follicles	High expression in winter skin, but no difference across hair growth stages	Rd
F08F12-470-1[1]	Not known	Present in early proanagen	Stronger in winter skin and one telogen sample collected 3 days PMI; and even stronger in anagen follicles	Dt
F08G04-450-3	Not known	Very low in winter skin, increased gradually as follicle growth progressed	Not in winter skin, very strong in one telogen sample collected 3 days PMI; similar for other samples	Dt

[1] Differential display conducted with only three representative samples.

Table 7-4 Comparison of expression patterns for 1998 ESTs

Name of ESTs	Identity	DD pattern (days 0, 0.5, 1, 2.5, 6 & 10 PMI)	Northern blot pattern (days 0, 0.5, 2.5, 6 PMI)	Classi-fication
F08G01-620-1	Colgn α2I	Decreased gradually	Increased progressively	Rd
F09G02-377-3	CoA ligase	Very low in one D0 sample, increased at D6	Very low in D0, increased after receiving MLT	Md
F10F11-355-1	MGP	Increased D0.5-6 , but maximal at D2.5	Increased from D0.5 to D6 , but maximal at D2.5	Md
F09G02-528-1	TIED	Increased D0.5-6, maximal at D6	Low in D0, increased from D2.5 to D6	Md
F09G04-370-3	GS	High in D0, lower at D6 & D10, but not in D1 & D2.5	Decreased from D0.5 to D2.5, recovered at D6	Md
F09G04-227-1	UBL3	Increased from D1 and remained unchanged afterwards	Decreased at D0.5 and D2.5, recovered at D6	Rd
F09G04-301-1[1]	LAR	Highest at D10 and high at D0. Lowest at D0.5 and low at D2.5	Much stronger in the D1 sample and not observable after D2.5	Rd
F10G02-355-1	Not known	In anagen follicles and the density of band correlates with the percentages of late proanagen follicles	Strong expression only in the D6 samples which had 62% late proanagen follicles	Md
F09G02-344-2	Not known	Present in D0.5, 2.5 and 10, but not in D0 and D6	Increased from D0.5 to D6	Md

[1] Northern blots with more samples from each treatment.

Table 7-5 Summary of expression pattern comparison

	Matched pattern	Reversed pattern	Different pattern	Total
1996 samples	4 (23.5%)	7 (41.2%)	6 (35.3%)	17
1998 samples	6 (66.7%)	3 (33.3%)	0 (0)	9
Total	10 (38.4%)	10 (38.4%)	6 (23.1%)	26

7.3.3 Preliminary study of reverse RNA dot blot hybridisation

Two different methods of making probes for RNA dot blots were employed. Reverse transcription with a poly T primer followed by PCR (using a primer set as for differential display), generated smeared bands in the acrylamide gel. Reverse transcription with oligo dT anchored primer and PCR under the same conditions

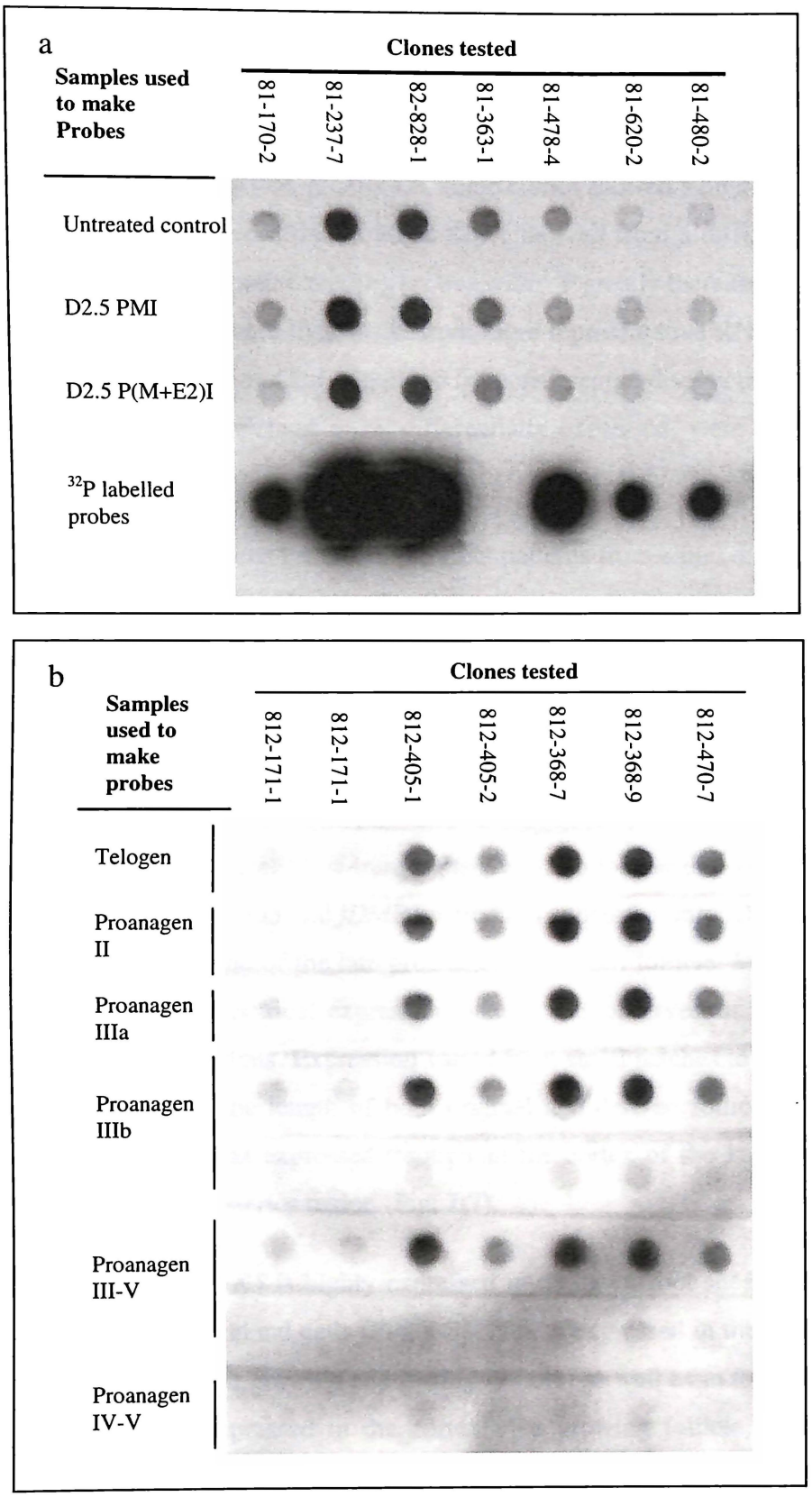


Fig. 7-5 Preliminary results of reverse RNA dot blot

P(M+E2)I: days after melatonin and oestradiol implantation.

produced distinct bands as in differential display. When the ^{33}P-labelled probes made from primer set F08G01 by the second method were hybridised to cloned DNA dotted on the nylon membrane, some clones showed a higher signal strength (Fig. 7-5a). However, clone F08G02-828-1 derived from a different primer, also showed a strong signal. Labelling probes with ^{32}P greatly increased the strength of the signal. Probes were then made from more representative RNA samples using another set of primers. Clones derived from differential display using the same set of primers and confirmed to be differentially expressed, were used to produce DNA which were dotted onto the matrix.

However, the dot blots generated different patterns from either differential display or Northern blot hybridisation (Fig. 7-5b). Some probes or clones did not generate any signal and the signal intensity varied considerably.

7.3.4 Localisation of gene expression

Of 17 clones examined, skin sites of expression were localised for 9. These genes were associated with hair follicles, either in a particular compartment or throughout the follicle. Most transcripts were also localised to other parts of skin (Fig. 7-6 to 7-11). *fHa8* and *fDMP1* were both expressed uniquely in the cortex of the keratinising zone of the late proanagen or anagen follicle. Most interestingly, for *fHa8* an asymmetrical expression pattern was observed in both longitudinal and transverse sections. Expression varied from small patches to almost the entire cortex and along the length of both original and derived follicles (Fig. 7-6). In contrast, DMP1 was expressed throughout the cortex of the keratogenous zone, starting from the exterior region (Fig. 7-7).

Epidermal keratin K5 is highly expressed in the outer-root sheath, the epidermis and the sebaceous gland cells (Fig. 7-8). *Titin* is expressed in the inner and outer-root sheaths of both growing and resting follicles as well as in the epidermis (Fig. 7-9). It is also expressed in the cortex of a growing follicle, but not in non-keratinised cells in the non-proliferative zone of the growing follicle. However, *titin* was also heavily expressed around the brush end and all over the lower portion of the telogen follicle, including the dermal papilla. The mRNA of an unknown gene (F10F12-396-5) was mainly localised in the inner-root sheath of

the growing follicle, but also at lower levels in the epidermis, the outer-root sheath and the sebaceous gland. However, its expression was largely absent from resting follicles (Fig. 7-10). *RPLA* and RPL7 were found to be widely expressed in the follicle inner-root sheath, outer-root sheath, cortex and medulla, and in the epidermis. Four other transcripts cloned from the same batch of differential display, eg, TIED, GnTI, 96F10G03-346-1 and 96F10G02-380-2.3.6 could not be detected in skin.

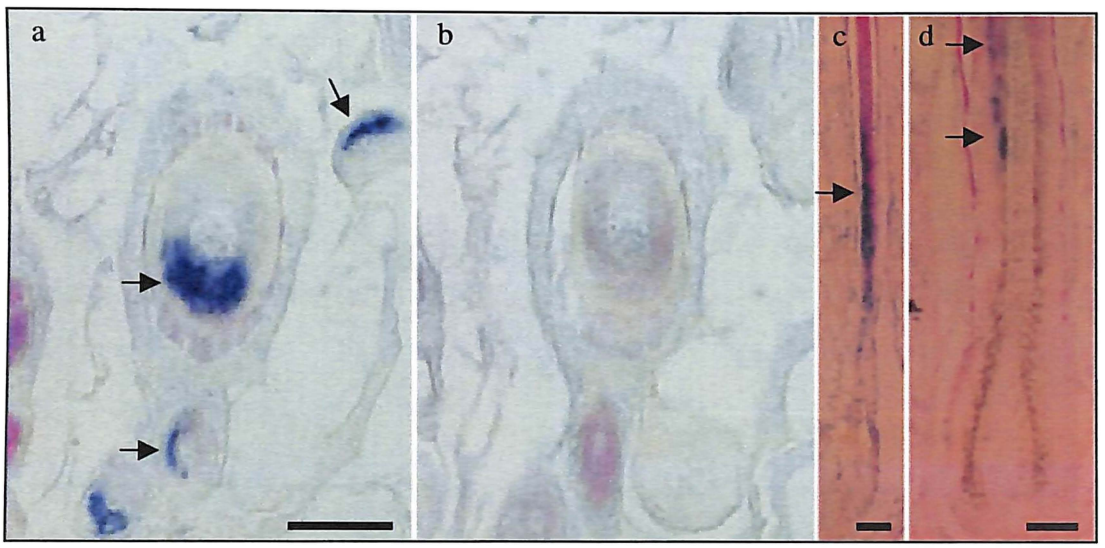


Fig. 7-6 Expression localisation of *fHa8*

a: Asymmetric expression in transverse section; b: Sense probe control; c: Asymmetric expression along the keratinising region; d: Expression appears to commence in a small number of cells in the inner cortex; Arrows point to sites of expression; Bars represent 50 μm.

Five clones derived from the 1998 samples were also examined by *in situ* hybridisation using skin sections from ferrets treated with melatonin in the 1996 trial. EST F10F12-255-2 was localised to nuclei of cells from all compartments of growing or resting follicles, as well as the epidermis and sebaceous glands (Fig. 7-11). The expression was stronger in the samples with hair follicles in advanced proanagen (Figs. 7-11b and d vs c) The expression of the remaining four transcripts, *CIR*, *TM9SF3* and two unidentified ESTs could not be visualised.

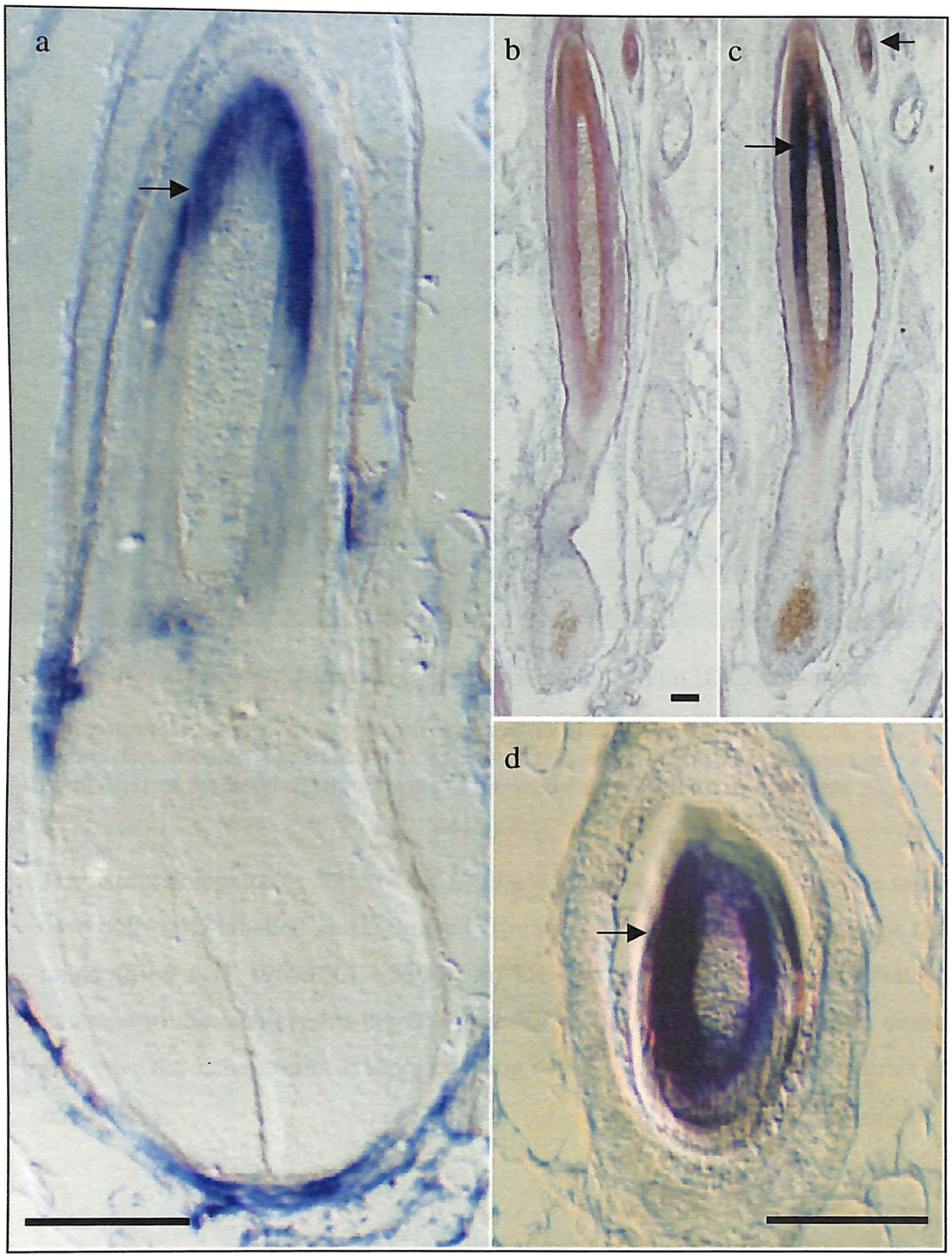


Fig. 7-7 Expression localisation of *fDMP1*

a and c: *fDMP1* expression in cortex of the keratinising zone in longitudinal section; b: Sense probe control; d: Expression in cortex of a transverse section; Arrows point to the site of expression; Bars represent 50 µm. a and c were taken with differential interference contrast (DIC) microscope.

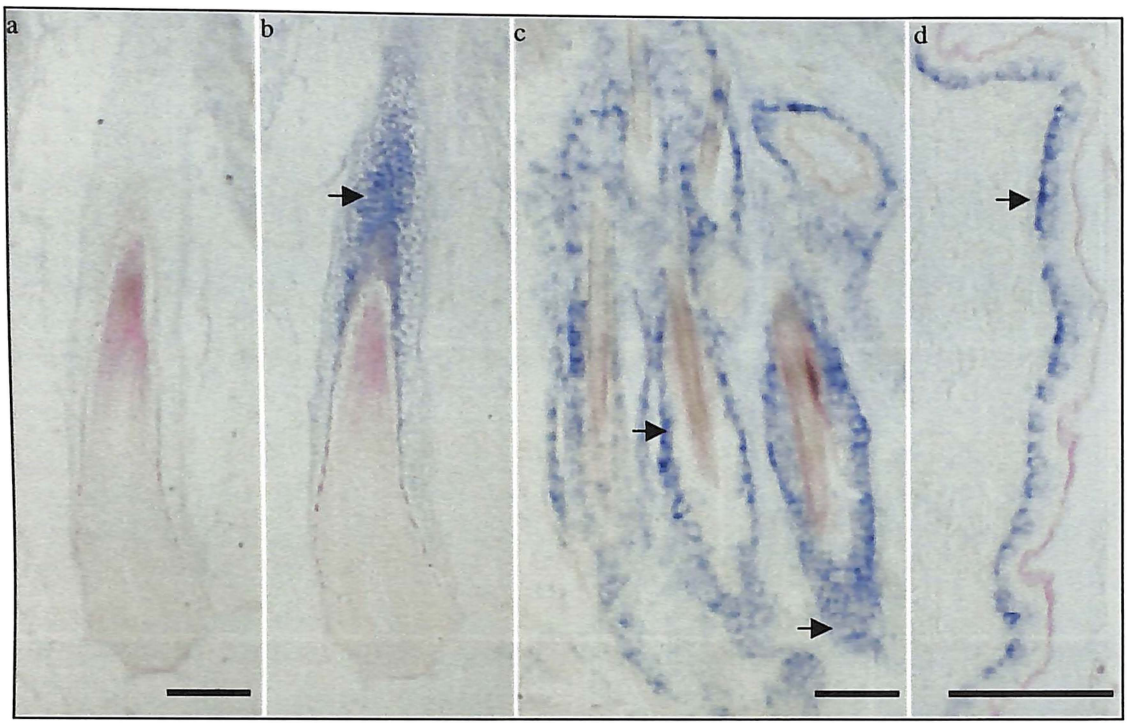


Fig. 7-8 Expression localisation of ferret epidermal keratin II K5 homologue

a: Sense probe control; b: expression in outer root sheath of a growing follicle; c: Expression in outer root sheath of a bundle of telogen follicles; d: Expression in basal layer of epidermis; Arrows point to the site of expression; Bars represent 50 μm.

The expression patterns detected for *fHa8* and *epidermal keratin k5* were the same with both DIG-labelled and ^{35}S-labelled *in situ*. No signal was detected for the unidentified EST (96F10G02-380-2.3.6) after exposure for a week, a similar outcome to that achieved using DIG-labelled probe. DIG-labelled stratifin probe also gave the same results as reported using radioisotope labelled probe (Rufaut *et al.*, 1999b).

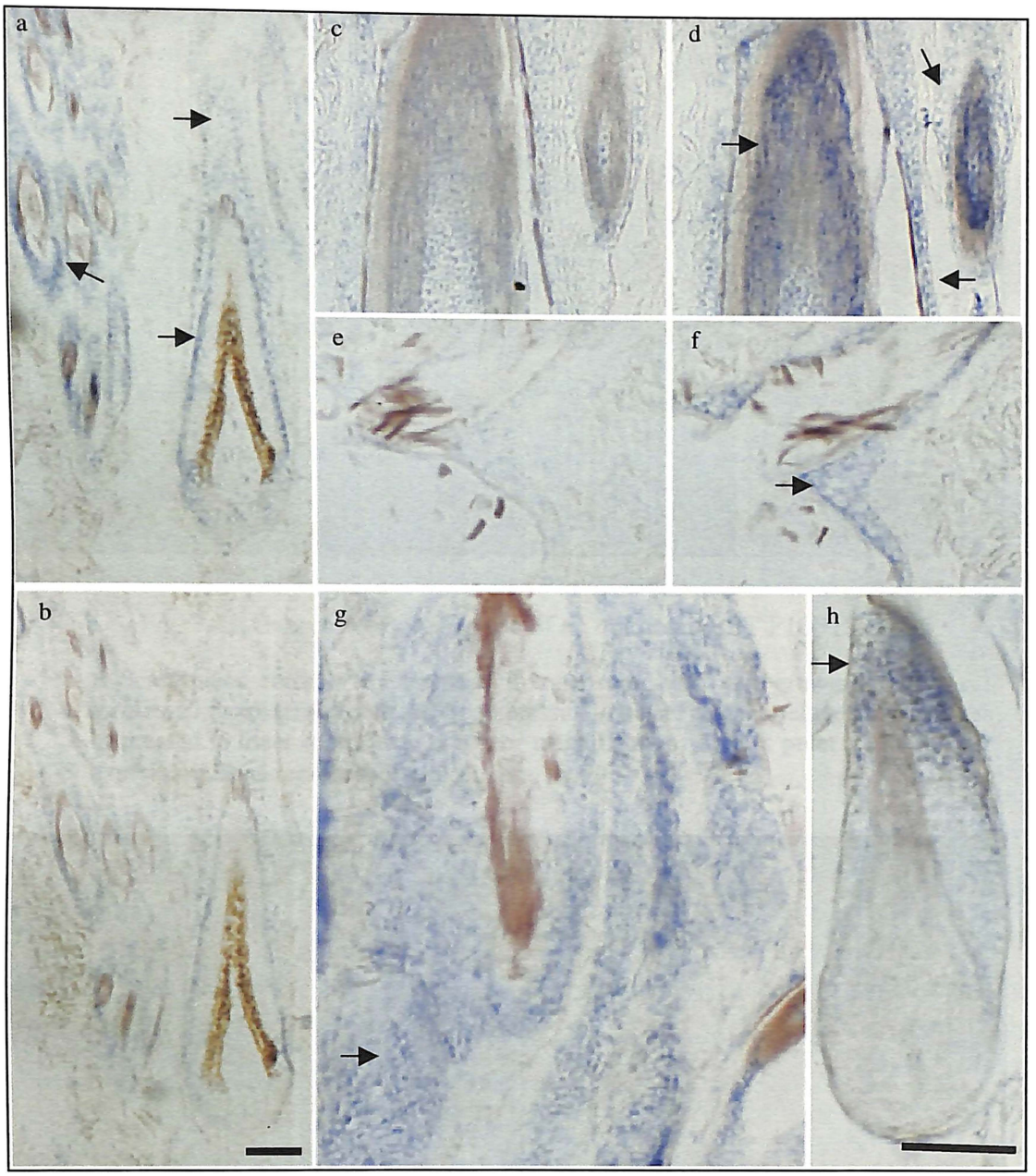


Fig. 7-9 Expression localisation of ferret *titin* homologue

a: Expression in the outer and inner root sheathes of a growing follicle; b and c and e: Sense probe control; d: Expression in keratinised cortex; f: Expressed in epidermal cells; g: Heavily expressed in lower portion of telogen follicles; h: Expressed in the outer root sheath at a sheep hair follicle; Arrows point to the site of expression; Bars represent 50 µm.

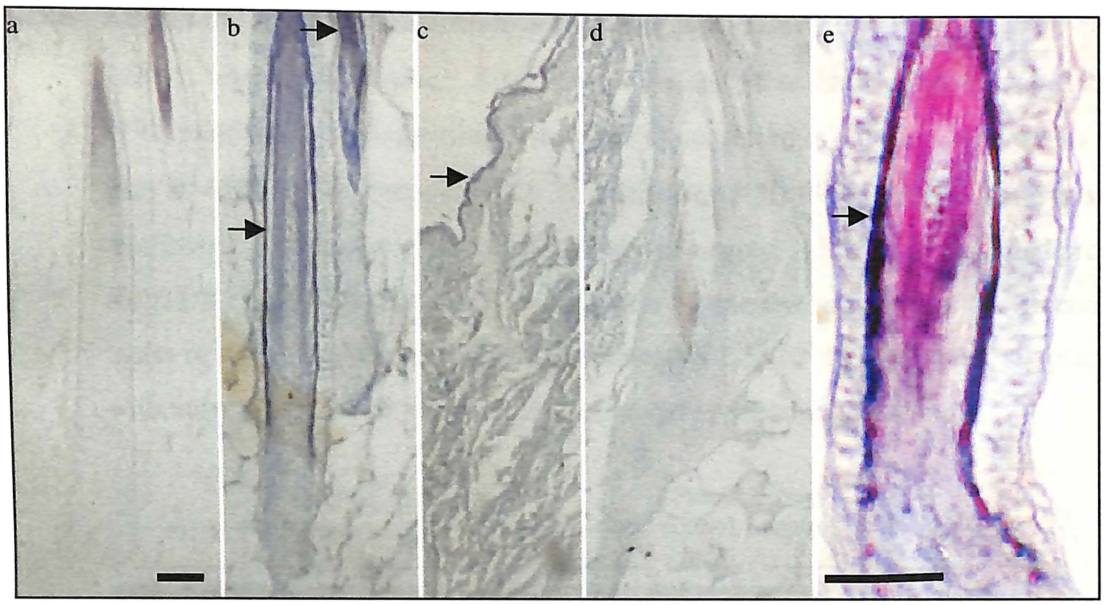


Fig. 7-10 Expression localisation of an unknown EST F10F12-396-5

a: Sense probe control; b: Expression in inner root sheath of a growing follicle; c: Expressed in epidermal cells; d: No expression in a late catagen-telogen follicle; e: Expressed in inner root sheath at higher magnification; Arrows point to the site of expression; Bars represent 50 μm.

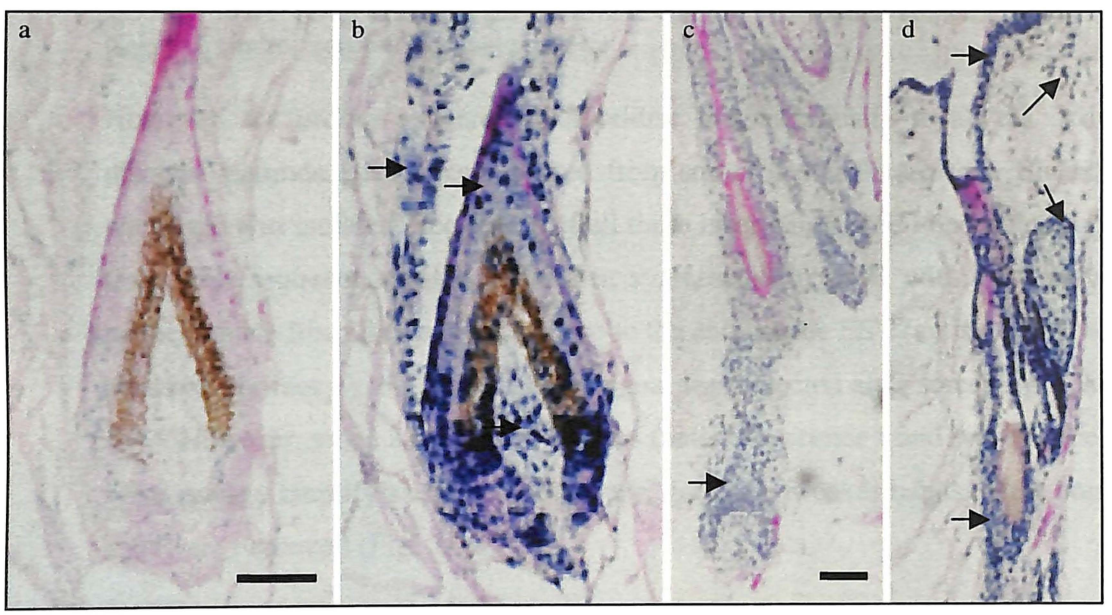


Fig. 7-11 Expression localisation of an unknown EST F10F12-255-2

a: Sense probe control; b: Expressed ubiquitously in cell nuclei in the lower portion of an anagen follicle; c: Expressed in an early proanagen follicle; d: Heavy expression around a club end of telogen follicle, epidermis, sebaceous gland cells and some inter-follicular cells. Arrows point to sites of expression; Bars represent 50 μm.

7.3.5 Additional sequence information for *fHa8* and *fDMP1*

cDNA library screening: After amplification, the library contained $3x10^{11}$ PFU/ml of lambda phage virus. The library was stored at 4°C for three weeks and then at -75°C in 7% (v/v) DMSO. The titre was $1.2x10^{10}$ PFU/ml when a tube of the library was thawed and used for library screening. Initially, ^{32}P labelled probes for *fHa8* (EST F10G02-355-1) were made by random priming of the linearised insert of the clone. By screening approximately $2.5x10^5$ PFU plaques, six plaques with weak signals were isolated and subjected to secondary screening.

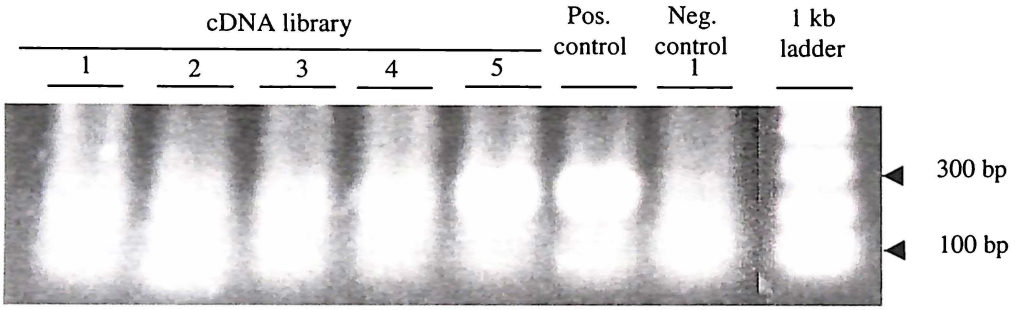


Fig. 7-12 Gradually decreased band density in serial diluted cDNA library

1-5: 10^{-5} to 10^{-1}serial diluted library; Positive control: 10^{-3} diluted cloned cDNA; Negative control: reaction without template.

However, the probes appeared to hybridise non-specifically to all the derived plaques. Lambda DNA was prepared from some of the plaques and Bluescript phagemid was isolated. Sequences of all these inserts indicated good homology to the vector sequence. Probes were then synthesised by PCR with gene specific primers made from the sequences close to the ends of the EST (antisense primer 1 and sense primer 1). No hybridisation signal was detected after screening $2.5x10^5$ PFU. However, when a new set of primers (antisense primer 1 and sense primer 2) were used to amplify serial diluted library, gradually weaker bands of the correct size were produced (Fig. 7-12).

RACE:

fHa8: The fragment cloned from differential display was 348 bp in length with limited homology to the 3' UTR of hHa8 mRNA. Primers lying within the sequence from differentially displayed cDNA tag (D-D tag) were made for both 5' and 3' RACE (Fig. 7-13). A fragment close to 850 bp was amplified with sense primer 2 and nested sense primer 2 (Section 3.10.2) and cloned (Figs. 7-13 and 7-

14a). The sequence contained a poly A tail and overlapping sequence to the original, and the original sequence was extended for 645 bp at the 3' end.

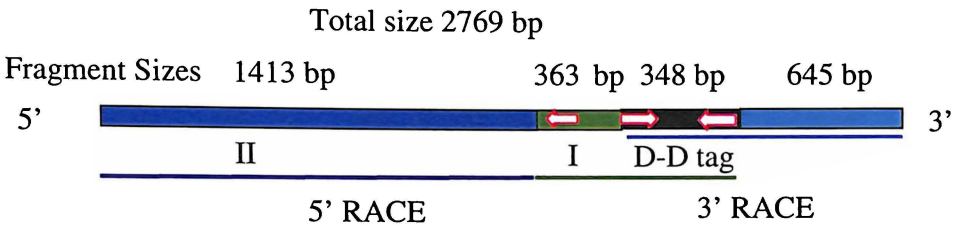


Fig. 7-13 Generation of additional sequence for *fHa8* by RACE

Antisense primer 1 and nested antisense primer 1 made from the original sequence amplified bands of approximately 700 and 300 bp in 5' RACE I (Fig. 7-13 and 7-14b) and the 700 bp fragment extended the original sequence by 363 bp. However, the extended sequence was still located outside of the coding region. Antisense primer 2 derived from the extended sequence information was used to reverse-transcribe total RNA at 50°C, instead of the recommended 42°C, to overcome mRNA secondary structure which might have prevented complete transcription. It amplified a fragment of approximately 1.6 kb which was subsequently cloned. This extended the sequence into the coding region of hHa8 and gave the transcript a total length of 2769 bp (Appendix I).

7.3.6 Comparison of *fHa8* and *fDMP1* sequences with their homologues

The extended sequence of *fHa8* has good DNA sequence homology to the 5' UTR and the whole coding region of the hHa8 mRNA (Appendix I-A). Despite a longer 3' UTR for *fHa8*, most of the sequence in this region is also reasonably conserved between the two species. A poly-adenylation signal is located at position 2728, followed by a stretch of adenine at position 2747. The size of the *fHa8* (2747 bp) is also similar to that of hHa8 mRNA (2837 bp). To a lesser degree, *fHa8* also has good sequence similarity to hHa7 mRNA (2728 bp) (Appendix I-B). In contrast to a longer 5' UTR in hHa7, *fHa8* has a much longer 3' UTR.

fDMP1: The original cloned fragment was only 181 bp and showed very high homology to a region close to the 5' end of both human and mouse *DMP1* mRNA. Primers for 5' and 3' RACE were made based on this sequence. 5' RACE with antisense primer 2 and nested antisense primer 2 produced a band of

approximately 800 bp (lane 1 in Fig. 7-15). This band extended the sequence to 760 bp in length at the 5' end. 3' RACE consistently generated a weak band around 900 bp (lane 2 in Fig. 7-15), but this was unable to be cloned.

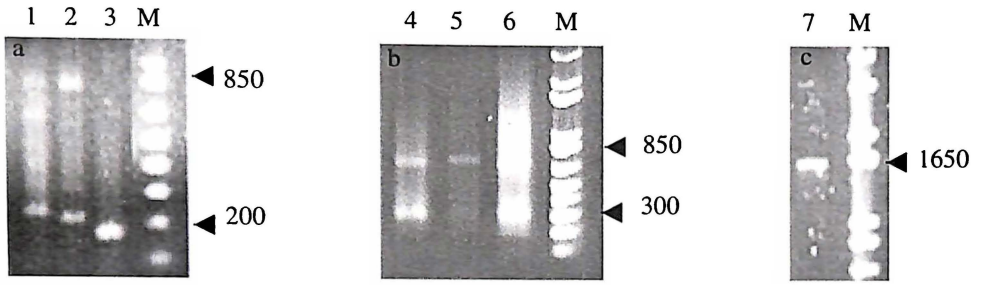


Fig. 7-14 *fHa8* RACE products visualised in agarose gel

a: 3' RACE with primer set 2; b: 5' RACE with primer set 1; c: 5' RACE with primer set 2; 1: AUAP + sense primer 2; 2: AUAP + nested sense primer 2; 3: cloned fragment control; 4: AUAP + nested antisense primer 1; 5: AAP + antisense nested primer 1; 6: AUAP + antisense nested primer 1 (20 µl reaction); 7: UAP + antisense nested primer 2; Arrows point to marker (M) sizes (bp).

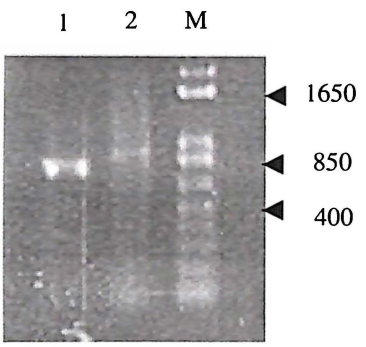


Fig. 7-15 *fDMP1* RACE products visualised in agarose gel

1: 5' RACE; AUAP+ sense primer 3; 2: 3' RACE; AUAP+ antisense nested primer 2; M: 1 kb marker; arrows point to the sizes of marker (in bp).

At amino acid level, *fHa8* had good homology to the entire 456 amino acid sequence of hHa8 (identities 76% and positives 83%) (Appendix I-C). The homology to hHa7 (471 amino acids) is lower (identities 70% and positives 82%), especially at the carboxyl terminus where hHa7 has a few additional amino acids (Appendix II-D).

The extended sequence of *fDMP1* has a start codon at the 232 position and shows excellent DNA sequence homology to part of the human DMP1 (94% identity from 45 to 804 bp) (Appendix II-A). Bases from 219 to 760 had 91% DNA

sequence homology to 235-776 bp of mouse DMP1. At amino acid level, this sequence showed only one amino acid difference to the first 176 amino acids of human DMP1 (identities and positives of 98% and 99%, respectively) (Appendix II-B), while homology to mouse DMP1 is also very high (identities and positives of 94% and 99%, respectively) (appendix II-C).

7.4 Discussion

7.4.1 Northern blot and differential display expression patterns

Confirmation of differential expression is an essential component of gene discovery using differential display. Verification by Northern blot was recommended by the inventor (Liang *et al.*, 1993), but different protocols have subsequently been used. Purified poly A RNA^+ was used to replace total RNA in some methods (Kallin *et al.*, 1991). This significantly increases the sensitivity. However, it requires large amounts of RNA and extra purification steps. Probes can also be synthesised from isolated bands (Liang and Pardee, 1992; Mou *et al.*, 1994), or cloned cDNA (Linskens *et al.*, 1995). However, as a band on a differential display gel can represent more than one cDNA species (Liang *et al.*, 1995; von der Kammer *et al.*, 1999) and bands of different sizes are quite often produced by amplification, probe synthesis directly from isolated bands can lead to subsequent specificity problems. Although a probe made from an individual cloned cDNA may not necessarily be derived from the differentially expressed gene of interest, the probability is increased if the cloned band is of the correct size. Therefore, all the probes for Northern blot analysis in this study were made from cloned cDNA fragments.

Of a total of 35 distinct ESTs examined by Northern blot hybridisation, 23 were confirmed to be expressed in ferret flank skin. More than 90% of these were differentially expressed during hair follicle growth initiation. Many were not expressed, or expressed at much lower levels, in tissues lacking hair follicles, which corroborates the close association of these transcripts with hair growth. This high percentage of differential expression and localisation in structures associated with hair growth initiation is encouraging. It indicates a higher specificity of the optimised differential display method, compared to the use of short primers and low annealing temperature in the original protocol (Liang and

Pardee, 1992; Bauer *et al.*, 1994). It also suggests that most other ESTs which have not been tested are likely to be differentially expressed and hair growth-related. The more changeable patterns of ESTs derived from the 1998 samples also demonstrated that the use of multiple, serially collected samples was particularly effective in detecting genes for which expression was gradually or transiently up or down regulated. The fact that a higher percentage of ESTs derived from the 1996 samples was verified to be expressed in skin is interesting. As many of these were structural genes related to hair follicle growth (Chapter 6), a reasonably high expression level could be anticipated. A lower percentage of confirmation for clones generated with the 1998 samples, which were in the very early stages of proanagen, is possibly due to lower levels of expression. Many of those that did not produce any signal from hybridisation were homologous to transcription factors or regulatory molecules, often expressed at lower levels than structural genes. More sensitive methods, such as Northern blot with poly A^+ RNA, real-time PCR or RNA protection assay may be required to confirm their expression.

Genes that were expressed exclusively, or predominantly, at a particular hair follicle growth stage are especially interesting. They might be growth stage-specific structural genes, like *fHa8*, or putative growth stage-specific regulators, like *fDMP1* (see below). EST F10G02-380-3 showed low homologies to transcripts of three different genes: *DIF2*, *IEX1* and *IEX1L*. The increased expression level of this EST in anagen follicles might suggest that it is not *DIF2*, as the expression of this gene has been found to decrease in differentiated cells (Pietzsch *et al.*, 1997). The expression of *IEX1* and *IEX1L* are associated with cell survival (see Chapter 8) (Kondratyev *et al.*, 1996; Wu *et al.*, 1998). The higher, but relatively small changes in collagen expression in skin containing anagen follicles agreed with previous reports (Ito and Sato, 1990; Makela *et al.*, 1990).

As *keratin II K5* is specifically expressed in mitotically active basal cells of different stratified epithelia (Moll *et al.*, 1982; Lersch *et al.*, 1989), it was, therefore, not surprising to find that its expression did not change significantly during hair follicle cycling and its expression was not detected in muscle and liver. The very high level of *titin* expression in muscle explains why it is much

better characterised in this tissue (Labeit and Kolmerer, 1995). However, the much smaller size of mRNA detected in ferret skin points to a different isoform (Eilertsen and Keller, 1992).

MGP has been found to be expressed at different levels in a number of tissues, but not in liver (Fraser and Price, 1988; Hale *et al.*, 1988) as observed in this study. Nil, or very low expression, detected in adrenal and muscle and suppression by oestradiol provide additional information on its tissue-specific expression and regulation.

UBL3 is a novel member of the ubiquitin-like proteins, highly conserved between species (Chadwick *et al.*, 1999). Structurally it is similar to *SUMO1* in that it lacks a lysine residue at the position corresponding to K48 of ubiquitin. Transcripts of various sizes (2.0-4.5 kb) have been detected at different levels in human adult tissues (Chadwick *et al.*, 1999). A signal of 3.0 kb was seen in various adult mouse tissues (Chadwick *et al.*, 1999), but the expression in skin tissues was not tested. In this study, a weak signal of 2.0 kb was detected in ferret skin and its expression was down-regulated by melatonin.

The expression of *LAR* has been confirmed by Northern blot hybridisation in a number of human tissues (Longo *et al.*, 1993), including lung, liver, brain, heart, muscle, kidney and pancreas. The expression level varied and transcripts of different sizes also detected, with the most common message being around 8 kb in most tissues. In ferret skin, a signal of approximately 7.1kb was only clearly seen in the sample collected one day after melatonin administration, indicating *LAR* expression level was transiently increased by this hormone. However, this stimulatory effect appeared to be suppressed by co-administration of oestradiol or progesterone with melatonin, as no signal was detected in these samples.

A few unidentified ESTs were also found to be expressed in a hair cycle-specific manner. More often, they showed higher expression in anagen follicles, but some had higher levels at other cycle stages. These transcripts may be associated with growth stage-specific structures or have a regulatory function. Therefore, results from Northern blots have not only confirmed the expression of many ESTs, but

provided useful information on candidates for further investigations. However, studies by others have demonstrated that Northern blot hybridisation cannot detect the expression of many differentially displayed genes (Bauer *et al.*, 1994; Linskens *et al.*, 1995). The low throughput and requirement for large amounts of RNA are also limiting.

As reverse dot/slot blots using probes made from reverse transcription may not be adequately sensitive for detecting rare transcripts (Callard *et al.*, 1994; Zhang *et al.*, 1996a), the use of probes made in the differential display PCR reaction should increase sensitivity. Screening for cDNA dotted onto a membrane with these probes is attractive both for its sensitivity and its potential for simultaneously screening multiple ESTs (Vogeli-Lange *et al.*, 1996).

In this study, to overcome the potential problem of mixed species of cDNA in a differentially displayed band, cloned cDNA amplified from plasmid was used as described (Callard *et al.*, 1994; Vogeli-Lange *et al.*, 1996). However, the results generated suggest optimised conditions are required. These would include ensuring an excess of template to probe and maintenance of the relative concentration of probes after amplification and labelling. However, the variation in expression levels and the likely differential amplification efficiency for different transcripts using the same set of primers may not conform with these requirements. Another potential problem is the cross-reactivity resulting from simultaneous synthesis of many probes. Half of the positive clones screened by a similar method failed to generate any signal in Northern blot analysis (Vogeli-Lange *et al.*, 1997). Differential expression patterns for five out of twelve clones derived from arrayed hybridisation, using probes made similarly, were not confirmed by RT-PCR (Trenkle *et al.*, 1999). Therefore, although the potential high sensitivity and throughput are attractive, the specificity of reverse RNA dot blot signals still needs to be established. Furthermore, as this method does not provide information on the size of messages, which can be useful for determining the identity of the transcript, this approach was not pursued further.

When the expression patterns detected by differential display and Northern blot hybridisation were compared, two thirds were similar between the two methods for the 1998 ESTs. This shows that the band density detected with differential

display often reflects the level of mRNA existing in the cells, when the differential display reactions were conducted in duplicate as for the 1998 samples. However, the expression patterns detected by the two methods were not always consistent. Differential display using the 1996 samples was conducted in two different formats and without duplication. Four out of the six ESTs which showed different patterns in the two methods, came from differentially displayed bands generated with only three mRNA samples. Hence, expression patterns generated with a limited number of samples may not be accurate. However, even in the case of differential display with the 1998 samples, where duplicate differential display reactions were used, a third of the expression patterns detected were opposite to that generated by Northern blots. Differential display was not considered to be quantitative with short primers and high PCR cycle numbers (40) (Bauer *et al.*, 1994). The reason why this happened when the cycle number was reduced to 22, as in this study, is not known. This could result from preferential amplification due to differences in priming and amplification efficiency of the different bands. It could also be due to the early saturation of abundant mRNA in PCR. It is also possible that cDNA from some individual differentials is comprised of mixed species with different expression patterns (von der Kammer *et al.*, 1999).

7.4.2 Localisation of expression sites

The identification of expression sites for a number of transcripts with the DIG-labelled RNA probe and a comparison with ^{35}S labelled probes confirm the usefulness and reliability of the method. The main advantages of DIG *in situ* hybridisation are speed (results often within two days) and avoidance of radioisotopes. Furthermore, DIG probes stored at -75°C for at least 12 months were found to perform well. However, hybridisation conditions, including the dilution of probes and hybridisation temperature, need to be optimised for each probe. Failure to detect the expression site for eight ESTs, particularly the 1998 ESTs, was possibly due to sub-optimal reaction conditions. However, some of these negative results could also reflect low expression levels. Alternatively, as skin sections from the 1996 trial were used, treatment-related or transiently expressed genes isolated from 1998 samples may not have been matched to appropriate skin sections.

As numerous interactions between the follicular and inter-follicular compartments are anticipated during the hair growth cycle (Hardy, 1992; Paus, 1996; Paus *et al.*, 1997b), gene expression in inter-follicular cells can have important regulatory roles in hair growth. The results of the *in situ* hybridisations have demonstrated that, while alterations in gene expression in ferret skin have centred on the hair follicle, many genes were also expressed in inter-follicular tissues. This reinforces the advantages of using whole skin as in this study.

All genes whose sites of expression could be localised were expressed differentially across the hair growth cycle. However, the temporal and spatial expression patterns varied. The expression of *fHa8* and *fDMP1* were localised to the keratinising zone of late proanagen or anagen hair follicles (Sections 7.4.5 and 7.4.6). The other ESTs examined were expressed more widely in hair follicles and skin. *Titin* is a giant sarcomeric protein responsible for the elasticity of striated muscle that may also function as a molecular scaffold for myofibrillar assembly (Maruyama, 1994; Trinick, 1994). The identification of a *titin* homologue in skin was a surprise. However, its expression in skin and hair follicles was confirmed by both Northern blot and *in situ* hybridisation, although at a much lower level compared with muscle. Cellular isoforms of *titin* have been reported in the terminal web region of intestinal brush border (Eilertsen and Keller, 1992) and more recently it has also been revealed as a chromatin protein (Machado *et al.*, 1998). As the size of the mRNA detected was only 13 kb, much smaller than its muscle homologue, both its relationship with *titin* and the role it serves in skin remain to be established. The higher level of expression in the lower telogen follicles might link to its role in chromatin structure (Machado *et al.*, 1998), as telogen cells are relatively inactive.

Although the expression of *keratin K5* did not alter markedly across the hair growth cycle, the slightly higher expression in samples with anagen follicles agrees with its sites of expression: the basal layer of epidermis and the outer root sheath. *Keratin K5* is one of the most abundantly expressed intermediate filament in keratinocytes and it pairs with K14 (Eckert and Rorke, 1988; Fuchs, 1988). These two proteins have attracted much attention as mutations have been found to be responsible for different forms of severe congenital disorders (Chan *et al.*,

1993). Furthermore, suppressed expression of K14 has been linked to transformation of human breast epithelial cells (Chen *et al.*, 1990). The expression of this protein may be also essential for normal function of the outer root sheath.

TI1 is a growth arrest-specific and transforming growth factor β regulated protein, originally found in a mink lung epithelial cell line (Ralph *et al.*, 1993). *TI1* shows homology to a family of transmembrane glycoproteins in lymphocytes and tumour cells (Kallin *et al.*, 1991). It is down-regulated by TGF-β and serum in quiescent cells and rapidly and transiently induced by TGF-β in actively growing cells. However, this gene was found to be widely expressed in the nuclei of many cell types, including dermal papilla cells of growing and resting follicles.

7.4.3 cDNA library screening

Screening a cDNA library is a common method to obtain longer sequence for a sequence tag generated by differential display. However, as the 3' non-coding sequence is usually less conserved than the coding sequence, probe made from this cDNA can cross hybridise to other genes, causing false positives and increasing the screening effort. And while screening of a cDNA library is straightforward, it is time consuming. Rare transcripts expressed only in a subset of cells or specific cycle stage may also be diluted out in a library, requiring screening a large number of plaques (Calvet, 1991). A serially diluted library was used to amplify *fHa8* (likely to be present in high copy number) as an assessment of the library. The density of the band was proportional to the dilution and a clearly visible band was amplified even when the library was diluted 10,000 fold. Based on this preliminary evidence, the cDNA library is believed to be of useful quality, although further successful screening, with both abundant and rare messages, is required to fully validate it. The unsuccessful screening of this library with probes made from *fHa8* sequence tag was mainly due to the design of the original primers derived from the ends of the original EST. One of the primers was not adequately accurate to generate specific probe. This primer also did not generate useful sequence in RACE.

7.4.4 RACE

RACE is a rapid procedure for obtaining longer sequence from a cDNA fragment and it bypasses the need for cDNA library construction (Frohman, 1993). It suits

cDNA fragments cloned from differential display in particular, as most of the fragments are expected to be located at the 3' end of the mRNA. With proper primers designed from the known sequence, 5' RACE, under optimised conditions, should generate all the upstream sequences for these ESTs.

However, with the two ESTs chosen for RACE, this is not the case. Although *fHa8* is located in the 3' UTR of the mRNA, it did not originate from the 3' end. Instead, it was derived from a stretch of adenines a few hundred bp upstream. The cloned *fDMP1* cDNA fragment showed homology to the internal coding regions of human and mouse homologues. Hence, both 5' and 3' RACE were required to obtain full sequence for these transcripts. For *fHa8*, the first 5' RACE with reverse transcription conducted at 42°C generated two bands of approximately 700 and 300 hundred bp and only the longer fragment extended the sequence. With a new primer from the extended 5' sequence and reverse transcription conducted at elevated temperature, a much longer fragment was amplified and extended the sequence to cover the whole *hHa8* mRNA. This outcome suggests that reverse transcription at a higher temperature aids in overcoming secondary structures which prevent synthesis of complete sequences.

For *fDMP1*, 5' RACE generated a sequence tag of 760 bp in total. It contained the first 176 amino acids and all the 5' UTR of human homologue except the first 34 bp. As human and mouse homologues are 3767 and 2903 bp, respectively, attention was focused on the 3' extension of large fragments of a few hundred bp or longer. A weak band of approximately 850 bp was repeatedly produced by 3' RACE, but it was not able to be cloned. However, this band may have been an artefact given that the size of mRNA detected was only approximately 900 bp by Northern blots, much smaller than its homologues in human and mouse.

7.4.5 *fHa8* and its homologues

Human hair keratin intermediate filaments (IFs) are classified into two subfamilies according to their isoelectric points: hair acidic (Ha) or type I hair keratins, and hair basic (Hb) or type II hair keratins. All the IF proteins are predicted to share common secondary structures (Steinert and Roop, 1988; Fuchs and Weber, 1994) with a central α-helical domain (rod domain), which is flanked by non-helical head (amino-end) and tail (carboxyl-end) domains. There was

originally thought to be only 10 hair keratins, five type I and five type II (Heid *et al.*, 1986; Heid *et al.*, 1988). However, nine type I hair keratins (Rogers *et al.*, 1998) and six type II hair keratins (Rogers *et al.*, 2000) have now been identified. On the basis of sequence homologies, human type I keratin genes can be divided into three groups. The first group consists of highly related hHa1, hHa3-I, hHa3-II, and hHa4. The second group comprises the novel genes hHa7 and hHa8, as well as pseudogene φhHaA, while the structurally less related genes hHa6, hHa5, and hHa2 are constituents of the third group (Rogers *et al.*, 1998). The amino acid homologies (positives/identities) between hHa8 and hHa7 are more than 98%/96% for both the head and rod domains (Langbein *et al.*, 1999), but lower in the tail region.

The expression patterns of all the nine type I keratins have been established (Langbein *et al.*, 1999). Two different groups of type I hair keratins were distinguished: a strongly expressed, coomassie-stainable series containing hair keratins hHa1, hHa3-I/II, hHa4, and hHa5, and a weakly expressed, immunodetectable series comprising hHa2, hHa6, hHa7, and hHa8. *In situ* hybridisation and immunohistochemical expression studies on scalp follicles show that two hair keratins, hHa2 and hHa5, define the early stage of hair differentiation, i.e. hHa5 expression in hair matrix and hHa2/hHa5 coexpression in the early hair cuticle cells. Matrix cells sequentially express hHa1, hHa3-I/II, and hHa4, supplemented by hHa6 at an advanced stage of cortical differentiation, and hHa8, which is expressed heterogeneously in cortex cells. Thus, six type I hair keratins are involved in the terminal differentiation of anagen hairs. The expression of hHa7 conspicuously differs from that of the other hair keratins in that it does not occur in the large anagen follicles of terminal scalp hairs, but only in central cortex cells of the rarer and smaller follicle type which gives rise to vestigial (vellus) hairs. In human scalp hair follicles, the expression of hHa8 and hHa7 was found to be very weak at the mRNA level (Langbein *et al.*, 1999). Although the heterogeneous onset of *fHa8* expression appears to be similar to both hHa8 and hHa7, its expression commences in follicles at proanagen IIIb and is at a much higher level during anagen. This ferret transcript is considered to be the hHa8 orthologue as it has a higher identity and is expressed in both original and derived hair follicles. However, there are some differences between *fHa8* and

its human orthologue. For instance, the expression of *fHa8* is distinctly asymmetric and in only part of the fibre cortex. As the expression of hHa8 was identified at protein level, it is possible, although unlikely, that the human gene is transcribed in a similar pattern, but the protein expression is further restricted.

The uneven distribution of *fHa8* is particularly interesting, as it may be linked to specific cell types comprising the hair shaft and contribute to important fibre properties such as crimp frequency. The biological basis of crimp in hair is not understood. It is thought to arise from the bilateral configuration of orthocortical cells on the outside of the crimp curve with a cluster of paracortical cells on the inside of the curve (Fraser and Rogers, 1955; Fraser, 1964). The orthocortex and paracortex of wool are also associated with the localised presence of keratin-associated proteins (although not, to date, keratin IFs) (Powell *et al.*, 1991). However, staining using Janus Green could not differentiate between cortical cell types in ferret hair follicles, as in sheep wool follicles (Fraser, 1964; Clarke and Maddocks, 1965). In addition, ferret fibre lacks crimp. Hence, the correspondence of *fHa8* expression with the formation of orthocortical or paracortical cell types awaits confirmation. Differential cortical cell keratinisation in ferret hair with no apparent curvature also raises interesting questions about the nature of fibre crimping. Interestingly, the human pseudogene φhHaA is a functional gene in both the chimpanzee and gorilla. Significantly, both φhHaA and hHa1 are mutually exclusively expressed in one side of the chimpanzee hair cortex, of which also lacks crimp (M. A. Rogers, 2000; personal communication).

7.4.6 *DMP1*

DMP1 (cyclin D-interacting myb-like protein) is a transcription factor originally identified in the mouse (Hirai and Sherr, 1996). Its mRNA and protein are ubiquitously expressed throughout the cell cycle in mouse tissues and in representative cell lines. It is also present in all the human and mouse tissues tested, although the levels of mRNA in human peripheral tissues and different sites of the brain vary (Bodner et al., 1999). *DMP1* has three myb-homology repeats in tandem and binds specifically to nanomeric DNA consensus sequences CCCG(G/T)ATGT to activate gene expression. A subset of its binding sites containing the GGA core are also bound by ets transcription factors, which

compete with *DMP1* for binding (Hirai and Sherr, 1996; Inoue and Sherr, 1998). Flanking the central DNA binding domains are two acidic transactivation domains.

The most intriguing feature of this protein is that it binds to type D cyclins and enables cyclin D-dependent kinases to regulate gene expression in a pRb-independent pathway (Hirai and Sherr, 1996). Cyclin D is found to repress *DMP1* transactivation activity specifically by preventing it binding to DNA and transcribing targeted genes (Inoue et al., 1998). The effects of *DMP1* on gene expression do not rely on CDK mediated phosphorylation (Inoue and Sherr, 1998). More importantly, mouse *DMP1* negatively regulates cell cycle progression by arresting mouse fibroblast in G1 phase and preventing entry into S phase (Inoue and Sherr, 1998; Bodner et al., 1999; Inoue et al., 1999). Disruption of its activity or deletion of the gene results in compromised function of the ARF locus and, facilitates cell immortalisation, transformation and tumorigenesis (Sherr, 1996; Bodner et al., 1999; Inoue et al., 2000).

Therefore, *DMP1* normally serves to maintain cells in a quiescent state by regulating cell cycle progression-related genes. One of these genes is $p19^{ARF}$ encoded by the INK4a/ARF locus (Inoue et al., 1999; Inoue et al., 2000). After p53, the INK4a/ARF locus is perhaps the second most commonly disrupted locus in cancer cells (Ruas and Peters, 1998). $p19^{ARF}$ stabilises and activates p53 to promote either cell cycle arrest or apoptosis. $p19^{ARF}$ binds to Mdm2, a p53 inhibitor, to promote MDM2 degradation and suppress its transcription, thus potentiating the transcriptional activity of p53. In return, p53 binds to a single canonical recognition site in the ARF promoter to activate gene expression. Therefore, high levels of $p19^{ARF}$ synthesis induced by *DMP1* causes p53-dependent cell cycle arrest, but not programmed cell death (Inoue et al., 1999).

The unique expression of *fDMP1* in the highly differentiated cortical region of late proanagen and anagen hair follicles, but not in other tissues tested and other parts of the skin, is interesting. As cell division is suppressed in the area of the follicle where it is expressed, the expression of *fDMP1* may be associated with cortical cell growth arrest, thus facilitating differentiation. Although the sequence obtained is highly homologous to both the human and mouse transcripts, the size

of the ferret homologue is much smaller than those in the other two species. Based on its restricted expression in late proanagen and anagen follicles and its smaller size, the ferret homologue is possibly a new member or an alternatively spliced form with specific functions. Two very recent GenBank entries (Accession Nos. AF202144 and AF202145) which encode shorter isoforms of *DMP1* appear to support this conclusion, although no report has been published on their functions. Further extension of the *fDMP1* sequence at the 3' end and analysis of its effects by functional interruption or overexpression would define the relationship with the human and mouse homologues and aid in deciphering a role in hair follicle growth.

7.5 Conclusion

Differential expression of approximately 90% of the detectable ESTs was confirmed by Northern blot analysis. For many of these, expression levels were different (usually absent or low) in tissues lacking hair follicles. All the ESTs whose expression was confirmed by *in situ* hybridisation were localised to hair follicles. Some were expressed in only certain growth stages, others in all growth stages, but at different levels. This close association of expression with the hair follicle and hair follicle growth suggests that most of the other ESTs derived from differentially displayed bands are also likely to be related to hair growth. The lower proportion of the 1998 ESTs confirmed by Northern blot hybridisation, and the greater number of regulatory molecules identified, suggested low expression levels for many ESTs during early proanagen.

The asymmetric expression of *fHa8* may link it to formation of specialised cortical cells. The differences in expression patterns and protein size for *fHa8,* compared with its human orthologue, may suggest a novel function in ferret hair follicles. *fDMP1* is likely to be an alternatively spliced form of *DMP1* with a role in regulating cortical cell differentiation.

CHAPTER 8 BIOLOGICAL ROLES OF THE IDENTIFIED TRANSCRIPTS

8.0 Abstract

The transcripts identified in this study were classified into functional categories, including transcription regulation and signalling, growth and differentiation, extracellular matrix (ECM), cell adhesion (CAM) and other structural molecules, fatty acid and cholesterol synthesis, metabolic enzymes and ribosomal constituents. Current knowledge of the biology of these molecules in other tissues suggests that many could have similar or related functions in hair growth cycling. Transcripts involved in cell structure/motility comprised of approximately one third of all the identified transcripts. This percentage is not only higher than that has been reported in human tissues generally and especially in normal human skin, but is also higher than that in cultured dermal papilla cells of rat vibrissae. Strikingly, a significant number of these transcripts encode proteins previously found to be associated with cancer cell metastasis. Some of the genes which were differentially expressed, depending on whether the treatments were stimulatory or inhibitory to hair growth, could point to key molecules controlling hair follicle cycling. Further work to confirm differential expression for other cloned ESTs and the elucidation of their functions will not only lead to better understanding of hair growth cycling, but may have potential application to other normal and cancerous growth processes.

8.1 Introduction

The earliest events in hair follicle regrowth include changes in the morphology of dermal papilla cells (Young and Oliver, 1976; Young, 1980; Adelson *et al.*, 1992). This is followed by increased DNA synthesis in the dermal papilla (Pierard and Brassinne, 1975; Silver and Chase, 1977) and hair germ cells (Silver and Chase, 1970; Silver and Chase, 1977) thought to be derived from the follicle stem cells in the follicle bulge region (Cotsarelis *et al.*, 1990; Wilson *et al.*, 1994; Lyle *et al.*, 1999; Taylor *et al.*, 2000). The proliferation of these transiently amplifying cells in the germinal matrix and the subsequent upward movement and differentiation of their daughter cells form the different structures of the anagen hair follicle. During this stage, the hair follicle gradually extends further into the dermis (Chase, 1954;

Young, 1980; Hansen *et al.*, 1984). Simultaneous changes in various interfollicular cell populations accompany these processes.

Apoptosis has been associated with hair follicle regression during catagen (Seiberg *et al.*, 1995; Lindner *et al.*, 1997). In this phase of the cycle, the dermal papilla and cells in the upper bulb and matrix translocate much closer to the epidermis (Straile *et al.*, 1961). Cells in the dermal papilla and the germinal matrix, as well as those in some other locations also show marked morphological changes during different hair cycle stages (Straile *et al.*, 1961; Silver *et al.*, 1969; Young, 1980). Thus, the current view of some of the biological processes involved in hair growth cycle can be summarised as in Fig. 8-1. More details on the processes involved in hair follicle cycling is provided in Section 2.1.

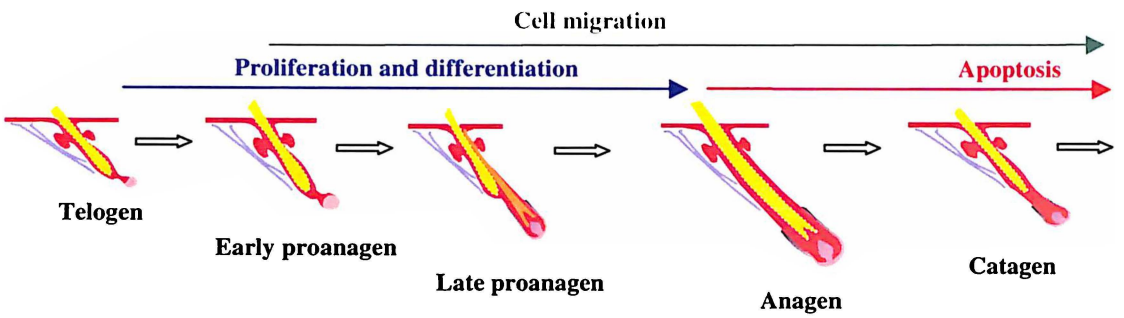


Fig. 8-1 Known biological processes associated with the hair growth cycle

However, no experimental data on the global gene expression patterns during hair growth initiation have been reported to complement these anatomical and physiological observations. In this study, two sets of samples were used for studying the patterns of gene expression underlying hair growth initiation. The 1996 sample set covered all proanagen stages and was suitable for discovering genes associated with structural formation of the fibre, as well as the regulation of all stages of proanagen. Most of the 1998 samples contained no histologically identifiable new hair follicle growth, but the commencement of cell proliferation was confirmed by increased expression of PCNA (Chapter 4). Some cell differentiation also occurred, as a small proportion of hair follicles observed later in the experiment had entered proanagen II and III.

8.2 Classification of transcripts by general function

The transcripts which showed sequence homology to GenBank entries were putatively classified according to their roles in other species or tissues. The categories were transcription regulation and signalling, proliferation and differentiation, ECM, CAM and other structural, metabolic enzymes, and protein synthesis and degradation (Table 8-1). Although some transcripts have multiple functions, they are listed in only one category for simplicity.

To enable an easier appreciation and systemic discussion of their potential involvement in hair growth, these transcripts are reclassified into more specific biological or biochemical processes (Table 8-2), on the basis of reported functions in other species and tissues. Transcripts with alternative functions are multiply classified. The putative functions of these groups of transcripts, their interrelationships and relevance to hair follicle growth are discussed in the following sections.

8.3 Transcription regulation and signalling molecules

Six transcription regulators were identified. These were *TBP, BTEB SREBP1, Sox9, MSSP1/2* and *CIR.* These proteins exert their effects by modulating the transcription of other genes. Only the two general transcription factors, *TBP* and *BTEB* and two signalling molecules, *LAR* and *MEKK1* will be discussed in this section. The other transcription factors are covered in the following sections in the context of their roles in other specific biological processes.

Two ESTs from differentially displayed bands showed homology to *TBP*, an evolutionarily conserved protein that exists primarily as a subunit of several large complexes. The polymerase II-specific complex, termed TFIID, consists of *TBP* and 10-12 TBP-associated factors (TAFs) (Buratowski, 1994; Hahn, 1998). The binding of *TBP* to the TATA box of a promoter initiates the formation of transcription machinery. A strong correlation between *TBP* binding and transcription activity has been found and *TBP* occupancy can be modulated by other factors (Kuras and Struhl, 1999; Li *et al.*, 1999). Although a single *TBP* is sufficient for TATA element recognition and subsequent assembly of other basal transcription factors to form a

Table 8-1 A general classification of identified transcripts: possible roles and time of initial expression alteration

	Name	Primary Functions and references	First change[1]
Transcription regulators and signalling molecules	BTEB1	A GC box binding transcription factor (Imataka *et al.*, 1992; Kikuchi *et al.*, 1996)	0.5 (2)[2]
	LAR	Homologous to neural cell adhesion molecule (N-CAM) whose intracellular domain is a receptor-linked tyrosine phosphatase (Pot *et al.*, 1991). It regulates cell growth by dephosphorylating proteins involved in tyrosine kinase signal transduction through cell-cell or cell-matrix interactions (Schaapveld *et al.*, 1995)	0.5
	MEKK1	An important component to relay extracellular signals to MAPK pathway which regulates a variety of processes, including gene expression, metabolism and cytoskeleton structure (Davis, 1993; Cobb and Goldsmith, 1995; Schlesinger *et al.*, 1998). Cleavage of MEKK1 is required for its participation in apoptosis (Schlesinger *et al.*, 1998).	0.5
	MSSP1/2	Regulation of c-myc transcription and replication (Niki *et al.*, 2000a; Niki *et al.*, 2000b) and induction of apoptosis (Iida *et al.*, 1997)	0.5
	SREBP1	Regulation of biosynthesis and homeostasis of fatty acid and cholesterol (Brown and Goldstein, 1997; Osborne, 2000) and involved in apoptosis (Wang *et al.*, 1996)	0.5
	TBP	A basic transcription factor of TIID complex, responsible for transcription initiation though binding to the TATA box motif (Buratowski, 1994; Kuras and Struhl, 1999)	0.5
	CIR	A corepressor linking the DNA binding factor CBF1 to the histone deacetylase complex (Hsieh *et al.*, 1999)	1
	PCDC5RP	Regulation of G2/M entry (Ohi *et al.*, 1994; Bernstein and Coughlin, 1998) and transduction of mitogenic signalling (Bernstein and Coughlin, 1997)	1
	Sox 9	Involved in male phenotype formation (De Santa Barbara *et al.*, 1998; Koopman, 1999), chondrogenesis (Wright *et al.*, 1995; Murakami *et al.*, 2000) and regulation of collagen production (Bell *et al.*, 1997)	T1

Proliferation and differentiation	CRIP	A LIM domain protein associated with proliferation/differentiation of specific cell types (Sadler *et al.*, 1992; Tsui *et al.*, 1994; Khoo *et al.*, 1997)	0.5
	TM9SF3	A membrane protein with 9 potential transmembrane segments (accession No. AF2691500)	0.5
	Latexin	Expressed in a cell type-specific manner in both central and peripheral nervous system as well as other tissues. Its expression is related to development of neocortical cells (Hatanaka *et al.*, 1994; Arimatsu *et al.*, 1999a).	2.5
	RTP/Ndr1 /Drg1/TDD5	RTP/Drg1/Ndr1 probably represent the same transcript and are widely distributed and up-regulated by various agents. Ligands of nuclear transcription factors involved in cell proliferation and differentiation are among the inducers of this novel protein. (Kokame *et al.*, 1996; Okuda and Kondoh, 1999; Piquemal *et al.*, 1999; Shimono *et al.*, 1999). Expression of TDD5, a spliced variant, is differentially repressed by testosterone and DHT (Lin and Chang, 1997)	2.5
	TI1	A growth arrest-specific and TGF-β-regulated epithelial gene (Kallin *et al.*, 1991)	P-IIIa
	DMP1	Arrests mouse fibroblasts in the G1 phase and prevents S phase entry in a pRb-independent manner (Inoue and Sherr, 1998; Inoue *et al.*, 1999). Disruption of its activity is associated with transformation and tumorigenesis (Sherr, 1996; Inoue *et al.*, 2000).	P-IIIb
ECM, CAM & other structural molecules	Ezrin/radixin /Meosin	A cytoskeletal-membrane linker protein (Sato *et al.*, 1992) involved in migration and morphogenesis in epithelial cells (Crepaldi *et al.*, 1997)	0.5
	IGSF4	Cell adhesion molecule of immunoglubulin superfamily (Charpin *et al.*, 1998; Gomyo *et al.*, 1999)	0.5 (2)
	MGP	A vitamin K-dependent protein expressed in bone, cartilage, lung, heart and kidney in a discrete tissue-specific cell type (Fraser and Price, 1988; Cancela *et al.*, 1990)	0.5 (2)
	MHP	Linker of cytochrome c-c_1 complex (Kim and King, 1983) and regulates the respiratory chain (Kim *et al.*, 1987); Overexpression enhances apoptosis (Okazaki *et al.*, 1998)	0.5 (2)
	MyHC IIa	A highly conserved ubiquitous actin-based motor protein that drives a wide range of motile processes (Weiss *et al.*, 1999). Expression modulation is linked to the process of fibrosis as well as malignant epithelial transformation (Chiavegato *et al.*, 1995).	0.5
	Titin	A giant protein responsible for muscle ultrastructure and elasticity (Labeit and Kolmerer, 1995) also found in intestinal brush border cytoskeleton (Eilertsen and Keller, 1992) and the chromosome (Machado *et al.*, 1998)	0.5 (2)

ECM, CAM and other structural molecules	Decorin	A member of the expanding small leucine-rich proteoglycans family. It is known to interact with collagen and growth factors and may play key roles during cell growth ontogenesis, tissue remodelling, and cancer (Yamaguchi *et al.*, 1990; Fleischmajer *et al.*, 1991; Vuorio *et al.*, 1991; De Luca *et al.*, 1996). Targeted disruption of decorin leads to abnormal collagen fibril morphology and skin fragility (Danielson *et al.*, 1997).	T1
	Fibulin1D	The fibulins are an emerging family of extracellular matrix and blood proteins presently having two members designated fibulin-1 and –2. Where epithelial-mesenchymal interactions occur, fibulin-1 mRNA and its corresponding protein are detected relatively uniformly around mesenchymal cells. Fibulin-1 protein is also located in some embryonic epithelial basement membranes and the epidermal layer of brain (Argraves *et al.*, 1989; Tran *et al.*, 1995; Zhang *et al.*, 1996b)	1
	Colgn α2I	The most abundant skin and fibrillar collagen is formed by two α1 (I) and one α2 (I) chains. High and low concentrations of collagen are associated with anagen and telogen, respectively (Valtonen and Blomstedt, 1988).	2.5
	GAKIN	GAKIN is a novel member of the kinesin superfamily of motor proteins which are important for intracellular trafficking and cytoskeletal reorganisation (Hanada *et al.*, 2000)	2.5
	Keratin K5	An epidermal keratin paired with K14. Expression of this and other keratins are associated with cell differentiation (Eckert and Rorke, 1988; Fuchs, 1993)	P-IV
	TIED	A novel integrin-related molecule. Integrins are a family of cell adhesion molecules that bind to different cell layers to form complex tissues and are mediators of morphogenesis (Berg *et al.*, 1999; Brown *et al.*, 2000), including hair follicle morphogenesis, keratinocyte differentiation and proliferation and integrity of skin and hair follicles (Brakebusch *et al.*, 2000)	P-V
Metabolic enzymes	ACL	A key enzyme for the synthesis of acetyl-CoA for fatty acid and cholesterol synthesis (Pearce *et al.*, 1998)	0.5
	GS	Catalyst of the synthesis of glutamine from ammonia and glutamate. Glutamine is an important neurotransmitter and mediates the positive feedback effects of ovarian steroids on gonadotrophin secretion at the hypothalamus (Herbison, 1998)	0.5
	GAPDH	An enzyme controlling glycolysis; regulated by 1,25-dihydroxyvitamin D3 (Desprez *et al.*, 1992) and p53 (Chen *et al.*, 1999); inducible in endothelial cells (Rimarachin *et al.*, 1992) and involved in apoptosis (Chen *et al.*, 1999; Shashidharan *et al.*, 1999) as well as many other biological processes	2.5
	SCD	A key rate-limiting enzyme in the synthesis of unsaturated fatty acids by insertion of a cis-double bond in the δ-9 position of fatty acid substrates (Ntambi, 1999)	6 (3)

Metabolic enzymes	GnTI	A key enzyme in the synthesis of N-linked carbohydrate (N-glycans) (Kumar *et al.*, 1990; Sarkar *et al.*, 1991) which is important during development and differentiation (Muramatsu, 1988)	T2
	CoA ligase	A mitochondrial protein for xenobiotic/medium-chain fatty acid: CoA ligase form XL-III encoded by a nuclear mRNA. It is important for detoxification (Raunio *et al.*, 1999; Paakki *et al.*, 2000) and is also involved in apoptosis (Pallardy *et al.*, 1999)	6
Protein synthesis and degradation	RPL7	A ribosomal protein associated with the large subunit of eukaryotic ribosome which can act as a co-regulator of nuclear receptor-mediated transcription and translation (von Mikecz *et al.*, 1999; Savelsbergh *et al.*, 2000).	0.5/NC
	HSPC	A subunit of proteasome which is a major degradation complex for protein and mRNA (Coux *et al.*, 1996; Hershko and Ciechanover, 1998; Laroia *et al.*, 1999). Proteasome is also involved in cell cycle progression (King *et al.*, 1996), apoptosis (Jones *et al.*, 1995) and many other biological processes.	1
	UBL3	A new member of the ubiquitin-like protein family and structurally similar to SUMO-1, another member of the family (Chadwick *et al.*, 1999; Jentsch and Pyrowolakis, 2000). Ubiquitination of proteins or mRNA link them to proteasome for degradation, a crucial step for regulation of many biological processes (Hershko and Ciechanover, 1998)	1
	RPL11	A highly conserved ribosomal protein involved in interactions between ribosomal proteins and a domain of large subunit rRNA. It is essential for efficient protein synthesis (Xing and Draper, 1996).	1
	RPL14	L14 is one of the most conserved ribosomal proteins and appears to have a central role in the ribonucleoprotein complex. Studies have indicated that L14 occupies a central location between the peptidyl transferase and GTPase regions of the large ribosomal subunit (Davies *et al.*, 1996).	1
	SAE1C	A subunit of the initiating enzyme in SUMO-1 (small ubiquitin-related modifier-1) ubiquitination pathway which lead to degradation, targeting or stabilisation of proteins (Hershko and Ciechanover, 1998; Jentsch and Pyrowolakis, 2000).	1
	SRP72	The signal recognition particle (SRP) of eukaryotic cells is a cytoplasmic ribonucleoprotein machine which arrests the translational elongation of nascent secretory and membrane proteins and facilitates their transport into the endoplasmic reticulum (Politz *et al.*, 2000). SRP72 is cleaved during apoptosis (Utz *et al.*, 1998).	6
	RPL4	A ribosomal protein which can inhibits protein translation in *E. coli* (Yates and Nomura, 1980; Worbs *et al.*, 2000).	P-IIIa

[1] First change in expression detected by differential display or Northern blot hybridisation (results from Northern blots supersede those from differential display). Numbers indicate day post-melatonin implantation. The letters indicate follicle growth stages where first change in expression were detected by Northern blots. NC: no change detected by Northern blot hybridisation.

[2] Number in bracket indicates the number of ESTs obtained for the molecule.

functional complex, the association with TAFs enables interactions with other transcription regulators for fine control of transcription (Buratowski, 1994).

Furthermore, it now appears that the "basal" transcriptional machinery also contributes to specificity via tissue-specific versions of TAFs and tissue-specific TBP-related factors (TRFs). The different promoter specificity of these factors may be responsible for regulation of a select subset of genes (Stolinski *et al.*, 1997; Rabenstein *et al.*, 1999). These findings suggest that multiple *TBPs* have evolved to accommodate the increase in gene expression during development and cellular differentiation. However, the mechanism of expression regulation of *TBP* itself is not well understood (Cho *et al.*, 1998; Oh *et al.*, 1999). Therefore, the significance of *TBP* gene expression alterations observed during the early stages of hair growth needs to be confirmed and assessed in this context.

Table 8-2 Transcripts involved in selected biological processes

Classification	Homologues of identified ferret ESTs				
Transcription regulation and signalling	*BTEB*	*CIR*	*MSSP1/2*	*SREBP1*	*Sox9* *TBP*
	MEKK1	*LAR*			
Cell proliferation and differentiation	*PCDC5RP*	*CIR*	*CRIP*	*Decorin*	*DMP1*
	Ha8	*Keratin K5*	*Latexin*	*LAR*	*MEKK1*
	MSSP1	*HSPC*	*RTP/Drg1*	*SAE1C*	*Sox9*
	TI1	*UBL3*	*DIF2**		
Cell structure, interaction and movement	*Colgn α21*	*Decorin*	*Ezrin*	*Fibulin1D*	*GnTI*
	GAKIN	*IGSF4 MGP*	*LAR*	*Lysozyme*	*MEKK1*
	MyHC IIa	*TIED*	*Titin*	*EVA**	
Apoptosis	*GAPDH*	*MEKK1*	*MHP*	*MSSP1/2*	*HSPC*
	SAE1C	*SREBP1*	*UBL3*	*SRP72*	*IEX1L**
Fatty acid and cholesterol synthesis	*ACL*	*GAPDH*	*SREBP1*	*SCD*	
Ribosomal functions	*RPL4*	*RPL7*	*RPL11*	*RPL14*	*SRP72*

* Putatively identified with comparatively high E values (e-4 to e-9).

The GC box sequence is another widely distributed promoter element in cellular and viral genes (Mitchell and Tjian, 1989). Sp1 was long thought to be a unique protein capable of binding to the GC box and regulating gene expression. However, a new series of GC box binding proteins, designated BTEBs have now been identified (Imataka *et al.*, 1992; Sogawa *et al.*, 1993; Martin *et al.*, 2000b). Although BTEB2 is

only expressed in testis and placenta, *BTEB1* and BTEB3 are widely expressed. The expression of *BTEB1* is found to be controlled at the translation level due to the existence of multiple upstream AUG sequences in the 5' UTR (Imataka *et al.*, 1994). However, as its expression is targeted by c-myc and Jun/Fos, transcriptional regulation is also expected (Shirasuna *et al.*, 1999). As for *TBP*, alterations in the expression of *BTEB1* are thought to affect the expression of many other genes, due to the common occurrence of its binding elements. However, the effects on different genes are likely to vary according to cellular environment, especially the availability of other transcription factors and topology of the target genes. Although the roles of the general transcription factors *TBP* and *TBEB* in hair growth are currently not clear, they certainly modulate the expression of the genes with the corresponding motifs. Hair keratin genes usually possess both the TATA box and CAAT box (Rogers *et al.*, 1998), and are likely target genes of these transcription factors. Collagens are abundant ECM proteins which are also expressed in hair follicles (Section 8.5). The expression of some collagens, if not all, is also regulated by *TBEB* (Chen and Davis, 2000).

Both *LAR* and *MEKK1* are important signalling molecules, but they have not previously been identified in hair follicles, or other skin cell populations. *Leukocyte common antigen-related molecule (LAR)* contains two intracellular domains homologous to protein tyrosine phosphatases (PTPases) (Pot *et al.*, 1991; Itoh *et al.*, 1992). PTPases regulate many biological processes, including proliferation, differentiation and apoptosis through dephosphorylation of proteins involved in tyrosine signal transduction (Fischer *et al.*, 1991; Tillmann *et al.*, 1994; Sorby and Ostman, 1996; Yang *et al.*, 1996; Radha *et al.*, 1997). Receptor-like protein tyrosine phosphatases (rPTPases) represent a family of transmembrane proteins that consist of a cytoplasmic catalytic region, a single transmembrane segment and an extracellular, putative ligand-binding domain (Itoh *et al.*, 1992; Gebbink *et al.*, 1993). At least 15 distinct transmembrane rPTPases have been cloned (Fischer *et al.*, 1991; Schaapveld *et al.*, 1995) subsequent to the prototype, leukocyte common antigen (CD45) (Trowbridge, 1991). The membrane-proximal domain 1 of *LAR* has enzymatic activity, while the membrane-distal domain 2 has a regulatory function. However, the extracellular region of *LAR* is larger than that of CD45 and contains three Ig-like domains and eight fibronectin-III domains (Longo *et al.*, 1993). As *LAR* and other

rPTPases can respond to environmental cues by interacting with molecules through their extracellular domains, they can regulate cell growth and differentiation through cell-cell or cell-matrix interactions, although their ligands have yet to be identified (Trowbridge, 1991). The *LAR* gene has been localised to a chromosomal region (1p32-33) frequently deleted in neuroectodermal tumours and its expression is altered in some tumours (Yang *et al.*, 1999; Yang *et al.*, 2000). In the central nervous system, *LAR* expression is developmentally regulated in a region-dependent manner. Changes in *LAR* expression were also found during nerve growth factor-induced cell differentiation and with contact-mediated inhibition of fibroblast growth. Therefore, *LAR* represents an additional mechanism regulating neural development (Longo *et al.*, 1993). However, the expression of *LAR* in skin is possibly quite low, as its expression was detected only in a skin sample taken one day after melatonin treatment. The significance of this transient up-regulation of *LAR* in hair growth initiation remains to be ascertained.

In contrast to PTPases, protein kinases phosphorylate proteins to control their activities. Many extracellular signals, including growth factors, hormones, cytokines and stress activate members of the mitogen-activated protein (MAP) kinase (MAPK) pathway comprised of a cascade of kinases (Kyriakis *et al.*, 1992; Davis, 1993; Schlesinger *et al.,* 1998). MAPKs are serine/threonine-protein kinases activated by dual phosphorylation of both serine and threonine residues. The MAPK family acts through three different pathways: the MAPK/ERK (extracellular signal-regulated kinase) pathway, the JNK (c-jun N-terminal Kinase)/SARP (stress-activated protein kinases) pathway and the p38 (osmotic imbalance responsive kinase)/Hog1 pathway (Cobb and Goldsmith, 1995).

MEKK1 is an upstream component of the MAPK pathway. Cloning of this molecule revealed other pathways activated by extracellular stimuli that were responsible for ERK activation. Since then, three more MEKK family members have been cloned adding further diversity to the regulation of the MAPK signalling (Blank *et al.*, 1996; Gerwins *et al.*, 1997). Among them, the biological role of *MEKK1* is best characterised. *MEKK1* is regulated by low molecular weight GTP binding protein (LMWG) and Ras (an oncogene originally found in rat sarcoma viruses) (Crews and Erikson, 1993; Fanger *et al.*, 1997). *MEKK1* activates MEK (MAP/ERK kinase) family members, which then induce serine/threonine phosphorylation of ERKs. The

activation of ERKs is associated with a myriad of biological events, both inside the cytoplasm and, after translocation into nuclei, through phosphorylation of c-fos, c-jun, c-myc, ets1, myb and other transcription factors (Blenis, 1993; Chen *et al.*, 1993; Marshall, 1995). However, *MEKK1* is a more efficient activator of the JNK/SAPK signalling cascade than of the MAPK pathway, by phosphorylation and activation of JNK kinase or MKK4, which then potentiates JNK/SAPK (Minden *et al.*, 1994; Cobb and Goldsmith, 1995; Xia *et al.*, 1995; Xu *et al.*, 1996; Xu and Cobb, 1997). *MEKK1* is also an important link in the p38/HOG1 kinase pathway (Han *et al.*, 1994; Cobb and Goldsmith, 1995). As MAPK signal transduction plays an important role in many biological processes, the alteration of *MEKK1* expression can affect different processes involved in hair growth, as discussed in the following sections.

Prolactin is implicated in ferret winter hair growth initiated by melatonin (Nixon *et al.*, 1992), subsequent to binding to its receptor (Choy *et al.*, 1995; Choy *et al.*, 1997; Nixon *et al.*, 1999b). Prolactin is known to activate both MAPK pathways (Cheng *et al.*, 1992; Erwin *et al.*, 1995; Das and Vonderhaar, 1996; Das and Vonderhaar, 1997) and the JAK-STAT (signal transducer and activator of transcription) signalling pathway (Rillema *et al.*, 1992; Rui *et al.*, 1994a; Rui *et al.*, 1994b). Although little is known about the specific genes targeted by the signalling pathways, prolactin is known to affect cell proliferation, differentiation and apoptosis (Cheng *et al.*, 1992; Clevenger and Plank, 1997; Bole-Feysot *et al.*, 1998; Clevenger *et al.*, 1998; Olazabal *et al.*, 2000). Although *MEKK1* has not been reported to be affected directly by prolactin, the complicated interactions between the signalling pathways and the resultant changes in cells and their environment is likely to alter the status of *MEKK1* (Erwin *et al.*, 1995; Bole-Feysot *et al.*, 1998; Schlesinger *et al.*, 1998).

8.4 Regulation of cell growth and differentiation

The most important checkpoint for cell cycle control is the restriction point called G0, in late G1 phase. When cells are at G1, they are subject to regulation by a variety of factors, including both mitogens and anti-proliferation signals. This checkpoint is mainly controlled by the phosphorylation activity of CDKs consequent upon binding to cyclin D and E. Hypophosphorylated pRb (retinoblastoma protein) arrests cells in G1 phase, while hyperphosphorylation by CDKs relieves this inhibition by releasing E2F and other transcription factors. These transcription factors activate expression of

the genes necessary for cell cycle progression. After passing this checkpoint, cells are committed to DNA synthesis for the next round of growth and are no longer affected by many of the regulatory factors (Hunter and Pines, 1994; Weinberg, 1995).

One of the other critical, but less understood, checkpoints occurs prior to entry into mitosis. The entry into mitosis is signalled by the activation of the primary mitotic kinase CDC2, which is maintained in an inactive phosphorylated form as cyclin B-CDC2 complex. The accumulation of this inactive complex during S and G2 phase triggers dephosphorylation by CDC25C phosphatase and enables the cell to enter mitosis (Hunter and Pines, 1994; King *et al.*, 1994).

Exit from the cell cycle also facilitates maturation and differentiation (Pardee, 1989). Physiological signals that favour cell proliferation, cell cycle arrest or differentiation act through the regulation nodes, by changing the level or state of one or more of these key factors, either directly or indirectly. For instance, oestrogen promotes cell growth by directly increasing the expression of both myc and cyclin D1 (Sutherland *et al.*, 1992; Sutherland *et al.*, 1998) (Chapter 2). In contrast, progestin can either inhibit or accelerate cell cycle progression, depending on the circumstances, through regulation of myc, cyclin D1 and cyclin E expression (Sutherland *et al.*, 1992; Sutherland *et al.*, 1998).

A number of transcripts identified in this study appear to have a role in the regulation of cell growth cycles and cell differentiation. *Pombe CDC5 related protein (PCDC5RP)* is a myb-related transcription factor whose activity is required for cell cycle progression during G2 (Ohi *et al.*, 1994). *PCDC5RP* contains two tandem repeats of a helix-turn-helix DNA binding motif, four consensus nuclear localisation signals, and a hydrophilic, proline-rich central region similar to the transcriptional activating domain in Myb family members (Hirayama and Shinozaki, 1996; Bernstein and Coughlin, 1997). Overexpression of human *PCDC5RP* in mammalian cells shortens G2 and reduces cell size. A dominant negative mutant of human *PCDC5RP* lacking the carboxyl-terminal activation domain slows G2 progression and delays entry into mitosis (Bernstein and Coughlin, 1998). Thus, human *PCDC5RP* is the first transcriptional regulator shown to affect G2 progression and mitotic entry in mammalian cells. It is also a putative transcription factor implicated

in mitogen-activated signalling (Bernstein and Coughlin, 1997) and is also involved in pre-mRNA splicing in eukaryotic cells (Burns *et al.*, 1999). In a prolactin-dependent cell line Nb2-11C, the expression of *PCDC5RP* is transiently suppressed by prolactin (Too, 1997). The suppression period concurs with G1 phase of the cell cycle, suggesting that this transcript may regulate prolactin-stimulated entry of the quiescent cells into the cell cycle. As low prolactin levels derived from melatonin administration has been linked to winter hair growth in mustelids (Martinet *et al.*, 1984; Nixon *et al.*, 1992; Nixon *et al.*, 1995), *PCDC5RP* might be expected to play a role in hair growth. As *PCDC5RP* expression detected by differential display showed similar patterns in both the stimulatory and inhibitory hormonal treatments, the function of this molecule in hair cycling merits further study.

In contrast to *PCDC5RP*, *DMP1 (cyclin D-interacting myb-like protein)* arrests cell growth at G1 in a novel CDK-independent manner (Hirai and Sherr, 1996; Inoue and Sherr, 1998), through the regulation of tumour suppresser gene expression (Inoue *et al.*, 1999). The restricted localisation of *DMP1* to the keratogenous zone of growing follicles suggests an important role in regulation of cortical cell differentiation (see Chapter 7).

Proto-oncogene c-myc is another important regulator of cell growth, as well as other processes (Chin *et al.*, 1996; Dang, 1999). Formation of heterodimer with Max is essential for c-myc activity (Amati *et al.*, 1993), while its activity is counteracted by Mxi1 (Zervos *et al.*, 1993). Through regulation of the transcription of other genes, such as the previously mentioned CDC25, c-myc affects cell cycle progression (Galaktionov *et al.*, 1996; Dang, 1999). In human epidermis, c-myc acts selectively on stem cells, initiating a premature differentiation program and driving them into the transient amplifying compartment (Gandarillas and Watt, 1997). The expression of c-myc has been identified in hair follicles (Rumio *et al.*, 2000) and skin (Pelengaris *et al.*, 1999) and c-myc is believed to play similar roles as in other tissues (Chin *et al.*, 1996). Although c-myc was not discovered in this study, a c-myc binding protein, *MSSP* was detected. Two isoforms of *MSSP* exist, *MSSP1* and *MSSP2,* which could not be distinguished from the sequence obtained in this study. *MSSPs* have been shown to participate in cell growth control by enhancing c-myc expression. *MSSPs* bind to the replication origin and transcriptional enhancer in the c-myc gene, interacting with a catalytic subunit of DNA polymerase α and

stimulating its polymerase activity (Negishi *et al.*, 1994; Takai *et al.*, 1994; Haigermoser *et al.*, 1996; Niki *et al.*, 2000a). Similarly, *MSSP1* stimulated DNA replication initiation of SV40 virus containing the *MSSP1* binding consensus sequence, further suggesting its involvement in regulation of cell cycle progression, especially from the G1 to S phase (Negishi *et al.*, 1994). Actually, binding of *MSSPs* to c-myc leads to cell transformation (Iida *et al.*, 1997). The transformation activity is parallel to the DNA replication activities of *MSSPs* (Niki *et al.*, 2000b).

Proteoglycans (PGs) are complex carbohydrates that contain one or more glycosaminoglycan chains covalently bound to a core protein, to which N- and O-linked oligosaccharides are also often attached. Two small chondroitin sulfate (CS) proteoglycans, biglycan (PGs1) and *decorin* (PGs2), are the products of two different genes (Fisher *et al.*, 1989). Both biglycan and *decorin* interact with collagen (Whinna *et al.*, 1993), fibronectin (Schmidt *et al.*, 1991) and TGF-β (Hildebrand *et al.*, 1994; Yamada *et al.*, 1999) in a similar way. Biglycan consists of a core protein and two CS chains, while *decorin* has a core protein of similar size and one CS (Iozzo and Murdoch, 1996). Eighty per cent of the core protein consists of 10 repeats of a leucine-rich sequence of 24 amino acids (Iozzo and Murdoch, 1996). Expression of high levels of *decorin* in Chinese hamster ovary cells has a dramatic effect on their morphology and growth properties (Yamaguchi and Ruoslahti, 1988). This effect is due, at least in part, to the ability of *decorin* to bind TGF-β, an autocrine factor that has a profound effect on cell growth in general (Moses *et al.*, 1990; Mann *et al.*, 1993; Kingsley, 1994) and hair follicle growth and cycling in particular (Welker *et al.*, 1997; Foitzik *et al.*, 1999). Synthesis of *decorin* is not only induced by TGF-β and other growth factors (Yamada *et al.*, 1999), but *decorin,* in turn, regulates the expression of growth factors (Ruoslahti, 1989; Yamaguchi *et al.*, 1990; Mauviel *et al.*, 1995), and thus cell growth and differentiation. For instance, binding of *decorin* to TGF-β inhibits the activity of this growth factor (Yamaguchi *et al.*, 1990), which in turn regulates the expression of *decorin.* Moreover, the expression of *decorin* is closely linked to p21 expression. Abrogation of *decorin* leads to suppression of p21 and restoration of cell division (De Luca *et al.*, 1996). Expression of this proteoglycan is also decreased or suppressed in cancer cells (Adany *et al.*, 1990) and induced by quiescence (Mauviel *et al.*, 1995). Therefore, *decorin* is likely to be a

component of the cell growth regulating system (Ruoslahti, 1989; Yamaguchi *et al.*, 1990).

TGF-β also blocks the phosphorylation of pRb by increasing the level of CDK inhibitors and reducing the level of cyclins, thus keeping the restriction point gate closed and preventing cell proliferation (Weinberg, 1995). *TI1*, another TGF-β-regulated gene, is considered a growth arrest-specific gene in epithelial cells (Kallin *et al.*, 1991; Ralph *et al.*, 1993). *TI1* expression is down-regulated by TGF-β and serum in quiescent cells. In actively growing cells, the *TI1* gene is rapidly and transiently induced by TGF-β, and it is overexpressed in the presence of protein synthesis inhibitors (Kallin *et al.*, 1991). However, how *TI1* plays a role in cell growth regulation has not been determined. Human UPK1B, which has 93% amino acid homology with *TI1* protein, is highly expressed in normal human urothelium. The expression of UPK1B mRNA was undetectable or markedly reduced in most transitional-cell bladder carcinoma tissue and in all five bladder carcinoma cell lines examined, also suggesting a role in cell growth or differentiation (Finch *et al.*, 1999). However, localisation of *TI1* to nuclei of various follicle and skin cell populations (Chapter 7) does not concur with its established relationship to transmembrane proteins (Kallin *et al.*, 1991).

Notch is a conserved transmembrane receptor. Notch-ligand interaction and the resultant signalling pathway act as an arbiter of cell fate, regulating proliferation, differentiation and apoptosis (Miele and Osborne, 1999). Studies of Notch overexpression in hair follicles have suggested that Notch may function in cell-cell interactions, either by directing the differentiation of follicular cells or assisting cells in interpreting a biochemical gradient emanating from the dermal papilla (Lin *et al.*, 2000). CBF1 is involved in Notch signalling as a transcription regulator. More recently, the effect of CBF1 has been found to be mediated though *CBF1 interacting corepressor (CIR)* (Hsieh and Hayward, 1995; Hsieh *et al.*, 1999). *CIR* binds to histone deacetylase and SAP30, a component of the histone deacetylase complex, and serves as a linker between CBF1 and the histone deacetylase complex (Hsieh *et al.*, 1999). The change of histone acetylation status alters DNA conformation, and thus gene transcription (Sommer *et al.*, 1997; Kao *et al.*, 1998; Zhang *et al.*, 1998). However, the link between *CIR* action and Notch-mediated cell fate is not known. Alternatively, CBF1 targets two transcription coactivators: dTAFII110, a subunit of

TFIID, and TFIIA, to repress transcription (Olave *et al.*, 1998). It is not yet known whether this pathway also involves *CIR*.

While the expression of cell growth regulators is important, selective degradation of these molecules, especially cell growth inhibitors, such as p16, p21, p27 and p53, through ubiquitination and the proteasome pathway, is also crucial for the modulation of cell growth (King *et al.*, 1994; King *et al.*, 1996; Hershko and Ciechanover, 1998). Mutation in proteasome subunits can lead to cell growth arrest (Ghislain *et al.*, 1993; Gordon *et al.*, 1993) and proteasome inhibition leads to up-regulation of specific members of transcription factor families controlling stress responses and proliferation (Zimmermann *et al.*, 2000). A *proteasome subunit HSPC* was identified in this study together with two ubiquitin-like proteins, *UBL3* and *SAE1C (E1C subunit of SUMO-1 ubiquitin-activating enzyme). SUMO-1* catalyses the initial step of ubiquitination, which eventually leads to marking of proteins or mRNA for degradation (Hershko and Ciechanover, 1998; Gong *et al.*, 1999; Okuma *et al.*, 1999). In response to external signals, IκBα, an inhibitor of transcription factor NFκB/Rel, is phosphorylated, ubiquitinated and degraded by the proteasome pathway, enabling NFκB/Rel to activate transcription (Desterro *et al.*, 1998). Furthermore, genetic approaches in yeast have underscored the importance of *SUMO1* for cellular regulation, in particular for cell cycle progression. Deletion of SMT3 encoding an orthologue of fission yeast *SUMO1*, is lethal, and yeast cells depleted for UBC9, another equivalent accumulate at the G2-M boundary of the cell division cycle (Seufert *et al.*, 1995). Similarly, inactivation of the expression of a protease which processes and activates *SUMO* leads to severe cell cycle defects (Li and Hochstrasser, 1999). Indeed, the levels of *SUMO* conjugates in yeast fluctuate strikingly during the cell cycle (Li and Hochstrasser, 1999).

UBL3 is a novel member of ubiquitin-like protein with similar structure to *SUMO-1* (Chadwick *et al.*, 1999). The mRNA level of *UBL3,* as detected by Northern blot hybridisation in skin, appears to be reduced by administration of either melatonin plus oestradiol or progesterone. Hence, the identification of *UBL3*, *proteasome HSPC* and *SUMO-1 subunits* suggests the involvement of ubiquitinated degradation (Hershko and Ciechanover, 1998; Okuma *et al.*, 1999; Jentsch and Pyrowolakis, 2000) in the control of hair follicle growth. Already, ubiquitinated degradation

through the proteasome pathway has been found to participate in keratin turnover (Ku and Omary, 2000).

As has been mentioned earlier, the regulation of phosphorylation levels of a large variety of molecules is one of the most important ways of modifying their activities in many biological processes, including cell proliferation and differentiation (Hunter, 1989; Schlessinger and Ullrich, 1992). Interestingly, the two signalling molecules identified in this study, *LAR* and *MEKK1*, are involved in two signalling pathways with opposing phosphorylation effects. *LAR* expression is not only developmentally regulated in a region-dependent manner, but changes in *LAR* expression were also found during nerve growth factor-induced PC12 pheochromocytoma cell differentiation and with contact-mediated inhibition of fibroblast growth (Longo *et al.*, 1993). Activation of ERK1 and ERK2 are often associated with proliferative signals (Pages *et al.*, 1993; Seger and Krebs, 1995). However, ERKs have also been shown to inhibit proliferation in a few systems (Casillas *et al.*, 1993; Bornfeldt *et al.*, 1997), and sustained ERK activity is required for differentiation (Qui and Green, 1992).

The identification of a number of transcripts associated with cell growth progression concurs with the expected biochemical status of the skin samples used in differential display. Most samples from ferrets treated with melatonin or melatonin plus progesterone contained proliferating cells from the hair follicle germinal matrix, while those from ferrets treated with melatonin plus oestradiol were prevented from entering the new cycle of hair growth (Chapter 4). All these hormones have been shown to affect cell growth (Lemus Wilson *et al.*, 1995; Sutherland *et al.*, 1998; Anisimov *et al.*, 2000). The identification of a number of genes involved in cell cycle control appears to confirm the importance of this process during early proanagen.

Differentiation is a complex process involving growth arrest, exit from the cell cycle and expression of differentiated cell-type specific functions. Thus, cell growth and cell differentiation are different, but closely related processes. However, the factors that facilitate cell cycle arrest and exit may not be sufficient to promote cell differentiation (Hauser *et al.*, 1997; Harvat *et al.*, 1998). Some transcripts involved in cell differentiation, or expressed in particular cell types or growth stages were also

identified. These are *CRIP*, *RTP/Drg1/Ndr1/TDD*, *latexin*, *Sox9*, *keratin K5* and *Ha8*.

CRIP is a cysteine-rich protein containing a double zinc-finger motif LIM domain, which is important for protein-protein or protein-DNA interactions (Schmeichel and Beckerle, 1994). Most proteins with the LIM domain influence cell signalling, proliferation and differentiation (Birkenmeier and Gordon, 1986; Sadler *et al.*, 1992; Crawford *et al.*, 1994). *CRIP* was first identified in the mouse small intestine through its pattern of developmental regulation in the neonate (Birkenmeier and Gordon, 1986), then in rats (Okano *et al.*, 1993; Levenson *et al.*, 1994) and humans (Khoo *et al.*, 1997). It is also present in human peripheral blood mononuclear cells (Khoo *et al.*, 1997) and heart (CRHP), where it is also developmentally regulated (Tsui *et al.*, 1994) as in mouse intestine (Birkenmeier and Gordon, 1986) and various chicken tissues (Crawford *et al.*, 1994). Dexamethasone has been found to affect the developmental expression of *CRIP* (Levenson *et al.*, 1993) in the neonatal rat, indicating that its expression can be modified by steroids.

Sequences of *RTP/Ndr1/Drg1/TDD5* are highly homologous, but they have no homologies to other molecules with established functions. They are widely expressed and the conditions in which the mRNAs are up-regulated suggest a role for the protein in cell growth arrest and terminal differentiation (Kokame *et al.*, 1996; Lin and Chang, 1997; van Belzen *et al.*, 1997; Kurdistani *et al.*, 1998; Okuda and Kondoh, 1999; Piquemal *et al.*, 1999; Shimono *et al.*, 1999). Their expression is regulated by ligands of nuclear transcription factors, down-regulated in tumour cells and up-regulated in cells which cease to proliferate and commence differentiation (Piquemal *et al.*, 1999).

There exists an inverse relationship between the expression of N-myc and *Ndr1* in various developing embryonic tissues. In the early stages of differentiation *Ndr1* expression is low or undetectable, when N-myc expression is high. When N-myc activity diminishes, *Ndr1* expression is augmented concomitantly with the occurrence of terminal differentiation (Shimono *et al.*, 1999). C-myc, a homologue of N-myc, converts stem cells into transient amplifying cells and it is thought to co-ordinate and link keratinocyte proliferation and initiation of terminal differentiation (Gandarillas and Watt, 1997; Gandarillas, 2000), in addition to the its roles in

proliferation, as discussed earlier. *Ndr1* promoter activity has also been found to be repressed by c-myc as by N-myc (Shimono *et al.*, 1999).

The expression of *RTP* is also found to cycle with cell division, peaking at G1 and G2-M, and reduced in S phase (Kurdistani *et al.*, 1998). Transfection of its cDNA into human cancer cells reduces cell growth both *in vitro* and in nude mice. Moreover, analysis of a tetracycline-regulated p53-inducible system in null-p53 cell lines shows that *RTP* mRNA expression increases concomitantly with that of p53 and followed a similar time course (Kurdistani *et al.*, 1998). The expression of *Drg1* in human intestine epithelia occurs late during differentiation and just before apoptosis and shedding into the colon lumen (van Belzen *et al.*, 1997).

TDD5 was isolated from a 5α-reductase-deficient T cell hybridoma by differential display (Lin and Chang, 1997) and is considered to be a spliced variant of the other transcripts in this group as it differs significantly in the C-terminal region. Northern blot analysis has demonstrated that the expression of *TDD5* is differentially repressed by testosterone and DHT at the mRNA level. Because of the early response of *TDD5* to androgens and also the transcriptional regulatory nature of androgen receptors, this molecule might be a factor in regulating differential cell growth or proliferation responses to testosterone over DHT. As DHT generally has higher biological activity than testosterone, the function of this molecule is of interest, particularly when the conversion of testosterone to DHT is blocked, as in the case of 5α-reductase deficiency, which seriously retards androgen-dependent hair growth (Chapter 2). Overall, the expression of this group of molecules is linked to terminal differentiation and cessation of proliferation.

Latexin, a carboxypeptidase A inhibitor, is expressed in a cell type-specific manner in both central and peripheral nervous systems (Hatanaka *et al.*, 1994; Miyasaka *et al.*, 1999; Uratani *et al.*, 2000). In the neocortex, a specific subpopulation of neurons in layers V and VI express *Latexin*. In the primary sensory ganglia, expression is restricted to smaller diameter neurons likely to be excitatory projection neurons. Therefore, the expression of *latexin* is associated with differentiation of neuronal cells (Arimatsu, 1994; Arimatsu *et al.*, 1999a; Arimatsu *et al.*, 1999b). The identification of transcription factor binding sites suggests that the cell type-specific expression of *latexin* is tightly regulated (Miyasaka *et al.*, 1999). *Latexin* is also

expressed in some other tissues, including lung, spleen and mast cells (Hatanaka *et al.*, 1994; Uratani *et al.*, 2000). Although the expression or function of *latexin* in skin has not been previously reported, hair follicles are well-innervated and both neural mechanisms (Paus *et al.*, 1997b) and mast cells (Paus *et al.*, 1994b) have important roles in hair growth. In this study, *latexin* appeared to be down-regulated two and half days after the hormone treatments in differential display, but increased subsequently. However, as its expression was regulated in a similar way by both hair growth stimulatory and inhibitory treatments, it may be peripheral to hair growth cycle regulation.

Sox9 is a transcription factor required for testis determination in mammals (De Santa Barbara *et al.*, 1998; Koopman, 1999). For proper male sexual differentiation, anti-Mullerian hormone (AMH) must be tightly regulated during embryonic development to promote regression of the Mullerian duct and testis formation. Two transcription factors *Sox9* and SF-1 (steroidogenic factor 1) are involved in the expression of the AMH gene in Sertoli cells (De Santa Barbara *et al.*, 1998). As *Sox9* is expressed in many other embryonic or adult tissues (Wright *et al.*, 1995), it can have additional roles in development. For example, *Sox9* is also essential for chondrocyte differentiation (Healy *et al.*, 1996; Lefebvre and de Crombrugghe, 1998; Bi *et al.*, 1999). The expression of *Sox9* is regulated by fibroblast growth factors (FGFs) through MAPK pathway (Murakami *et al.*, 2000) and bone morphogenetic protein-2 (BMP2) (Healy *et al.*, 1999; Zehentner *et al.*, 1999). As both FGFs (Hebert *et al.*, 1994; Martin *et al.*, 1994; Pethö-Schramm *et al.*, 1996) and BMP2 (Lyons *et al.*, 1990a; Lyons *et al.*, 1990b; Wilson *et al.*, 1999) are implicated in hair follicle development and cycling, *Sox9* is also likely to play a role in hair growth.

DIF2 is highly homologous to a mouse gene Gly96 which is rapidly induced by serum growth factors during the G0/G1 transition, possibly through a protein kinase C (PKC) pathway (Charles *et al.*, 1993). However, Northern blot analysis revealed that the expression of an EST (96F10G02-380-3) with some homology to *DIF2* was up-regulated when hair follicles developed from telogen to anagen (Chapter 7), differing from *DIF2* which is down-regulated in differentiated monocytes (Pietzsch *et al.*, 1997). Together with the limited homology (e-4), the expression pattern suggests that this EST is a different molecule. Actually, the same EST also showed some homology to transcripts of a radiation early response gene *(IEX1)* and an

apoptosis inhibitor gene *(IEX1L)*. *IEX1* cDNA showed 68% homology to gly96 and its expression is also regulated by PKC and other signal transduction pathways (Kondratyev *et al.*, 1996). *IEX1L* is the same as *IEX1* except that it has an in-frame insertion of 111 nucleotides in the coding region of *IEX1*, which makes it 37 amino acids longer (Wu *et al.*, 1998). However, longer sequence is required in order to establish the identity of this EST and to understand its role in hair growth.

Keratin intermediate filaments (IFs) can be classified into epidermal keratins or cytokeratins (soft keratins) and hair/wool keratins (hard keratins). Cytokeratins are expressed in epithelial cells, while hard keratins are present in hair, wool and nails. Like hair keratin IFs, cytokeratin IFs are also divided into type I and type II keratins (Fuchs, 1988; Steinert and Roop, 1988), and both are essential for filament formation (Fuchs and Weber, 1994). The expression of cytokeratins and hair keratins are tightly regulated according to the requirements for cell function during development and differentiation (Moll *et al.*, 1982; Fuchs *et al.*, 1987; Fuchs, 1988; Fuchs, 1993; Langbein *et al.*, 1999). *Type II keratin K5* is paired with type I keratin K14 in forming protofilament and they are coexpressed in mitotically active and relatively undifferentiated basal keratinocytes of epithelia and epidermal keratinocytes (Eckert and Rorke, 1988; Casatorres *et al.*, 1994). When the basal cells differentiate, and move to the suprabasal compartment, the synthesis of this pair is reduced or terminated, while the production of other keratin IFs is turned on (Fuchs, 1988). Results from this study agree with these data. The expression of *K5* was localised only to the basal layer of skin and mid to upper outer root sheath cells. Interestingly, the expression of *K5* was equally strong in the upper outer root sheath of both anagen and telogen follicles, indicating that these root sheath cells are similar in their differentiation status. However, heavy expression of *K5* in lower portion of telogen follicles was observed, compared with no expression in lower portion of anagen follicles (Fig. 7-8). As epidermis and hair follicle stem cells are thought to be located in bulge region (Cotsarelis *et al.*, 1990; Taylor *et al.*, 2000), outer-root sheath cells in lower portion of anagen follicles are distant from the source of the stem cells. More importantly, the outer-root sheath is thinner in this region and cells have become flattened, also suggesting differentiation and loss of *keratin K5* expression.

Sequential expression of hair keratin IFs is also observed during hair follicle growth (Powell *et al.*, 1992; Langbein *et al.*, 1999). As in human hair follicles, the high

expression level of *fHa8* is a relatively late event in ferret hair growth. By using skin samples containing hair follicles of different stages, a low level of expression was first detected at P-IIIb by Northern blot. The mRNA level then increased and peaked at anagen, indicating that a higher proportion of cortical cells have differentiated into the stage of *Ha8* production.

The mechanism of differential expression of IFs is not well-understood. Besides TATA and CAAT motifs, a number of transcription factor binding sites have been found in keratin promoter regions (Rogers *et al.*, 1998). Some of these transcription factors have profound effects on keratin IF expression and hair growth (Casatorres *et al.*, 1994; van Genderen *et al.*, 1994; Zhou *et al.*, 1995; Dunn *et al.*, 1998). In this context, the identification of a homologue of human and mouse DMP1 solely in the keratinising cortex of a follicle is very interesting. Although the function of this molecule is not yet known, it is likely to have similar functions in regulating cortical cell growth or differentiation as in other tissues (Inoue and Sherr, 1998; Inoue *et al.*, 1999; Inoue *et al.*, 2000)(Chapter 7).

8.5 Cell interactions and movement

The extracellular matrix (ECM) is an intricate network of macromolecules responsible for holding cells and tissues together and providing an organised lattice within which cells can migrate and interact with one another. The ECM primarily consists of fibrous proteins embedded in a hydrated polysaccharide gel. These proteins are comprised of two types: mainly structural (such as collagen and elastin) and mainly adhesive (such as fibronectin and laminin). The hydrated polysaccharides are glycosaminoglycans and proteoglycans which form a gel-like ground substance. The ECM serves not only as scaffolding to stabilise the physical structure of tissues, but also plays an active and complex role in regulating behaviours of the embedded cells. ECM molecules influence cell development, proliferation, differentiation, shape and metabolic functions (Adams and Watt, 1993). In contrast to ECM molecules, cell adhesion molecules (CAM) are membrane-bound glycoproteins which are crucial for normal growth, tissue and organ formation and maintenance (Chothia and Jones, 1997; Humphries and Newham, 1998; Brown *et al.*, 2000). Both ECM and CAM molecules also play important roles in cell migration, which has a central role in many biological processes, ranging from gastrulation and development

of nervous system in embryogenesis, to leukocyte movement in inflammation and fibroblast and vascular endothelial cell migration in wound healing. In the case of cancer, cell movement (metastasis) spreads cancerous cells to other parts of the body and greatly decreases the prognosis. Cell migration is also believed to be a prominent process during the hair growth cycle. Although cell migration is still poorly understood, the components involved are being rapidly identified. Evidence accumulated so far suggests that cell migration is a multiple step process involving orchestrated actions of many molecules, especially those of ECM, CAM and cytoskeleton proteins (Lauffenburger and Horwitz, 1996; Banyard *et al.*, 2000; Clark *et al.*, 2000; Evers *et al.*, 2000; Ridley, 2000). A group of transcripts identified in this study encode ECM, CAM or cell motor proteins. These include *colgn α2I, decorin, TIEP, MGP, fibulin1D, GnTI, MyHC IIa, ezrin, GAKIN, titin* and quite likely *EVA*.

Collagens are the most abundant ECM protein. Collagen variants are not only present in different compartments of the hair follicle, but their expression is also growth stage-dependent, both in the skin and in hair follicles (Valtonen and Blomstedt, 1988; Jahoda *et al.*, 1992; Berthod *et al.*, 1997). The development of the winter hair coat is temporarily related to high concentrations of collagen. During catagen, the skin becomes thinner and the dermal content of collagen diminishes to a baseline in telogen (Valtonen and Blomstedt, 1988). Growth related mRNA levels and protein production have been reported in cultured skin fibroblasts (Makela *et al.*, 1990) and a mutation in collagen α1(I) gene is lethal for embryo development (Schnieke *et al.*, 1983). In support of these findings, *colgn α2I* and another EST (96F10G03-346-1) which showed some homology to collagen α1(I), were expressed at slightly higher level in late proanagen samples. *Colgn α2I* mRNA level was elevated by melatonin in association with hair growth initiation. However, oestradiol, which inhibited hair growth, decreased *colgn α2I* expression 10 days after co-administration with melatonin (Chapter 7). Furthermore, two regulators of collagen expression, *BTEB* (Chen and Davis, 2000) and *Sox9* (Bell *et al.*, 1997; Lefebvre *et al.*, 1997), were also identified.

As discussed earlier, the extracellular component *decorin* has important roles in cell proliferation and differentiation. This molecule also has a crucial role in cellular interactions (Scholzen *et al.*, 1994), through an association with collagen. The name

decorin was given in recognition of its ability to bind to collagen fibrils and to affect fibril formation (Vogel *et al.*, 1984). Disruption of *decorin* leads to abnormal collagen morphology and skin fragility (Danielson *et al.*, 1997). Differential expression of *decorin* and other proteoglycans has been identified during hair growth cycle (Couchman *et al.*, 1990; Westgate *et al.*, 1991b). Higher expression was detected in dermal papilla and follicle buds as they developed. Lower expression was found in catagen and it was undetectable in telogen follicles. While proteoglycans are implicated in the regulation of cell adhesion, growth, migration and differentiation, their specific roles in hair growth cycling requires further investigation (Couchman and du Cros, 1995).

N-glycans are asparagine-linked oligosaccharides and share a common core structure (Brockhausen *et al.*, 1988). Complex N-glycans have branches that are initiated by the action of the Golgi-localised enzyme *α-3-D-mannoside β-1,2-Nacetylglucosaminyltransferase I (GnTI)* (Kumar *et al.*, 1990; Sarkar *et al.*, 1991). N-glycans have been shown to have functions in a multitude of biological processes, including cell-matrix and cell-cell interactions (Varki, 1993; Fiedler and Simons, 1995; Laidler and Litynska, 1997).

Fibulin is a calcium binding, acidic protein of the ECM which interacts with cell adhesion molecules, including fibronectin and laminin (Argraves *et al.*, 1989; Sasaki *et al.*, 1995; Tran *et al.*, 1995; Gu *et al.*, 2000). However, adhesion of mouse fibulin2 to human cells containing the integrin β3 chain may suggest a new function of the protein in bone marrow cells (Gu *et al.*, 2000). *Fibulin1* was found to be up-regulated by oestrogen in ovarian cancer cells (Clinton *et al.*, 1996; Roger *et al.*, 1998). The amount of immunoprecipitated *fibulin1* secreted into the medium and present in the cell extract was increased up to 10-fold by oestradiol in three oestrogen-responsive ovarian cancer cell lines (Clinton *et al.*, 1996). As oestrogen receptor-positive breast cancer cells are generally poorly invasive and oestrogen induces up-regulation of *fibulin1*, it has been suggested that reduced cell migration is due to the blockade of fibronectin-mediated migration by *fibulin1* (Hayashido *et al.*, 1998). *Fibulin1D* is a 2.7 kb isoform which encodes a novel, alternatively spliced form of human *fibulin1* (Tran *et al.*, 1997). The deduced amino acid sequence of the D form is identical in its first 566 residues to the three known *fibulin1* variants (fibulin1 A-C). However, *Fibulin1D* has a unique 137 amino acid C-terminal segment encoded by the

alternatively spliced portion of its transcript. RNA hybridisation analysis showed that *fibulin 1D* is co-ordinately expressed with fibulin1C, both in tissues and in cultured cells. In differential display, the expression of *fibulin1D* appeared to be up-regulated by melatonin and melatonin plus oestradiol, but did not respond to melatonin and progesterone in both male and female ferrets. Although *fibulin1D* may have functions in cell migration and interaction, its role in hair growth initiation requires further investigation.

Matrix Gla protein (MGP) is a vitamin K-dependent protein initially isolated from bovine bone. *MGP* is also expressed at high levels in cartilage, heart, kidney, and lung and up-regulated by vitamin D in bone cells (Fraser and Price, 1988; Cancela *et al.*, 1990). Analysis of the *MGP* promoter revealed, in addition to the typical TATA and CAT boxes, the presence of a number of putative regulatory sequences homologous to previously identified hormone and transcription factor-responsive elements (Cancela *et al.*, 1990). In particular, two regions of the promoter were delineated containing possible binding sites for retinoic acid and vitamin D receptors. In the present study, the mRNA level of *MGP* was increased at 0.5 and 2.5 days after melatonin administration and 1 day after co-administration of melatonin and oestradiol. The very low mRNA level observed 10 days after simultaneous administration of melatonin and oestradiol suggests its expression was suppressed by oestradiol. Although the exact function of *MGP* is not known, the broad tissue distribution demonstrates that its function is not specific to connective tissues and it is unlikely to act solely by virtue of its accumulation in an extracellular matrix (Fraser and Price, 1988). As *MGP* has been found to be expressed in some osteoblast cell lines and not others, its expression is thought to be associated with the development of particular cell phenotypes (Fraser *et al.*, 1988). Furthermore, *MGP* has been found to be expressed in highly metastatic human breast carcinoma cells, in comparison to no expression or lower level expression in most non-metastatic breast cancer cell lines or normal mammary epithelial cell lines (Chen *et al.*, 1990). Therefore, *MGP* may be among those factors that are responsible for metastases (see also Section 8.10) (Chen *et al.*, 1990; Clark *et al.*, 2000).

Integrins are members of a superfamily of surface glycoproteins that act as ECM receptors and mediate the adhesive functions of different types of cells. Integrins enable cells to interact with one another and with ECM, they maintain tissue integrity

and are required for development and morphogenesis (Peltonen *et al.*, 1989; Judware and Culp, 1997; Brakebusch *et al.*, 2000; Brown *et al.*, 2000). *TIED (ten integrin EGF-like repeat domains protein)* is a novel protein with some structural similarity to β-integrin and sequence homology to *OSCP (osteoblast specific cysteine-rich protein)*, but its role is not yet determined. It is widely expressed, but not particularly abundant apart from in the human aorta (Berg *et al.*, 1999). In this study, the expression of this gene was stimulated by melatonin, but inhibited by oestradiol. Given the EGF-like domains it contains, it may participate in protein-protein or protein-cell interactions (Berg *et al.*, 1999) and may also possess mitogenic activity (Panayotou *et al.*, 1989).

IGSF4 (immunoglobulin superfamily 4) is a novel member of the immunoglobulin superfamily and shares strong homology with other IGSF members, including mouse *IGSF-B12* and neuron cell adhesion molecules NCAM1 and NCAM2 within their Ig-like C2-type domains (Gomyo *et al.*, 1999). IGSF4 was expressed in all the 15 tissues examined and located in a region of chromosome where loss of heterozygosity commonly occurred (Gomyo *et al.*, 1999), suggesting that it is a candidate tumour suppressor gene. The fundamental importance of NCAM for feather development has been demonstrated (Jiang and Chuong, 1992). A study of NCAM immunoreactivity during murine hair follicle development and regression detected expression on epithelial hair placodes, and later in selected keratinocytes in the distal outer root sheath. Mesenchymal NCAM immunoreactivity was noted on fibroblasts of the presumptive dermal papilla and the perifollicular connective tissue sheath. Fetal hair follicle elongation coincided with strong, ubiquitous dermal NCAM immunoreactivity, which remained strong until the follicles entered the first neonatal catagen. At this time, the interfollicular dermal NCAM level decreased substantially. During consecutive hair cycles, mesenchymal NCAM was seen exclusively on dermal papilla and perifollicular connective tissue sheath fibroblasts and on the trailing cells of regressing catagen hair follicles (Muller-Rover *et al.*, 1998). These highly restricted and developmentally controlled expression patterns suggest an important role for NCAM in hair follicle morphogenesis and cyclic remodelling.

EVA (epithelial V-like antigen) is another novel member of the immunoglobulin superfamily and is differentially expressed during thymocyte development and in

several epithelial cell populations during embryogenesis (Guttinger *et al.*, 1998). It mediates cell adhesion through homophilic interaction and it is possibly associated with the cytoskeleton. Two independent ESTs identified during early hair growth showed some homology to this molecule, although its expression in skin was too low to be confirmed by Northern blot hybridisation.

The changes in expression of ECM components and the resultant interactions between cells produce a myriad of signals which modify cellular activities (Adams and Watt, 1993). Some ECM proteins themselves possess intrinsic growth factor activity (Adams and Watt, 1993). However, for cells to respond to these environmental cues, these signals need to transduced into the cytoplasm and nuclei. The MEKK family members, including the identified *MEKK1*, are characterised as localised sensors that control cell responses at the level of gene expression, metabolism and the cytoskeleton (Blank *et al.*, 1996; Schlesinger *et al.*, 1998) (Section 8.3). In addition, *MEKK1* is also an effector of Rho GTPases which have major roles in the regulation of assembly and organisation of cytoskeleton (Bishop and Hall, 2000). In order to ensure that the kinase initiated signalling is transient, phosphorylated proteins need to dephosphorylated (Fischer *et al.*, 1991). Interestingly, the identified PTPase, *LAR*, has a large extracellular domain similar to NCAM. Through expression of a rPTPase in insect Sf9 cells using recombinant baculovirus, it has been demonstrated that the rPTPase dramatically promotes cell-to-cell adhesion in a homophilic, Ca^{2+} independent manner (Gebbink *et al.*, 1993). Thus, *LAR* may not only regulate cell signalling, but could also be involved in cell-cell interactions.

The identification of *ezrin, titin, myosin, GAKIN (guanylate kinase associated kinesin)* was also of interest, as all are involved in ECM-cytoskeleton interaction, cell-shape determination, movement and chromatid segregation. Their discovery in this study further emphasises the role these processes play in the hair cycle. The cytoskeleton of eukaryotic cells provides structural support to the plasma membrane and also contributes to dynamic processes such as endocytosis, exocytosis, and transmembrane signalling pathways. The ERM (ezrin-radixin-moesin) family of proteins, of which *ezrin* is the best studied member, play structural and regulatory roles in the assembly and stabilisation of specialised plasma membrane domains (Bretscher *et al.*, 1997; Tsukita and Yonemura, 1999). *Ezrin* and related molecules

are concentrated in surface projections such as microvilli, filopodia, retraction fibres and membrane ruffles where they link the microfilaments to the membrane (Bretscher *et al.*, 1997). Specific ablation of *ezrin* blocks membrane ruffling and motility (Lamb *et al.*, 1997). Furthermore, overexpression of these molecules appears to enhance cell adhesion (Martin *et al.*, 1995). There are many binding partners for ERM, including the regulatory subunit of protein kinase A and Rho, suggesting that *ezrin* is an integral component of these signalling pathways. Rho, one of the low molecular GTP-binding proteins, is now considered to be a general regulator of actin-based cytoskeletal organisation (Hall, 1998). A relationship between the Rho signalling pathway and the activation of ERM has been suggested by both *in vitro* and *in vivo* studies (Tsukita and Yonemura, 1999). Activated ERM proteins are associated directly with adhesion molecules, such as CD44 and ICAM1/2/3, and indirectly with other integral membrane proteins (Tsukita and Yonemura, 1999). *Ezrin* is also concentrated at cleavage furrows in dividing cells (Sato *et al.*, 1991) and serves as an effector of hepatocyte growth factor (HGF)-mediated migration and morphogenesis in epithelial cells (Crepaldi *et al.*, 1997).

In addition to thick and thin filaments, vertebrate striated muscle contains a third filament system formed by the giant protein *titin*. The *titin* filament contributes to muscle assembly and resting tension (Labeit and Kolmerer, 1995). *Titin* kinase domain has a novel regulatory mechanism and it probably acts as a 'protein ruler' to regulate the assembly of myosin and actin filaments precisely (Trinick, 1994; Trinick and Tskhovrebova, 1999). *Titin* has also been found in non-muscle tissues, like brush border cytoskeleton of intestine (Eilertsen and Keller, 1992). The identification of *titin* as a chromosomal component is consistent with its function as a molecular scaffold for chromosome structure and elasticity (Machado *et al.*, 1998). Although the expression level of *titin* in skin was found to be much lower than that in striated muscle, its pattern appeared to be associated with hair cycle. In growing follicles, it was mainly expressed in cortex and outer root sheath cells, which are not actively proliferating, but was not detected in the germinal matrix. Similarly, heavy expression was found in the non-proliferating cells in the lower portion of telogen follicles (Fig. 7-9). Hence, expression of *titin* was restricted to some populations of hair follicle cells, as well as cells in the basal layer of the epidermis. However, its role in hair growth remains to be established.

Eukaryotic cells rely on actin- and microtubule-based protein motors to generate intracellular movements (Walker and Sheetz, 1993). Microtubule motors have two basic types: kinesin and dynein. These motors use the energy of ATP hydrolysis to transport cellular freight (vesicular organelles, chromosomes, etc.) along microtubule pathways. Members of the kinesin superfamily and the dynein family have a variety of other functions, including organisation of cytoskeleton and control of signal transduction (Walker and Sheetz, 1993; Goldstein and Philp, 1999; Hanada *et al.*, 2000; Manning and Snyder, 2000). In addition, they are essential in all organisms for proper chromosome and spindle behaviours during chromosome segregation (Barton and Goldstein, 1996; Heald, 2000), The native form of most kinesin molecules is a tetrameric complex consisting of two kinesin heavy chains and two regulatory light chains. In comparison, dynein is larger in size and contains one or more high-molecular-mass heavy chains, which are the ATP-binding subunits (Ogawa, 1991). Interestingly, one clone generated by this study showed sequence homology to *GAKIN (guanylate kinase associated kinesin)*, a novel member of kinesin superfamily of motor protein, which shares significant sequence similarity with the Drosophila kinesin-73, a heavy chain of the motor protein (Hanada *et al.*, 2000). Northern blot analysis indicated that *GAKIN* mRNA was ubiquitously expressed with higher levels in some tissues (Hanada *et al.*, 2000). According to its functional link with membrane-associated guanylate kinase homologues (MAGUKs), it was proposed that *GAKIN* might couple the MAGUKs to microtubule-based cytoskeleton, and that this interaction may be functionally important for the intracellular trafficking of MAGUKs and associated protein complexes *in vivo* (Hanada *et al.*, 2000). Another EST (F09G01-204-1) showed some limited similarity to dynein heavy chain.

Myosin heavy chains are another class of ubiquitous actin-based motor proteins that drive diverse processes including cytokinetics, vesicular transport, and cellular locomotion in eukaryotic cells. In skeletal muscle, the expression patterns of myosin mRNA isoforms indicate that the corresponding genes are regulated in a tissue-specific and developmental stage-specific manner (Bober *et al.*, 1990). Myosin has been identified in a number of non-muscle tissues, including epidermis (Bhatnagar and Freedberg, 1979; Bhatnagar, 1981; Williams *et al.*, 1989). Myosin plays an important role in motility of cells and metastasis. The major mechanism of Ca^{2+}

sensitisation of smooth muscle contraction and non-muscle cell motility is through inhibition of the smooth muscle myosin phosphatase that dephosphorylates the regulatory myosin light chain. The active, GTP-bound form of RhoA activates a serine/threonine kinase, Rho-kinase, which phosphorylates the regulatory subunit of smooth muscle myosin phosphatase and inhibits phosphatase activity (Somlyo and Somlyo, 2000). In smooth muscle, Ca^{2+}-mediated myosin light chain kinase activity is sufficient to promote contraction (Parizi *et al.*, 2000). The main regulator of Ca^{2+} flush in this case is lysophosphatidic acid (LPA) (Parizi *et al.*, 2000). Furthermore, LPA has been showed to induce a rapid reappearance of focal adhesions and stress fibres in quiescent cells, accompanied by an increase in phosphotyrosine in focal adhesions (Chrzanowska-Wodnicka and Burridge, 1994). One EST (F08F12-265-7) had some homology to a *LPA receptor* which is required for the function of extracellular LPA (Contos and Chun, 1998; Chun *et al.*, 1999).

Finally, *lysozyme*, a key protease in the lysosome, may be also involved in cell-cell interactions or cell movement by modifying ECM or intracellular proteins involved, and has been linked to cancer metastasis (Clark *et al.*, 2000). However, the nature of *lysozyme* activity in these processes is not known.

The identification of a class of transcripts associated with ECM, cell adhesion, extracellular stimuli signalling, cell motility and chromosome segregation is intriguing. This suggests that expression alternation of a large number of genes involved in ECM and cell-cell interactions is required in the early stages of hair follicle reactivation. On the other hand, as many of these genes are involved in cell growth and differentiation, it supports the observations that these processes are prominent during early hair follicle growth (Chapter 2) (Silver and Chase, 1970; Pierard and Brassinne, 1975; Wilson *et al.*, 1994). As hair follicle stem cells have been identified to the bulge region of hair follicles (Cotsarelis *et al.*, 1990; Taylor *et al.*, 2000) and cell amplification mainly occurs in the germinal matrix at the bottom of the follicles, reactivation of a new hair cycle requires migration of stem cells from the bulge region to the germinal matrix and bulb structure reshuffling. More prominent cell migration is seen when large number of progenitor cells move from the germinal matrix up into other compartments and differentiate into specific follicle cell types. Cell movement requires the coordinate expression of numerous genes

(Lauffenburger and Horwitz, 1996). Thus, identification of genes associated with cell motility supports this picture of hair growth processes associated with proanagen.

8.6 Apoptosis during hair growth initiation

Apoptosis plays a central role in embryogenesis, morphogenesis and many other processes (Packham and Cleveland, 1995; Jacobson *et al.*, 1997). Five functions for apoptosis have been suggested: (1) sculpture of tissue structure; (2) deletion of unneeded structures; (3) control of cell number; (4) elimination of abnormal, misplaced, non-functional, or harmful cells; and (5) production of differentiated cells without organelles (Jacobson *et al.*, 1997). A number of transcripts involved in apoptosis have been identified in this study, including *MSSP1/2, MEKK1, MHP, proteasome HSPC subunit, SAE1C), UBL3, SREBP1, SRP72* and possibly apoptosis inhibitor *IEX1L.*

C-myc has a dual function in the induction of cell cycle progression (Section 8.4) and in the acceleration of apoptosis (Hoffman and Liebermann, 1998; Dang, 1999; Prendergast, 1999; Conzen *et al.*, 2000). C-myc can rapidly activate ARF and p53 gene expression and trigger replicative crisis by inducing apoptosis (Zindy *et al.*, 1998). *MSSP1/2* not only participates in apoptosis indirectly by regulating the expression of c-myc (Section 8.4) (Duncan *et al.*, 1994; Niki *et al.*, 2000a; Niki *et al.*, 2000b), but is also directly involved in apoptosis induction (Iida *et al.*, 1997). Both *MSSP1* and *MSSP2* induce apoptosis in a dose-dependent manner in the human Hela cell line. A region of *MSSP* containing one of the two RNA polymerase (RNP) consensus motifs, RNP1-B, is required for induction of apoptosis as well as specific DNA binding activity (Iida *et al.*, 1997).

As a signalling component (Section 8.3) and sensor of cellular environment (Section 8.5), *MEKK1* is an important molecule in the regulation of cell survival and apoptosis (Schlesinger *et al.*, 1998; Gibson *et al.*, 1999; Minamino *et al.*, 1999). *MEKK1* regulation of the JNK pathway is a critical response for protection against oxidative stress-induced apoptosis in cardiac myocytes (Minamino *et al.*, 1999) and stress-induced apoptosis in general (Xia *et al.*, 1995). On the other hand, the cleavage of *MEKK1* by caspases in turn stimulates caspase activity and leads to apoptosis following genotoxin treatment or disrupted integrin-mediated contact with the ECM (Cardone *et al.*, 1997). Thus, caspases, the most common executors of apoptosis, can

induce apoptosis by activating *MEKK1*. Intact *MEKK1* is generally involved in promoting cell survival (Yujiri *et al.*, 1998; Minamino *et al.*, 1999). Subcellular localisation appears to be the critical regulatory mechanism controling the survival-promoting versus apoptotic-inducing roles of *MEKK1*. The 196 kDa full length *MEKK1* is membrane-associated, but the cleaved 91 kDa kinase domain-containing fragment is localised to the soluble fraction of cytoplasm (Deak *et al.*, 1998). Tethering the 91 kDa form of *MEKK1* to the membrane prevents *MEKK1*-mediated apoptosis (Schlesinger *et al.*, 1998). *MEKK1* mediates survival versus apoptosis possibly via its ability to regulate transcription factors, the expression of death receptors and their ligands (Schlesinger *et al.*, 1998).

Mitochondria play an important role in apoptosis as one of the residing structures for both pro-apoptosis molecules, such as caspases, and apoptosis inhibitors such as Bcl-2. Release of cytochrome c and other pro-apoptotic molecules from mitochondria induces apoptosis (Green, 1998; Green and Reed, 1998; Jacotot *et al.*, 2000). *MHP*, a subunit of respiratory chain, is found to affect apoptosis by binding to cytochrome c and regulating its release (Okazaki *et al.*, 1998). Release of cytochrome c in turn modulates the activity of caspases (Green, 1998; Green and Reed, 1998). The function of *MHP* in hair growth remains to be determined, as its mRNA was found to be suppressed by melatonin or melatonin and oestradiol or progesterone in the present study.

HSPC is a subunit of the proteasome, the principal cellular machinery for protein degradation, which is required for apoptosis (Coux *et al.*, 1996). Proteasome inhibitors alter apoptosis activity (Kawakami *et al.*, 1999; An *et al.*, 2000; Huang *et al.*, 2000). The degradation of targeted proteins or mRNA are greatly enhanced by labelling with one or multiple ubiquitins (Hershko and Ciechanover, 1998). Increased ubiquitin expression and ubiquitin-dependent proteolysis are associated with apoptosis (Jones *et al.*, 1995). The identified *SAE1C*, a subunit of *SUMO1*, initiates ubiquitination of the targeted protein (Okuma *et al.*, 1999). Binding of *SUMO1* to Mdm2, a ubiquitin ligase for p53, abrogates self-ubiquitination and increases ubiquitination of p53, resulting in the inhibition of p53-mediated apoptosis (Buschmann *et al.*, 2000). *UBL3*, another ubiquitin-like protein was also identified, further suggesting the importance of ubitiquination and proteasome degradation

during proanagen. It should also be noted, in this context, that keratin turnover has been found to be effected through ubitiquination (Ku and Omary, 2000).

SREBP1 plays a central role in metabolism of fatty acids (see next section) (Mater *et al.*, 1999; Osborne, 2000). However, an involvement in apoptosis has also been reported (Wang *et al.*, 1996). It is cleaved into an active enzyme during apoptosis induced by different factors, although its exact functional significance has not been established.

Signal recognition particle (SRP) is a highly conserved complex composed of a structural RNA molecule and 6 polypeptides, including *SRP72*. It has three known functions: signal recognition, elongation arrest, and translocation promotion, all of which are needed for mediating the targeting of secretory proteins to the endoplasmic reticulum (ER) (Walter and Blobel, 1980; Walter and Blobel, 1982). Peptide of 72 kDa (*SRP72*) has been implicated in binding to SRP receptor and promoting the directional translocation of newly translated proteins bearing a signal sequence into the lumen of the ER (Siegel and Walter, 1988). However, *SRP72* undergoes post-translational modifications during programmed cell death (Utz *et al.*, 1998). Both serine phosphorylation and caspase cleavage of *SRP72* are observed in cells of human, dog, rat, and mouse origin. Cleavage of *SRP72* can be prevented by chemical and peptide caspase inhibitors, and by over-expression of Bcl-2, an inhibitor of apoptotic cell death. It was predicted that the cleavage of *SRP72* during apoptosis may inhibit binding of the complex to the SRP receptor on the ER membrane. This would result in a global prevention of the localisation of the secretory proteins to the ER and irreversible elongation arrest of newly translated proteins bearing signal sequence (Utz *et al.*, 1998). However, how this could affect hair follicle growth is currently unknown.

Apoptosis inhibitors are a rapidly growing family of molecules which play a critical part in apoptosis regulation, cell cycle progression and differentiation (Palestro *et al.*, 1997; Adams and Cory, 1998; Cory *et al.*, 1999; Grossman *et al.*, 1999; Porat and Simantov, 1999; Kanamori *et al.*, 2000). Inhibition of caspase activation or cytochrome C release is two of the many strategies by which apoptosis inhibitors promote cell survival (Adams and Cory, 1998). The induction of transcription of the apoptosis inhibitor *IEX1L* by TNF was decreased in cells with defective NFκB

activation, rendering them sensitive to TNF-induced apoptosis (Wu *et al.*, 1998). Transfection with *IEX1L* abolished apoptosis. In support of this observation, over-expression of antisense *IEX1L* partially blocked TNF-induced expression of *IEX1L* and sensitised normal cells to apoptosis (Wu *et al.*, 1998).

GAPDH, a glycolytic enzyme, has long been regarded as a house-keeping gene with relatively constant expression pattern. However, more recently, many different isoforms and functions of this molecule have been identified (Chuang and Ishitani, 1996; Chen *et al.*, 1999). Its expression is also inducible (Rimarachin *et al.*, 1992) and is involved in apoptosis of both neural cells and non-neural cells (Chuang and Ishitani, 1996; Chen *et al.*, 1999; Shashidharan *et al.*, 1999). For instance, apoptosis of cerebellar granule cells involves the expression of both *GAPDH* and p53, and similar to Bax, GAPDH is up-regulated by p53 after exposure to the apoptotic insult (Chen *et al.*, 1999). Further, increased expression and nuclear accumulation of *GAPDH* have been identified as an early and critical event in several forms of apoptosis (Shashidharan *et al.*, 1999).

Apoptosis has been identified in catagen hair follicles (Seiberg *et al.*, 1995; Cece *et al.*, 1996; Lindner *et al.*, 1997; Tobin *et al.*, 1998; Muller-Rover *et al.*, 1999). Apoptotic cells not only appear in the regressing epithelium of the proximal follicle but, surprisingly, are also seen in the central inner root sheath, in the bulge/isthmus region, and in the secondary germ, but never in the dermal papilla (Lindner *et al.*, 1997). Bcl-2, an apoptosis inhibitor, and Bax, an apoptosis promoter, show tightly regulated and hair cycle-dependent expression patterns (Muller-Rover *et al.*, 1999; Muller-Rover *et al.*, 2000). Apoptosis was not found in growing murine hair follicles during the first postnatal hair cycle conducted using the terminal deoxynucleotidyl transferase-mediated dUTP nick end-labelling (TUNEL), complemented with electrophoresis of DNA isolated from the hair follicles (Matsuo *et al.*, 1998). Therefore, the identification of nine molecules that participate in apoptosis from skin samples containing predominantly early proanagen hair follicles was surprising.

However, most of the skin samples used in differential display were derived from ferrets treated with melatonin or melatonin with oestradiol or progesterone. Apoptosis is modulated by all of these hormones. Oestradiol inhibits apoptosis in most circumstances (Alvarez *et al.*, 1997; Chen *et al.*, 2000; Perillo *et al.*, 2000). On

the contrary, progesterone often induces apoptosis, especially in the presence of oestrogen (Bu *et al.*, 1997; Formby and Wiley, 1998; Gaytan *et al.*, 2000). Melatonin usually prevents apoptosis (Sainz *et al.*, 1995; Osborne *et al.*, 1998; Yu *et al.*, 2000), although it induces apoptosis when delivered with retinoic acid (Eck *et al.*, 1998; Eck-Enriquez *et al.*, 2000), through the alteration in the expression of a number of apoptosis regulators.

The detection of apoptosis in the early stages of hair growth is possibly linked to the prominent cell proliferation and differentiation processes (Sections 4.3.5.3 and 8.4) in which apoptosis plays a critical role (Jacobson *et al.*, 1997; Jacotot *et al.*, 2000). Furthermore, some skin samples did contain a small number of growing juvenile follicles destined for growth regression prior to the first winter pelage development. These regressing follicles could be another source for the detection of apoptosis-associated gene expression. Confirmation of the sites of expression of these transcripts is now required to assess the role of apoptosis in proanagen initiation.

8.7 Fatty acid and cholesterol synthesis

De novo biosynthesis of fatty acids involves *ATP citrate lyase (ACL)*, acetyl CoA carboxylase, fatty acid synthase, *stearoyl CoA desaturase (SCD)* and lipoprotein lipase. In addition to the two enzymes *ACL* and *SCD*, the transcriptional regulator of all these genes, *sterol responsive element binding protein-1 (SREBP1),* was also detected in this study. *SREBP1* is a transcription factor and a key regulator of lipogenesis and cholesterol homeostasis (Brown and Goldstein, 1997; Swinnen *et al.*, 1997; Osborne, 2000). There are three members of *SREBP* coded by two different genes (Brown and Goldstein, 1997). *SREBP1* has two isoforms (*SREBP1a* and *SREBP1c*) arising through the use of alternate transcription start sites. *SREBPs* are membrane-bound and exist as inactive precursor proteins. They are activated by two sequential cleavages whereupon the active N-terminal is released and translocated into the nucleus where they bind to their target genes (Sato *et al.*, 1994; Brown and Goldstein, 1997). Genes regulated by *SREBPs* include those involved in cholesterol homeostasis, such as the low-density lipoprotein receptor (Briggs *et al.*, 1993; Wang *et al.*, 1993), HMG-CoA (3-hydroxy-3-methylglutaryl CoA) (Yokoyama *et al.*, 1993), HMG-reductase (Vallett *et al.*, 1996), farnesyl diphosphate synthase (Ericsson *et al.*, 1996; Jackson *et al.*, 1996) and sualene synthase (Guan *et al.*, 1995).

Additionally, *SREBP* also modulates several genes for fatty acid synthesis, including acetyl CoA carboxylase (Lopez *et al.*, 1996) and fatty acid synthase (Bennett *et al.*, 1995). Moreover, the effects of androgens on lipogenic gene expression are mediated via *SREBP* (Swinnen *et al.*, 1997), rather than the individual enzymes, further demonstrating the key role of *SREBP* in lipid metabolism.

ACL is a cytosolic enzyme that generates acetyl-CoA for cholesterol and fatty acid synthesis *de novo* (Pearce *et al.*, 1998). *SREBP1* and *2* transcriptionally regulate *ACL* enzyme activity, thus affecting both cholesterol and fatty acid synthesis (Sato *et al.*, 2000). Another enzyme, *SCD*, is a rate-limiting enzyme in the synthesis of mono-unsaturated fatty acid from saturated fatty acids. The ratio of stearic acid and oleic acid in phospholipids is critical for regulating cell membrane fluidity. Through the control of stearic acid production, *SCD* plays a pivotal role in determining membrane fluidity (Kim and Ntambi, 1999). *SCD* thus affects inter- and intracellular signalling, by controlling the movement of membrane-bound signal transduction molecules, including receptors (Gyorfy *et al.*, 1997) and ion channels (Perez *et al.*, 1997). The expression of *SCD* is regulated by a number of factors, including polysaturated fatty acids and cholesterol, hormones, temperature at the levels of transcription, and mRNA stability via *SREBP* and other transcription factors (Ntambi, 1995; Tiku *et al.*, 1996; Ntambi, 1999). Differential display band density for *SCD* gradually increased after all three hormone treatments in males and melatonin and progesterone treatment in females. Although the differential expression of these genes requires confirmation, fatty acid and cholesterol synthesis do appear to have an important role in hair cycling (see below).

The glycolytic enzyme *GAPDH* could also be involved in fatty acid and cholesterol synthesis by providing ATP or other substrates required for this process. Although the evidence for the identification of the *LPA receptor* is comparatively weak, the observation that LPA produced by adipocytes can serve as a paracrine mediator for pre-adipocyte growth and function (Pages *et al.*, 2000) may suggest a role for the differential expression of this gene.

The nature of the changes in fatty acid and cholesterol synthesis associated with the early stages of hair follicle growth is speculative. As cholesterol is the precursor of steroid synthesis (Chapter 2), alteration in the status of the genes involved in

cholesterol and fatty acid production might have resulted from steroid administration, resetting the requirement for cholesterol. Steroids, such as oestradiol and progesterone, may also modulate the expression of these genes, as has already been demonstrated for androgens (Swinnen *et al.*, 1997). Melatonin also affects cholesterol metabolism, although how this is achieved is unknown (Esquifino *et al.*, 1997).

The altered metabolism of lipids could modify the saturation status of membrane lipoprotein which affects membrane fluidity, and thus cell signalling, cell-cell interactions and cell migration, all processes which are prominent in the early stage of hair growth (Section 8.5). On the other hand, the skin structure changes drastically following hair growth initiation. Ten days after depilation in mouse skin, the corium and adipose layers increase to approximately 390 μM and 260 μM, respectively from 250 μM and 150 μM. Therefore, active new fatty acid synthesis and cholesterol metabolism are required to accommodate this expansion. Equally importantly, fatty acid and cholesterol synthesis has been found to be important for normal growth of skin and hair follicles cells. Overexpression of apolipoprotein C1 reduces the relative amounts of triglycerol and wax diesters in sebum of the epidermis. This is accompanied by a disorder of the skin identified by hair loss, epidermal hyperplasia and hyperkeratosis, and atrophic sebaceous glands (Jong *et al.*, 1998). Altered *SCD* expression is involved in abnormal hair growth in asebia (Zheng *et al.*, 1999; Sundberg *et al.*, 2000). Further, mono-unsaturated fatty acid can co-activate various isoforms of protein kinase C (Shirai *et al.*, 1998), which have important roles in growth and differentiation of epidermal cells and hair follicles (Dlugosz and Yuspa, 1993; Harmon *et al.*, 1995). All three *SCD* transcripts have been found in skin, including a novel species (*SCD3*) expressed in sebaceous glands and hair follicle matrix cells (Parimoo *et al.*, 1999; Zheng *et al.*, 1999). Furthermore, although the expression of *SCD1* is limited to sebaceous glands, its expression is regulated throughout the hair cycle, with higher levels during proanagen (Zheng *et al.*, 1999).

8.8 Ribosomal functions

The ribosome is the machinery which produces proteins, thus transforming genotype to phenotype. Ribosomes are major cell constituents. An actively growing *E. coli* cell contains about 15,000 ribosomes which account for about a quarter of the dry cell

mass. Each ribosome consists of one large subunit and one small subunit. Each of these subunits is a complicated multiple-component structure. For eukaryotic cells, the large subunit consists of 32 ribosomal proteins (RP) and three ribosomal RNAs (rRNA), while the small subunit has 21 ribosomal proteins and one rRNA. The rRNAs associated with the large subunit of eukaryotic cells are 28s, 5.8s and 5s, rather than 23s and 5s rRNA of prokaryotic cells. For the small subunit, 18s rRNA replaces the 16s in prokaryotic cells. Knowledge from the simpler, better studied prokaryotic ribosome indicate that all these components interact in a complicated manner to accomplish protein synthesis (Cate *et al.*, 1999; Ban *et al.*, 2000; Wimberly *et al.*, 2000). The translation elongation cycle depends on three fundamental processes: (1) aminoacyl-tRNA selection, (2) peptide bond formation, and (3) translocation of tRNAs from the A site to the P site. All of these require a fully assembled ribosome. Four ribosomal protein cDNAs and a 72 kDa component of signal recognition particle (Section 8.6) were cloned in this study. Interestingly, all the ribosomal proteins were from the large subunits and they were *RPL4, RPL7, RPL11* and *RPL14*. *RPL7* couples with RPL12 in forming the ribosome and they are the only two ribosomal proteins which have multiple copies. *RPL7*/12 appears to function by stabilising the GTPase transition state of elongation factor-G (Savelsbergh *et al.*, 2000). In addition, *RPL7* also acts as co-regulator of nuclear receptor-mediated transcription (von Mikecz *et al.*, 1999). However, its differential expression was not confirmed by Northern blot analysis. Although quiescent and senescent cells were found to synthesise protein at similar rates, senescent foetal lung and foreskin cultures showed a decline in mRNA levels for L7 and five other ribosomal proteins (L5, P1, S3, S6, and S10) (Seshadri *et al.*, 1993).

The structure and function of bacteria *RPL4* is much better understood. This ribosomal protein resides near the peptidyl transferase centre of the bacterial ribosome and may, together with rRNA and proteins RPL2 and RPL3, actively participate in the catalysis of peptide bond formation. *RPL4* of *E. coli* is also an autogenous feedback regulator of transcription and translation (Yates and Nomura, 1980; Worbs *et al.*, 2000). The expression of *RPL4* was found to be at a higher level in a skin sample containing mainly anagen follicles which have a higher rate of protein synthesis.

RPL11 interacts with a 58-nucleotide domain of the large subunit rRNA and stabilises its tertiary interactions, which are important for ribosome function (Xing and Draper, 1995; Xing and Draper, 1996). Studies have indicated that *RPL14* occupies a central location between the peptidyl transferase and GTPase regions of the large ribosomal subunit and functions to promote the folding and stabilisation of ribosomal RNA (Davies *et al.*, 1996). To understand the relevance of these ribosomal proteins to hair growth, the differential expression patterns of these genes need to be confirmed by other methods.

8.9 Genes potentially important for hair growth initiation

Melatonin, prolactin and steroid hormones exert their effects by binding to their cognate receptors on the cell surface or in the cytoplasm, which triggers still poorly understood signalling pathways (Chapter 2). In this way, they directly and indirectly modify the expression of many genes to achieve their respective biological functions. The simultaneous administration of melatonin and steroids is likely to further complicate the situation given the potential interactions of different signalling pathways. For instance, melatonin suppresses the expression of the oestrogen receptor gene in breast cancer cells (Molis *et al.*, 1994). Furthermore, as it has been shown in Chapter 4, oestradiol shuts down hair growth initiated by melatonin regardless whether it was given prior to, simultaneously or after melatonin. Other steroids have different effects on hair growth depending on whether they are co-administered with melatonin and the time of administration. Many of the biological processes identified during hair growth initiation are known to be affected by melatonin and steroids in skin or other tissues, however, no transcripts which are specifically linked to the metabolism or signal transduction of steroids were identified in this study. The limited number of primer sets used and the likely low levels of expression of such genes may have been factors in this outcome.

Identification of genes whose expression is specifically linked to early growth initiation or inhibition represents an important initial step towards understanding hair cycle control. Melatonin and melatonin plus progesterone were found to stimulate hair growth initiation in males and females respectively (Chapter 4). In contrast, both melatonin plus oestradiol and melatonin plus progesterone inhibited hair growth in males. Skin samples collected at various time points after these treatments were used

in differential display (Chapter 5). Differential display band density patterns indicated treatment-responsive genes. Some identified ESTs showed consistent differential display patterns depending on whether the treatment was stimulatory or inhibitory. Identified transcripts belonging to this category include *PCDC5RP, MSSP1/2, IGSF4, LAR, MHP* and *MyHC IIa.* These transcripts are more likely to be involved in central pathways for hair growth regulation, and thus represent good candidates for further studies.

8.10 Comparison of results with other studies

In an effort to characterise human gene expression patterns, more than 52 million nucleotides from 174,472 ESTs were generated from 300 cDNA libraries constructed from 37 organs and tissues (Adams *et al.*, 1995). These ESTs were combined with an additional 118,406 ESTs from the GenBank DBest database and subjected to shotgun sequence assembly. Of the resulting 87,983 distinct sequences, 10,214 had statistically significant sequence similarity to known genes in the available databases, with the remainder representing previously unknown genes. All the known transcripts were then classified into seven functional categories. In a very recent report, 5130 clones from a cDNA library derived from an early passage of dermal papilla cells of rat vibrissae were randomly sequenced and classified similarly (Sleeman *et al.*, 2000). For comparison, the ESTs generated in this study were reclassified according to the same criteria (Adams *et al.*, 1995). The percentages of genes in the seven different cellular roles across the 37 human tissues, in human and ferret skin and in cultured rat dermal papilla cells are summarised in Table 8-3.

Compared with both the total human pool and human skin ESTs, a higher percentage of transcripts in this study were associated with cell structure and motility. Ferret skin samples collected during hair follicle growth initiation showed more than three times as many transcripts in this category than reported in the human. Approximately a quarter of the ESTs from cultured dermal papilla cells had sequence similarity to proteins involved in cell structure and motility (Sleeman *et al.*, 2000). Although this percentage is also higher than that in normal human skin and human tissue overall, it is still significantly lower that seen in ferret skin during hair growth initiation. Expression alterations of such genes are likely to be a crucial part of early follicle reactivation.

Table 8-3 Comparison of transcript classification in different studies

Classification[1]	Human tissue overall[2]	Human skin[2]	Rat dermal papilla cells[3]	Ferret skin[4]
cell signalling & communication	16.5	12.7	11.6	11.5
gene & protein expression	29.1	35.0	30.2	34.6
cell division & DNA synthesis	5.9	5.3	3.5	5.8
cell structure & motility	10.8	10.9	24.4	32.7
defence & homeostasis	15.8	17.0	17.4	3.8
metabolism	21.8	19.1	12.8	9.6

[1] Cellular roles were assigned by Adams *et al.* (1995) to the following categories by examination of database annotations and literature cited therein: **Cell signalling/cell communication**, includes receptors, protein modification, hormone/growth factors, intracellular transducers, effectors/modulators, metabolism, cell adhesion and channels/transport proteins. **Gene/protein expression**, includes protein synthesis, translation factors, ribosomal proteins, post-translational modification/targeting, protein degradation, tRNA synthesis/metabolism; RNA synthesis, transcription factors, RNA polymerase, RNA processing; RNA degradation. **Metabolism**, includes amino acids, nucleotides, sugars, lipids, cofactors, protein modification, energy, and carriers proteins/membrane transport. **Cell division/DNA synthesis**, includes cell cycle, apoptosis, DNA synthesis/replication and chromosomal structure. **Cell structure/motility**, includes cytoskeletal, microtubule-associated proteins/motors, and extracellular matrix. **Cell/organism defence and homeostasis,** includes immunology, homeostasis, carrier proteins/membrane transport, stress response, and DNA repair.

[2] Adams *et al.*, 1995; [3] Sleeman *et al.*, 2000; [4] 42 genes identified by sequence homology in the current study.

Note: Genes with unknown function are excluded from the comparison. These comprised 24.8 % of the total and 25.1 % of the human skin ESTs (Adams *et al.* , 1995) and 14.1% in the rat dermal papilla cell culture (Sleeman *et al.*, 2000).

It is also interesting to note that only two ferret ESTs involved in host defence and homeostasis (4%) were detected in this study. The immune system and its components have been found to be involved in hair follicles and undergo cell cycle-associated remodelling (Gibson *et al.*, 1991; Westgate *et al.*, 1991a; Paus *et al.*, 1994b; Paus *et al.*, 1998). The maintenance of the homeostasis of hair follicle cells is also important to prevent skin tumours (Miller *et al.*, 1993b; Taylor *et al.*, 2000).

The low percentage of transcripts in this category suggests that the number of immune cells involved in hair growth is comparatively small and the consequential gene expression alterations were shadowed by changes in other cells. However, on the basis of this initial survey, the importance of the immune system to the hair growth cycle is questionable, particularly at the early stages of follicle reactivation. The lower percentage of ESTs for transcripts involved in metabolism in the present study may partially reflect the predominance of cell structural/motility ESTs. However, it is more similar to the percentage derived from cultured dermal papilla

cells. While this comparison has indicated some interesting differences between normal human tissues and ferret skin during hair growth initiation, the comparatively small number of ESTs generated in this study may not be sufficient for an accurate assessment. More importantly, the ferret ESTs were not randomly sampled, but were selected by differential display.

In another study, the expression profiles of 7,070 human genes and 6,347 mouse genes from three different human and mouse metastatic melanoma cell lines and their non-metastatic counterparts were compared by microarray to identify genes responsible for metastasis (Clark *et al.*, 2000). The expression levels of 16 human genes and 16 mouse genes were found to be significantly higher in the metastases than in the primary tumour. Overexpression of one of the identified genes, RhoC, was sufficient for metastasis in all three melanoma cell lines (Clark *et al.*, 2000). It is notable that five of these 32 metastasis transcripts were also discovered in the present study, and a further one was closely related to the hair cycle genes. These were *RTP/Ndr1, MGP, IEX1/Gly96, lysozyme, colgn α2I* and *biglycan.* Six others, fibronectin, ERK1, fibromodulin, TGF-β, keratin K14 and RhoC (Chen *et al.*, 1990; Clark *et al.*, 2000) are functionally associated with some genes identified in this study. This high overlap percentage suggests that many genes involved in the development of metastasis may also play important roles in normal hair growth cycles.

TGF-β is a strong inhibitor of cell proliferation and differentiation in many cell types (Massague, 1990; Pelton *et al.*, 1991). TGF-β has also been found to have profound effects on hair follicle growth and cycling (Green, 1984; Jones *et al.*, 1991; Blessing *et al.*, 1993; Sutton *et al.*, 1995; Lange *et al.*, 1996; Paus *et al.*, 1997a; Welker *et al.*, 1997; Foitzik *et al.*, 1999). Using a mink lung epithelial cell line as a model, a number of genes associated with TGF-β1-exerted cell growth arrest were identified (Kallin *et al.*, 1991; Ralph *et al.*, 1993). These included *collagen-α(I), TI1*, cyclin A, fibronectin, monocyte chemotactic cell-activating factor, osteonectin, plasminogen inhibitor 1, and an unknown transcript. Northern blot confirmed that most of these transcripts were differentially expressed during cell growth (Kallin *et al.*, 1991; Ralph *et al.*, 1993). Many of these molecules have functions associated with the

ECM, for which a significant number of related transcripts were detected in the current study.

In terms of the biological processes involved in hair growth, evidence generated in the current study confirms that cell proliferation and cell differentiation are prominent during early follicle reactivation. Numerous identified transcripts involved in cell interaction and movement link follicle cell migration to metastasis, suggesting that hair follicles could be an easily accessible model for the study of some aspects of tumour development. In addition, apoptosis also appears to be an important component of proanagen, which has not been established previously. Fatty acid synthesis and metabolism may have much more important implications in hair growth than has been realised. It is required to accommodate the above processes in hair follicles and the expansion of the adipose tissues in skin. Deregulation of gene expression involved in fatty acid synthesis and metabolism can cause serious hair growth problems as in asebia mutant mouse which has rudimentary sebaceous glands (Zheng *et al.*, 1999). Therefore, Fig. 8-1 can be modified to produce Fig. 8-2. Compared with Fig. 8-1, cell migration appears to be required even for the proanagen stage before histological changes can be seen. Cell proliferation and differentiation is confirmed to be essential during early proanagen. Apoptosis appears to be required not only during catagen, but is actively regulated during proanagen. The role of fatty acid synthesis and metabolism during follicle growth re-initiation has not previously been fully appreciated.

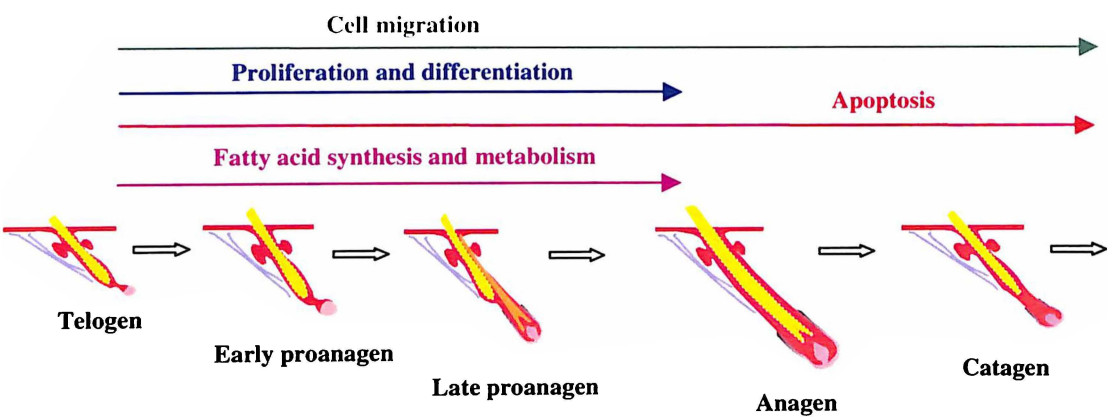


Fig. 8-2 Revised association of biological processes and the hair growth cycle

8.11 Conclusions

Differential display has been previously used in the detection of gene expression in a variety of tissues. By using a protocol which combines the advantages of single-base anchored longer oligo dT primer and a longer arbitrary primer in conjunction with two-stage PCR, 112 sequences were obtained from two sets of skin samples spanning the hair growth initiation process. Most of the ESTs were generated from the skin samples associated with early growth events. The use of complete skin has also aided in revealing of potential cellular interactions among and between follicular and interfollicular cells involved in hair follicle formation and cycling (Hardy, 1992; Stenn and Paus, 1999; Widelitz and Chuong, 1999).

Sequence homology searches revealed that, of the 112 ESTs, 42 were homologous to known transcripts in GenBank. These transcripts were tentatively grouped into functional categories, including cell growth and differentiation, cell interactions and motility, apoptosis, fatty acid and cholesterol synthesis, transcription and signalling and ribosomal constituents. The current knowledge of these molecules in skin, hair follicles and other tissues suggest that many of these molecules are likely to participate in hair growth initiation.

In comparison to general human gene expression patterns in skin and other tissues, the percentages of transcripts involved in cell structure/motility were markedly increased in the present study, indicating that cell-cell interaction and cell migration are especially prominent in hair follicle growth initiation. The unexpected identification of many transcripts linked to apoptosis and fatty acid metabolism demonstrates importance of these processes in the development of proanagen and has implications on an improved understanding of hair growth initiation.

CHAPTER 9 CONCLUSIONS

9.1 Ferret winter hair growth and the effects of steroids

The initiation of hair growth was synchronised by melatonin implantation in trials conducted in each of the three successive years. However, as not all hair follicles were in telogen at the time of melatonin administration, the hair regrowth was not as synchronised as previously reported (Nixon *et al.*, 1992; Nixon *et al.*, 1995). Nevertheless, these trials represented the first systemic histologically-based study of the effects of steroids on ferret winter hair growth initiation and generated suitable samples for analysis of gene expression underlying hair follicle reactivation.

The observed inhibitory effects of oestradiol, testosterone and dexamethasone on spontaneous hair growth in the male ferret were in general agreement with those documented in other species (Mohn, 1958; Stenn *et al.*, 1993; Paus *et al.*, 1994a; Chanda *et al.*, 2000). The profound inhibitory effects of oestradiol suggest that it is a useful tool to explore the control mechanisms of telogen induction and maintenance (Chanda *et al.*, 2000). The different effects of dexamethasone, testosterone and deoxycorticosterone on spontaneous and melatonin-initiated hair growth indicated that their effects were weaker and could be counteracted by exogenous melatonin. However, testosterone given seven days before melatonin modestly stimulated hair growth. A novel finding was the sexual dimorphic effect of progesterone. This steroid inhibited melatonin-initiated hair growth in male ferrets, but had no effect in females. Another potentially significant observation was an apparent permissive (or stimulatory) effect of some steroids on early proanagen, contrasting with an inhibitory effect in late proanagen. This growth stage-dependent effect could provide a useful model for dissecting the actions of these hormones.

With the exception of dexamethasone, steroid effects on hair growth initiated by melatonin did not differ when the steroids was administered simultaneously, or seven days earlier than melatonin, suggesting that steroids and melatonin work through independent pathways. The identification of steroid receptors in skin and in hair follicles over recent years not only allows for this possibility, but also suggests that some interactions of the steroids, melatonin and prolactin are likely to be

downstream from the events surrounding changes in circulating hormone levels and their receptor binding.

9.2 Gene expression patterns over hair growth initiation

Flank skin samples from the ferrets treated with melatonin in 1996 were representative of a progressive series of hair cycle stages to which differential display analysis of gene expression was applied. Using the protocol optimised in this study, it was estimated that approximately 6% of the total expressed genes in skin were screened. As 100 (7%) of the total displayed cDNA bands changed in density by a factor of two-fold or more in a set of samples covering all proanagen stages, the number of genes involved in hair growth reinitiation may be greater than a thousand. The most frequent changes in gene expression coincided with the stages at which hair follicle cells underwent proliferation, differentiation and follicle morphogenesis. Using liver and footpad skin containing no follicles as control tissues, it was found that up to a half of the mRNAs that changed in expression were present only in flank skin, and hence represent genes which are regulated by melatonin and likely to have follicle growth cycle-specific functions.

When the earliest growth initiation stages were studied using the 1998 skin samples, prior to morphological changes in the telogen follicles, gene expression alterations were most frequent in samples collected 12 hours after melatonin administration. Other skin samples collected after the simultaneous administration of melatonin and oestradiol or progesterone, revealed potentially complicated interactions between these hormones. Most genes appeared to be down-regulated in the skin biopsies derived from ferrets treated with steroids.

9.3 Genes identified by sequence homology

ESTs cloned from differentially displayed bands were usually between 200 and 500 bp in length. They were generally defined by the primer sets used and most ESTs derived from the same differential represented the same transcripts. The oligo dT anchor primers annealed to the poly A tails of mRNAs in approximately half of the identified ESTs, with the remainder originating from internal regions bounded by 3' adenine-rich sequences. While the internal binding of the anchor primers reduced the efficiency of differential display, it also allowed the identification of additional transcripts when a limited number of upstream arbitrary primers were used and

increased the chance of identifying sequence from the coding region. Differential display, as used in this study, proved to be an effective technique capable of screening gene expression in skin samples for multiple comparisons. Of the total of 112 unique ESTs discovered, 42 were homologous to GenBank entries. Of these, 18 were also identified by the predicted amino acid sequence homology, demonstrating that almost half were derived from the coding regions of their respective mRNAs. Few of these transcripts have been previously implicated in hair growth.

9.4 Gene expression underlying hair follicle growth

Twenty-one ESTs were confirmed to be differentially expressed by Northern blot hybridisation, although the expression patterns were not necessarily the same as detected by differential display. For most ESTs, expression in tissues lacking hair follicles was usually absent or reduced, supporting their association with hair follicle growth. Approximately half of the 1998 ESTs (early proanagen) were detected by Northern blot hybridisation, compared with more than 90% of those derived from the 1996 samples (early to late proanagen). Together with the greater number of regulatory molecules identified in the 1998 samples, this suggests low expression levels for many mRNAs during early proanagen.

All the ESTs whose expression was confirmed by *in situ* hybridisation were localised to restricted compartments of hair follicles, or more generally throughout the hair follicle and in some other skin cell populations. Some ESTs were only expressed in certain growth stages of hair follicles, others in all growth stages, but at different levels. These close associations of expression with the hair follicle and the growth cycle suggest that a high proportion of the remaining ESTs will also eventually be linked to hair growth.

Two ESTs, *fDMP1* and *fHa8*, were subjected to further studies. Both were highly expressed only in the keratinising zone of late proanagen or anagen follicles in which keratinisation had commenced. These transcripts can thus serve as growth markers for proanagen IIIb during hair follicle development. Previously *Ha8* had only been identified in human hair follicles. The human and ferret sequences are highly conserved, especially at the amino acid level, and the expression patterns are similar in that not all the cortical cells in the keratogenous zone express *Ha8*. However, there

are some significant differences. These include an apparent higher expression level of *fHa8* and a more distinct asymmetric expression pattern in follicle cortical cells.

In contrast, *fDMP1* mRNA expression was localised evenly across the fibre cortex. The 760 bp cDNA sequence generated for *fDMP1* had very high sequence homology to human and mouse DMP1 in the 5' UTR and the coding regions. The size of the *fDMP1* mRNA is similar to the sizes of the mRNAs encoding for the alternatively spliced α and β isoforms of human DMP1. As human and mouse DMP1 are widely expressed transcription factors involved in cell cycle regulation, the smaller size and restricted expression of *fDMP1* suggest that it is a truncated form specific to the hair follicle, possibly involved in the arrest of keratinocyte proliferation and terminal differentiation.

9.5 Biological significance of the identified transcripts

According to their reported functions in other systems, genes identified by homology were most likely to be associated with transcription and signalling, cell growth and differentiation, cell interactions and motility, apoptosis, fatty acid and cholesterol metabolism.

Using skin samples from two hair growth-stimulatory and two hair growth-inhibitory treatments, the expression of some genes was found to alter in a manner that could be associated with the hair growth outcome. These transcripts could represent key genes for hair growth control. ESTs belonging to this category included *PCDC5RP, MSSP1/2, IGSF4, LAR, MHP* and *MyHC IIa.* All appear to have important roles in the biological processes associated with hair growth initiation. The expression of *PCDC5RP* has previously been reported to be regulated by prolactin (Too, 1997) and *LAR* was also confirmed to be regulated by melatonin and steroids by Northern blot hybridisation in this study.

When ESTs from the present study were compared to the expressed gene populations across human tissues and in skin in particular (Adams *et al.*, 1995) and to rat cultured follicle dermal papilla cells (Sleeman *et al.*, 2000), a higher percentage was found to be transcripts associated with cell structure and motility. Comparison with the molecules responsible for metastasis also revealed some common transcripts, linking normal hair follicle growth development to cancer cell migration.

9.6 Future studies

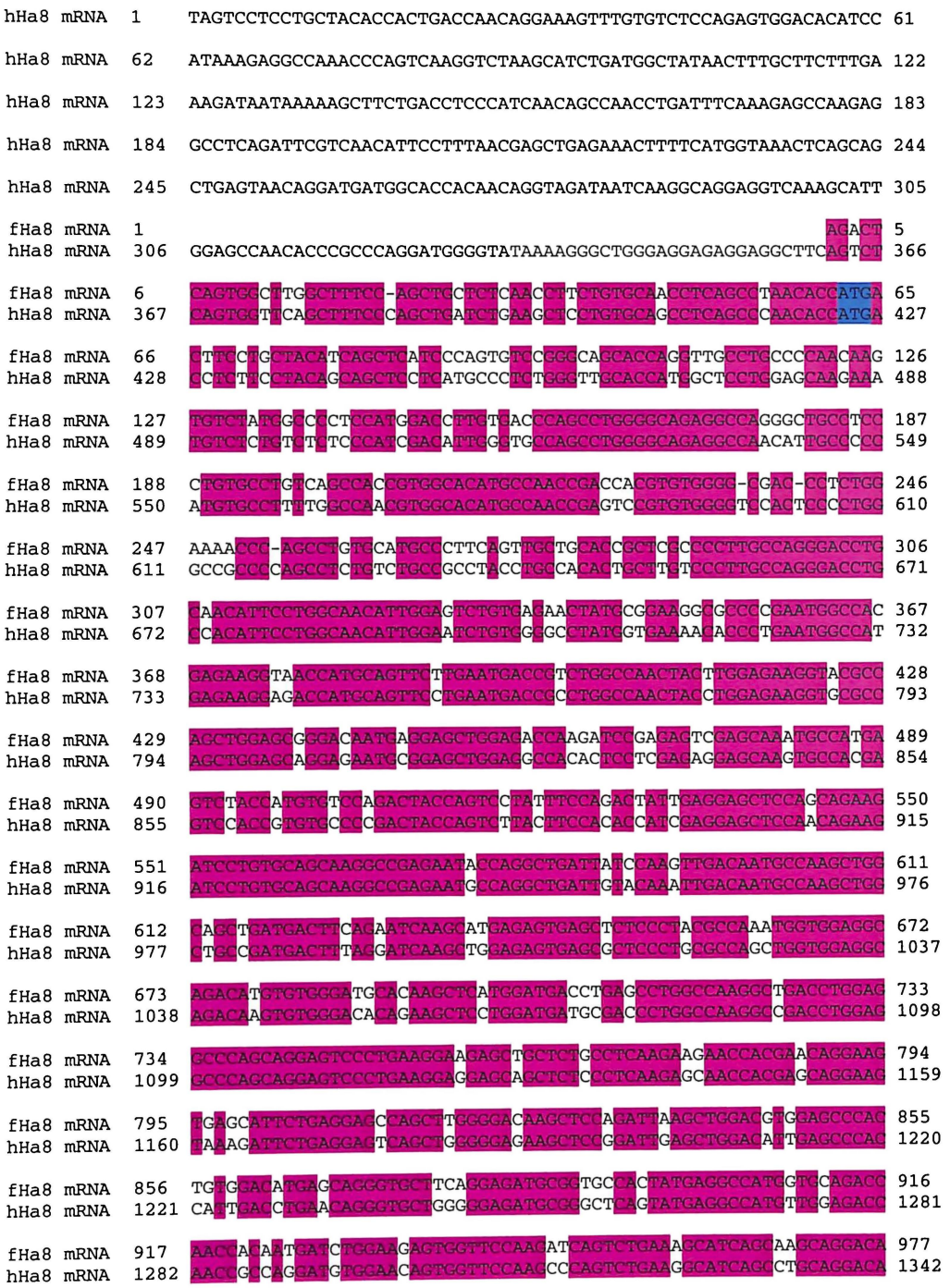
To my knowledge, this is the first systematic search for genes associated with hair growth initiation. Although the scale of the investigation was relatively small, the results were encouraging. It has not only confirmed the previously reported biological processes involved in hair follicle growth, but also has indicated that apoptosis and fatty acid synthesis may be more important than previously appreciated. It has provided 112 gene candidates for further studies. Furthermore, it has demonstrated the application of differential display methodology to identify genes associated with hair growth, and has laid down the groundwork for a more extensive search in the future. The recent rapid advancements in gene discovery and expression profiling technologies will also provide new options for detecting other genes associated with hair follicle growth initiation, particularly in genetically well-studied species like humans and mice. However, differential display will continue to be a useful tool especially in less well characterised species such as the ferret.

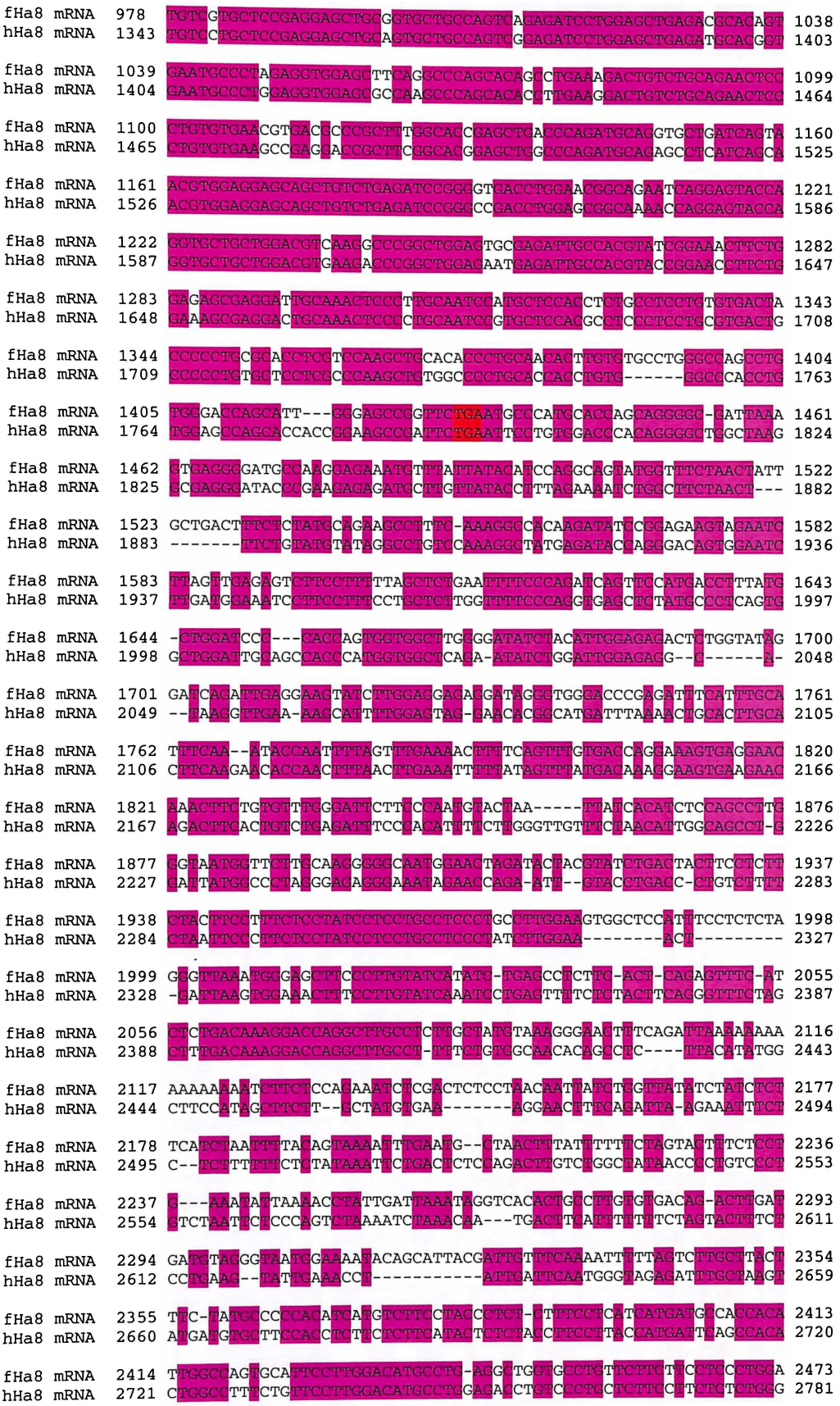
Although the expression patterns of some molecules have been established in this study, those of many more ESTs remain to be investigated. Confirmation of the detailed expression profiles across the hair cycle and functional studies to understand the roles of the genes already identified will provide a wealth of new information related to hair growth control and the mechanisms of melatonin and steroids on these processes. Those genes which showed distinct growth stage-specific expression or expression linked to inhibitory and stimulatory steroid treatments merit the highest priority. In this context, the completion of the human genome project, and the ongoing identification of new genes and their functions, will also provide unprecedented opportunities for hair biologists to resolve some of the many remaining enigmas in hair growth control.

Appendix I Alignment of *fHa8* to its homologues

I-A: Alignment of *fHa8* to hHa8 mRNA.

For nucleotide sequence alignment, purple-shading indicates homologous regions while no homologous sequence is in white background. Blue-shading is the translation start site and orange-shading is the translation stop codon. For amino acid sequence, green-shading indicates similar amino acids.




fHa8 mRNA 978 TGTCGTGCTCCGAGGAGCTGCGGTGCTGCCAGTCAGAGATCCTGGAGCTGAGACGCACAGT 1038
hHa8 mRNA 1343 TGTGCTGCTCCGAGGAGCTGCAGTGCTGCCAGTCGGAGATCCTGGAGCTGAGATGCACGGT 1403
fHa8 mRNA 1039 GAATGCCCTAGAGGTGGAGCTTCAGGCCCAGCACAGCCTGAAAGACTGTCTGCAGAACTCC 1099
hHa8 mRNA 1404 GAATGCCCTGGAGGTGGAGCGCCAAGCCCAGCACACTTTGAAGGACTGTCTGCAGAACTCC 1464
fHa8 mRNA 1100 CTGTGTGAACGTGACGCCCGCTTTGGCACCGAGCTGACCCAGATGCAGGTGCTGATCAGTA 1160
hHa8 mRNA 1465 CTGTGTGAAGCCGAGGACCGCTTCGGCACGGAGCTGGCCCAGATGCAGAGCCTCATCAGCA 1525
fHa8 mRNA 1161 ACGTGGAGGAGCAGCTGTCTGAGATCCGGGGTGACCTGGAACGGCAGAATCAGGAGTACCA 1221
hHa8 mRNA 1526 ACGTGGAGGAGCAGCTGTCTGAGATCCGGGCCGACCTGGAGCGGCAAAACCAGGAGTACCA 1586
fHa8 mRNA 1222 GGTGCTGCTGGACGTCAAGGCCCGGCTGGAGTGCGAGATTGCCACGTATCGGAAACTTCTG 1282
hHa8 mRNA 1587 GGTGCTGCTGGACGTGAAGACCCGGCTGGAGAATGAGATTGCCACGTACCGGAACCTTCTG 1647
fHa8 mRNA 1283 GAGAGCGAGGATTGCAAACTCCCTTGCAATCCATGCTCCACCTCTGCCTCCTCTGTGACTA 1343
hHa8 mRNA 1648 GAAAGCGAGGACTGCAAACTCCCCTGCAATCCGTGCTCCACGCCTCCCTCCTGCGTGACTG 1708
fHa8 mRNA 1344 CCCCCTGCGCACCTCGTCCAAGCTGCACACCCTGCAACAGTTGTGTGCCTGGGCCAGCCTG 1404
hHa8 mRNA 1709 CCCCCTGTGCTCCTCGCCCAAGCTGTGGCCCCTGCACCAGCTGTG------GGCGCACCTG 1763
fHa8 mRNA 1405 TGGGACCAGCATT---GGGAGCCGGTTCTGAATGCCCATGCACCAGCAGGGGC-GATTAAA 1461
hHa8 mRNA 1764 TGGAGCCAGCACCACCGGAAGCCGATTCTGAATTCCTGTGGACCCACAGGGGCTGGCTAAG 1824
fHa8 mRNA 1462 GTGAGGGGATGCCAAGGAGAAATGTTTATTATACATCCAGGCAGTATGGTTTCTAACTATT 1522
hHa8 mRNA 1825 GCGAGGGATACCCGAAGAGAGATGCTTGTTATACCTTTAGAAAATCTGGCTTCTAACT--- 1882
fHa8 mRNA 1523 GCTGACTTTCTCTATGCAGAAGCCTTTC-AAAGGCCCCAAGATATCCGGAGAAGTAGAATC 1582
hHa8 mRNA 1883 -------TTCTGTATGTATAGGCCTGTCCAAAGGCTATGAGATACCAGGGACAGTGGAATC 1936
fHa8 mRNA 1583 TTAGTTGAGAGTCTTCCTTTTTAGCTCTGAATTTCCCAGATCACTTCCATGACCTTTATG 1643
hHa8 mRNA 1937 TTGATGGAAATCCTTCCTTTCCTGCTCTTGGTTTCCCAGGTGAGCTCTATGCCCTCAGTG 1997
fHa8 mRNA 1644 -CTGGATCCC---CACCAGTGGTGGCTTGGGGATATCTACATTGGAGAGACTCTGGTATAG 1700
hHa8 mRNA 1998 GCTGGATTGCAGCCACCCATGGTGGCTCAGA-ATATCTGGATTGGAGAGG--C------A- 2048
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fHa8 mRNA 1762 TTTCAA--ATACCAATTTTAGTTTGAAAACTTTTCAGTTTGTGACCAGGAAAGTGAGGAAC 1820
hHa8 mRNA 2106 CTTCAAGAACACCAACTTTAACTTGAAATTTTTATAGTTTATGACAAAGGAAGTGAAGAAC 2166
fHa8 mRNA 1821 AAACTTCTGTGTTTGGGATTCTTCCCAATGTACTAA-----TTATCACATCTCCAGCCTTG 1876
hHa8 mRNA 2167 AGACTTCACTGTCTGAGATTTCCCACATTTTCTTGGGTTGTTTCTAACATTGGCAGCCT-G 2226
fHa8 mRNA 1877 GGTAATGGTTCTTTCCAAGGGGCCAATGGAAGTAGATACTACTGTATCTGACTACTTCCTCTT 1937
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fHa8 mRNA 1938 CTACTTCCTTTCTCCTATCCTCCTGCCTCCCTGCCTTGGAAGTGGCTCCATTTCCTCTCTA 1998
hHa8 mRNA 2284 CTAATTCCCTTCTCCTATCCTCCTGCCTCCCTATCTTGGAA--------ACT--------- 2327
fHa8 mRNA 1999 GGGTTAAATGGGAGCTTCCCTTGTATCATATG-TGAGCCTCTTC-ACT-CAGAGTTTC-AT 2055
hHa8 mRNA 2328 -GATTAAGTGGAAACTTTCCTTGTATCAAATCCTGAGTTTTCTCTACTTCAGGGTTTCTAG 2387
fHa8 mRNA 2056 CTCTGACAAAGGACCAGGCTTGCCTCTTGCTATGTAAAGGGAACTTTCAGATTAAAAAAAA 2116
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hHa8 mRNA 2444 CTTCCATAGCTTCTT--GCTATGTGAA-------AGGAACTTTCAGATTA-AGAAATTTCT 2494
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hHa8 mRNA 2554 GTCTAATTCTCCCAGTCTAAAATCTAAACAA---TGACTTCATTTTTTTCTAGTACTTTCT 2611
fHa8 mRNA 2294 GATCTAGGGTAATCGAAAATACAGCATTACGATTGTTTCAAAATTTTTAGTCTTGCTTACT 2354
hHa8 mRNA 2612 CCTGAAG--TATTGAAACCT-----------ATTGATTCAATGGGTAGAGATTTGCTAAGT 2659
fHa8 mRNA 2355 TTC-TATGCCCCCACATCATGTCTTCCTACCCTCT-CTTTCCTCATCATGATGCCACCACA 2413
hHa8 mRNA 2660 ATGATGTGCTTCCAGCTCTTCTCTTCATACTCTCTACCTTCCTTACCATGATTCAGCCACA 2720
fHa8 mRNA 2414 TTGGCCAGTGCATTCCTTGGACATGCCTG-ACGCTGGTGCCTGTTCTTCTTCCTCCTGGA 2473
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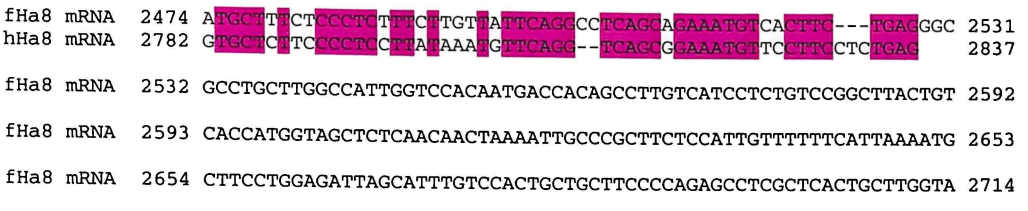
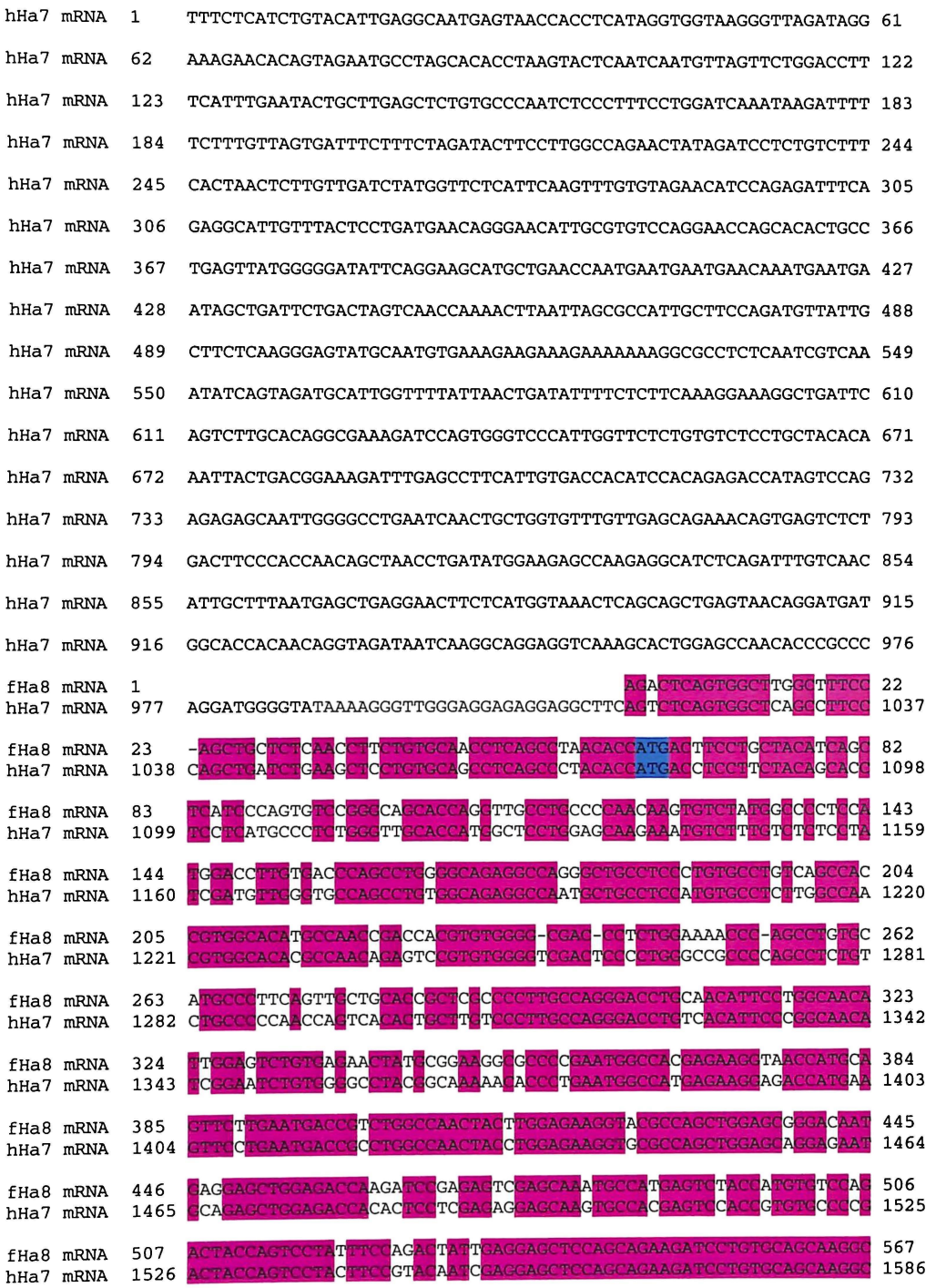
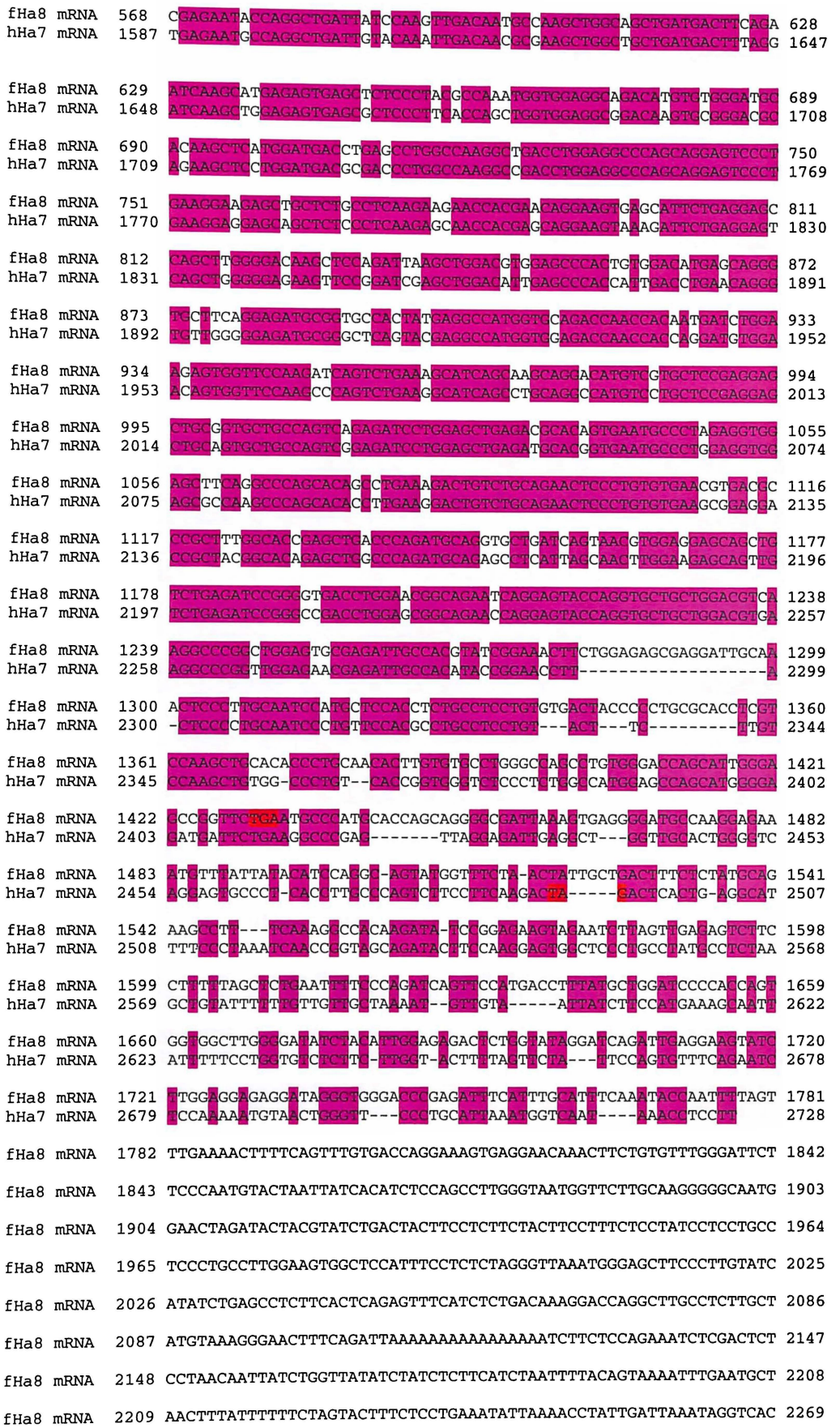


I-B: Alignment of *fHa8* to hHa7 mRNA

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fHa8 mRNA 629 ATCAAGCATGAGAGTGAGCTCTCCCTACGCCAAATGGTGGAGGCAGACATGTGTGGGATGC 689
hHa7 mRNA 1648 ATCAAGCTGGAGAGTGAGCGCTCCCTTCACCAGCTGGTGGAGGCGGACAAGTGCGGGACGC 1708
fHa8 mRNA 690 ACAAGCTCATGGATGACCTGAGCCTGGCCAAGGCTGACCTGGAGGCCCAGCAGGAGTCCCT 750
hHa7 mRNA 1709 AGAAGCTCCTGGATGACGCGACCCTGGCCAAGGCCGACCTGGAGGCCCAGCAGGAGTCCCT 1769
fHa8 mRNA 751 GAAGGAAGAGCTGCTCTGCCTCAAGAAGAACCACGAACAGGAAGTGAGCATTCTGAGGAGC 811
hHa7 mRNA 1770 GAAGGAGGAGCAGCTCTCCCTCAAGAGCAACCACGAGCAGGAAGTAAAGATTCTGAGGAGT 1830
fHa8 mRNA 812 CAGCTTGGGGACAAGCTCCAGATTAAGCTGGACGTGGAGCCCACTGTGGACATGAGCAGGG 872
hHa7 mRNA 1831 CAGCTGGGGGAGAAGTTCCGGATCGAGCTGGAGATTGAGCCCACCATTGACCTGAACAGGG 1891
fHa8 mRNA 873 TGCTTCAGGAGATGCGGTGCCACTATGAGGCCATGGTGCAGACCAACCACAATGATCTGGA 933
hHa7 mRNA 1892 TGTTGGGGGAGATGCGGGCTCAGTACGAGGCCATGGTGGAGACCAACCACCAGGATGTGGA 1952
fHa8 mRNA 934 AGAGTGGTTCCAAGATCAGTCTGAAAGCATCAGCAAGCAGGACATGTCGTGCTCCGAGGAG 994
hHa7 mRNA 1953 ACAGTGGTTCCAAGCCCAGTCTGAAGGCATCAGGCTGCAGGCCATGTCCTGCTCCGAGGAG 2013
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hHa7 mRNA 2136 CCGCTACGGCACAGAGCTGGCCCAGATGCAGAGCCTCATTAGCAACTTGGAAGAGCAGTTG 2196
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hHa7 mRNA 2258 AGGCCCGGTTGGAGAACGAGATTGCCACATACCGGAACCTT----------A 2299
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hHa7 mRNA 2300 -CTCCCCTGCAATCCCTGTTCCAGGCCTGCCTCCTGT---ACT---TC---------TTGT 2344
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fHa8 mRNA 2087 ATGTAAAGGGAACTTTCAGATTAAAAAAAAAAAAAAAAATCTTCTCCAGAAATCTCGACTCT 2147
fHa8 mRNA 2148 CCTAACAATTATCTGGTTATATCTATCTCTTCATCTAATTTTACAGTAAAATTTGAATGCT 2208
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fHa8 mRNA  2392 TCCTCATCATGATGCCACCACATTGGCCAGTGCATTCCTTGGACATGCCTGAGGCTGGTGC 2452

fHa8 mRNA  2453 CTGTTCTTCTTCCTCCCTGGAATGCTTTCTCCCTCTTTCTTGTTATTCAGGCCTCAGCAGA 2513

fHa8 mRNA  2514 AATGTCACTTCTGAGGGCGCCTGCTTGGCCATTGGTCCACAATGACCACAGCCTTGTCATC 2574

fHa8 mRNA  2575 CTCTGTCCGGCTTACTGTCACCATGGTAGCTCTCAACAACTAAAATTGCCCGCTTCTCCAT 2635

fHa8 mRNA  2636 TGTTTTTTCATTAAAATGCTTCCTGGAGATTAGCATTTGTCCACTGCTGCTTCCCCAGAGC 2696

fHa8 mRNA  2697 CTCGCTCACTGCTTGGTAAAGAGTAGATGCTTAATAAACATCTATTGAAAGAAAAAAAAAA 2757

fHa8 mRNA  2758 AAAAAAAAAAAA 2769
```

I-C: Alignment of *fHa8* to hHa8 amino acid sequence

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fHa8 aa   1   MTSCYISSSQCP-GSTRLPAPTSVYGPSMDLVTQPGAEARAASLCLSATVAHANRPRVG-RPSGR 63

hHa8 aa   65  PSLCLPPTCHTACPLPGTGHIPGNIGICGAYGENTLNGHEKETMQFLNDRLANYLEKVRQLEQEN 129
fHa8 aa   64  PSLCMPFSCCTARPLPGTCNIPGNIGVCENYAEGAPNGHEKVTMQFLNDRLANYLEKVRQLERDN 128

hHa8 aa   130 AELEATLLERSKCHESTVCPDYQSYFHTIEELQQKILCSKAENARLIVQIDNAKLAADDFRIKLE 194
fHa8 aa   129 EELETKIRESSKCHESTMCPDYQSYFQTIEELQQKILCSKAENTRLIIQVDNAKLAADDFRIKHE 193

hHa8 aa   195 SERSLRQLVEADKCGTQKLLDDATLAKADLEAQQESLKEEQLSLKSNHEQEVKILRSQLGSKLRI 259
fHa8 aa   194 SELSLRQMVEADMCGMHKLMDDLSLAKADLEAQQESLKEELLCLKKNHEQEVSILRSQLGDKLQI 258

hHa8 aa   260 ELDIEPTIDLNRVLGEMRAQYEAMLETNRQDVEQWFQAQSEGISLQDMSCSEELQCCQSEILELR 324
fHa8 aa   259 KLDVEPTVDMSRVLQEMRCHYEAMVQTNHNDLEEWFQDQSESISKQDMSCSEELRCCQSEILELR 323

hHa8 aa   325 CTVNALEVERQAQHTLKDCLQNSLCRAEDRFGTELAQMQSLISNVEEQLSEIRADLERQNQEYQV 389
fHa8 aa   324 RTVNALEVELQAQHSLKDCLQNSLCERQARFGTELTQMQVLISNVEEQLSEIRGDLERQNQEYQV 388

hHa8 aa   390 LLDVKTRLENEIATYRNLLESEDCKLPCNPCSTPPSCVTAPCAPRPSCGPCTTC--GPTCGASTT 452
fHa8 aa   389 LLDVKARLECEIATYRKLLESEDCKLPCNPCSTSASCVTTPCAPRPSCTPCNTCVPGPACGTS-I 452

hHa8 aa   453 GSRF 456
fHa8 aa   453 GSRF 456
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I-D: Alignment of *fHa8* to hHa7 amino acid sequence

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fHa8 aa   64  PSLCMPFSCCTARPLPGTCNIPGNIGVCENYAEGAPNGHEKVTMQFLNDRLANYLEKVRQLERDN 128
hHa7 aa   65  PSLCLPPTSHTACPLPGTGHIPGNIGICGAYGKNTLNGHEKETMKFLNDRLANYLEKVRQLEQEN 129

fHa8 aa   129 EELETKIRESSKCHESTMCPDYQSYFQTIEELQQKILCSKAENTRLIIQVDNAKLAADDFRIKHE 193
hHa7 aa   130 AELETTLLERSKCHESTVCPDYQSYFRTIEELQQKILCSKAENARLIVQIDNAKLAADDFRIKLE 194

fHa8 aa   194 SELSLRQMVEADMCGMHKLMDDLSLAKADLEAQQESLKEELLCLKKNHEQEVSILRSQLGDKLQI 258
hHa7 aa   195 SERSLRQLVEADKCGTQKLLDDATLAKADLEAQQESLKEEQLSLKSNHEQEVKILRSQLGEKFRI 259

fHa8 aa   259 KLDVEPTVDMSRVLQEMRCHYEAMVQTNHNDLEEWFQDQSESISKQDMSCSEELRCCQSEILELR 323
hHa7 aa   260 ELDIEPTIDLNRVLGEMRAQYEAMVETNHQDVEQWFQAQSEGISLQAMSCSEELQCCQSEILELR 324

fHa8 aa   324 RTVNALEVELQAQHSLKDCLQNSLCERQARFGTELTQMQVLISNVEEQLSEIRGDLERQNQEYQV 388
hHa7 aa   325 CTVNALEVERQAQHTLKDCLQNSLCEAEDRYGTELAQMQSLISNLEEQLSEIRADLERQNQEYQV 389

fHa8 aa   389 LLDVKARLECEIATYRKLLESE----DCKL-------PCN------PCSTSASCVTTPCAPRPSC 436
hHa7 aa   390 LLDVKARLENEIATYRNLTPLQSLFHACLLYFLSKLWPCHRWVSLWPWSQHGEMILKARIRRLRL 454

fHa8 aa   437 TPCNTCVPGPACGTSIGSRF 456
hHa7 aa   455 VALGSGVFSH-CPVFLQD   471
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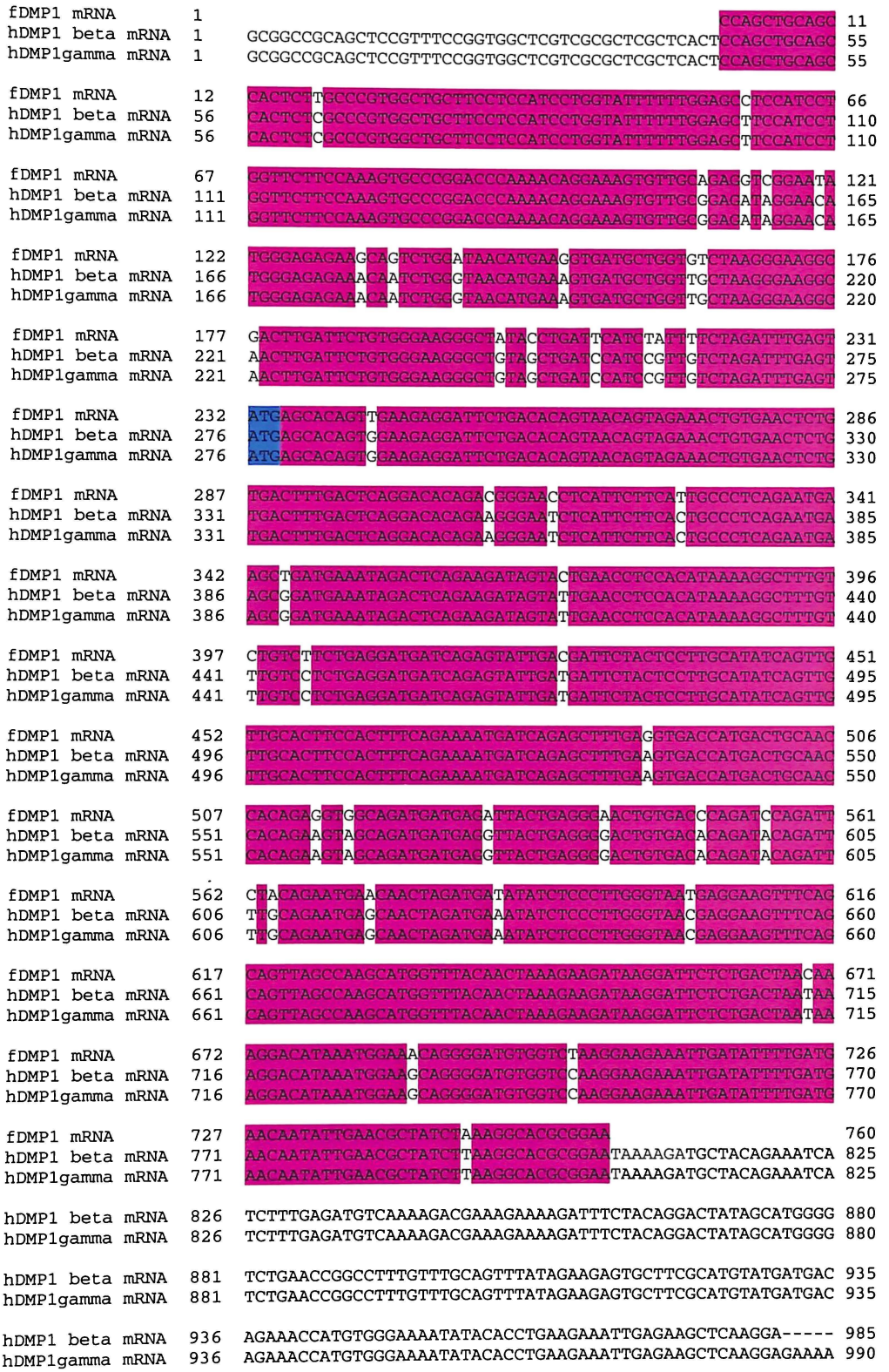
Appendix II Alignment of *fDMP1* to its homologues

II-A: Alignment of *fDMP1* to hDMP1 mRNA



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hDMP1gamma mRNA   1046 AGGCCACACTTTCAAACTTTGGCTCTCAAAGTATTGCTGCCCACAACTTCCAAAC 1100

hDMP1 beta mRNA   1062 CAGTCAAATGGGAAGAAGAAGAATGAAGAATAAATGATGAAATTAAAAGGGAGAA 1116
hDMP1gamma mRNA   1101 CAGTCAAATGGGAAGAAGAAGAATGAAGAATAAATGATGAAATTAAAAGGGAGAA 1155

hDMP1 beta mRNA   1117 AACAATTGAACCAAATGCTGATTGGCAGACTCTTGTTTTAGGCTCCGGATAAAGC 1171
hDMP1gamma mRNA   1156 AACAATTGAACCAAATGCTGATTGGCAGACTCTTGTTTTAGGCTCCGGATAAAGC 1210

hDMP1 beta mRNA   1172 ATGGCAATGACTGGGCAACAATAGGGGCGGCGCTAGGAAGAAGTGCATCTTCTGT 1226
hDMP1gamma mRNA   1211 ATGGCAATGACTGGGCAACAATAGGGGCGGCGCTAGGAAGAAGTGCATCTTCTGT 1265

hDMP1 beta mRNA   1227 CAAAGATCGGTGCCGACTGATGAAGGATACTTGCAACACAGGGAAGTGGACAGAA 1281
hDMP1gamma mRNA   1266 CAAAGATCGGTGCCGACTGATGAAGGATACTTGCAACACAGGGAAGTGGACAGAA 1320

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hDMP1gamma mRNA   1321 GAAGAAGAAAAGAGACTTGCAGAAGTGGTTCATGAGTTGACAAGCACTGAGCCAG 1375

hDMP1 beta mRNA   1337 GTGACATAGTCACACAGGGTGTGTCTTGGGCAGCTGTGGCTGAACGAGTCGGTAC 1391
hDMP1gamma mRNA   1376 GTGACATAGTCACACAGGGTGTGTCTTGGGCAGCTGTGGCTGAACGAGTCGGTAC 1430

hDMP1 beta mRNA   1392 CCGCTCAGAAAAGCAATGTCGTTCTAAATGGCTCAACTACCTGAATTGGAAACAG 1446
hDMP1gamma mRNA   1431 CCGCTCAGAAAAGCAATGTCGTTCTAAATGGCTCAACTACCTGAATTGGAAACAG 1485

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hDMP1gamma mRNA   1541 CAGAACTTGATGTAGCTGATGAAAATGACATTAACTGGGATCTGTTAGCTGAGGG 1595

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hDMP1gamma mRNA   1596 ATGGAGTAGTGTCCGTTCACCACAATGGCTACGAAGTAAATGGTGGACCATCAAA 1650

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hDMP1gamma mRNA   1651 AGGCAAATTGCAAACCATAAGGATGTTTCGTTCCCTGTCTTAATAAAAGGTCTTA 1705

hDMP1 beta mRNA   1667 AACAGTTACATGAGAACCAAAAAAACAACCCAACGCTTTTGGAGAATAAATCAGG 1721
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hDMP1 beta mRNA   1777 GTTGCCCGCTTGGAAGATAATACAGCCATCTCTTCTAGCCCCATGGCAGCATTGC 1831
hDMP1gamma mRNA   1816 GTTGCCCGCTTGGAAGATAATACAGCCATCTCTTCTAGCCCCATGGCAGCATTGC 1870

hDMP1 beta mRNA   1832 AGATTCCAGTCCAGATCACCCATGTTTCTTCAGCAGACTCTCCTGCTACCGTTGA 1886
hDMP1gamma mRNA   1871 AGATTCCAGTCCAGATCACCCATGTTTCTTCAGCAGACTCTCCTGCTACCGTTGA 1925

hDMP1 beta mRNA   1887 CTCAGAAACAATAACACTAAACAGTGGAACACTACAGACATTTGAGATTCTTCCC 1941
hDMP1gamma mRNA   1926 CTCAGAAACAATAACACTAAACAGTGGAACACTACAGACATTTGAGATTCTTCCC 1980

hDMP1 beta mRNA   1942 TCTTTCCATCTACAGCCCACTGGCACTCCAGGCACCTACCTACTTCAAACAAGCT 1996
hDMP1gamma mRNA   1981 TCTTTCCATCTACAGCCCACTGGCACTCCAGGCACCTACCTACTTCAAACAAGCT 2035

hDMP1 beta mRNA   1997 CAAGCCAAGGCCTTCCCCTAACTCTGACTGCTAGTCCCACAGTAACCCTGACAGC 2051
hDMP1gamma mRNA   2036 CAAGCCAAGGCCTTCCCCTAACTCTGACTGCTAGTCCCACAGTAACCCTGACAGC 2090

hDMP1 beta mRNA   2052 TGCTGCTCCTGCTTCTCCTGAACAGATTATTGTTCATGCTTTATCCCCAGAACAT 2106
hDMP1gamma mRNA   2091 TGCTGCTCCTGCTTCTCCTGAACAGATTATTGTTCATGCTTTATCCCCAGAACAT 2145

hDMP1 beta mRNA   2107 TTGTTGAACACAAGTGATAATGTTACAGTGCAGTGTCACACACCAAGAGTCATCA 2161
hDMP1gamma mRNA   2146 TTGTTGAACACAAGTGATAATGTTACAGTGCAGTGTCACACACCAAGAGTCATCA 2200

hDMP1 beta mRNA   2162 TTCAGACTGTTGCCACAGAGGACATCACTTCTTCCATATCCCAAGCAGAACTGAC 2216
hDMP1gamma mRNA   2201 TTCAGACTGTTGCCACAGAGGACATCACTTCTTCCATATCCCAAGCAGAACTGAC 2255

hDMP1 beta mRNA   2217 AGTCGATAGTGATATTCAGTCATCTGATTTTCCTGAGCCTCCAGACGCCCTAGAA 2271
hDMP1gamma mRNA   2256 AGTCGATAGTGATATTCAGTCATCTGATTTTCCTGAGCCTCCAGACGCCCTAGAA 2310

hDMP1 beta mRNA   2272 GCAGACACTTTCCCAGATGAAATTCATCACCCTAAGATGACTGTGGAGCCATCAT 2326
hDMP1gamma mRNA   2311 GCAGACACTTTCCCAGATGAAATTCATCACCCTAAGATGACTGTGGAGCCATCAT 2365

hDMP1 beta mRNA   2327 TTAATGATGCTCATGTATCCAAATTCAGTGACCAAAATAGCACAGAACTGATGAA 2381
hDMP1gamma mRNA   2366 TTAATGATGCTCATGTATCCAAATTCAGTGACCAAAATAGCACAGAACTGATGAA 2420
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hDMP1 beta mRNA  2437 GAATCACCCTCTGATTTAGCCAGTGCTTATGTTACTGAGGGTTTAGAGTCTCCCA 2491
hDMP1gamma mRNA  2476 GAATCACCCTCTGATTTAGCCAGTGCTTATGTTACTGAGGGTTTAGAGTCTCCCA 2530

hDMP1 beta mRNA  2492 CTATAGAAGAACAAGTTGATCAAACAATTGATGATGAAACAATACTTATCGTTCC 2546
hDMP1gamma mRNA  2531 CTATAGAAGAACAAGTTGATCAAACAATTGATGATGAAACAATACTTATCGTTCC 2585

hDMP1 beta mRNA  2547 TTCACCACATGGCTTTATCCAGGCATCTGATGTTATAGATACTGAATCTGTCTTG 2601
hDMP1gamma mRNA  2586 TTCACCACATGGCTTTATCCAGGCATCTGATGTTATAGATACTGAATCTGTCTTG 2640

hDMP1 beta mRNA  2602 CCTTTGACAACACTAACAGATCCCATACTCCAACATCATCAGGAAGAATCAAATA 2656
hDMP1gamma mRNA  2641 CCTTTGACAACACTAACAGATCCCATACTCCAACATCATCAGGAAGAATCAAATA 2695

hDMP1 beta mRNA  2657 TCATTGGATCATCCTTGGGCAGTCCTGTTTCAGAAGATTCAAAGGATGTCGAAGA 2711
hDMP1gamma mRNA  2696 TCATTGGATCATCCTTGGGCAGTCCTGTTTCAGAAGATTCAAAGGATGTCGAAGA 2750

hDMP1 beta mRNA  2712 TTTGGTAAACTGTCATTAGAATAATTCTTAGAAATAGGCAGTTCAAGCAAAGAAG 2766
hDMP1gamma mRNA  2751 TTTGGTAAACTGTCATTAGAATAATTCTTAGAAATAGGCAGTTCAAGCAAAGAAG 2805

hDMP1 beta mRNA  2767 GCACACTGTTAATTACAACCTCTTCAAAGAAATAGGAGCAAACCCCCAAGAGGCT 2821
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hDMP1 beta mRNA  2822 TAATTTACCAATTTAAATAGCCACAGTCCTTAAGCCACACACATTGTTGCTGCTA 2876
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hDMP1 beta mRNA  2987 GAATAAGGAATTTCATTCACTTCAGCCACTAAGAAAAGTTTAGAATCACGAAAGC 3041
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hDMP1gamma mRNA  3576 CGAGAGAATAGTTTGTCATCCACTTAGTGTGTTAGCTGGTGGGGTACAATATAAC 3630

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hDMP1gamma mRNA  3631 CTCTCATCTCAGGCTATTTTAAAAAAACAATATTTGCTTCTATAACAAAAGGAAA 3685

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hDMP1gamma mRNA  3686 CAAATCTAAGAATCATTCCTGTACTACAGAAGGGTTAAGGCAAAGGTAGCCTTTT 3740

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hDMP1gamma mRNA  3741 GGGCTTTTTAATGAATATGACCCCTATAGAAAAGTCAAGAAAAAAAAACCCTTGT 3795

hDMP1 beta mRNA  3757 ATAAATTATTTTATTTATTATTGTAATTAGATCTTCACAAAGTTGTCTTTTCACT 3811
hDMP1gamma mRNA  3796 ATAAATTATTTTATTTATTATTGTAATTAGATCTTCACAAAGTTGTCTTTTCACT 3850
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```
hDMP1 beta mRNA   3812 GTGTTTTGTCAACGTGAAATTAAATTGTAGTTATAAGCAAAAGTTGGTTGCCTAG 3866
hDMP1gamma mRNA   3851 GTGTTTTGTCAACGTGAAATTAAATTGTAGTTATAAGCAAAAGTTGGTTGCCTAG 3905

hDMP1 beta mRNA   3867 GGAACAATTGTATATTCAGTTTAACAGAAATAAAAGAATATTTGTCTTAAAAAAA 3921
hDMP1gamma mRNA   3906 GGAACAATTGTATATTCAGTTTAACAGAAATAAAAGAATATTTGTCTTAAAAAAA 3960

hDMP1 beta mRNA   3922 AAAAAAAAAAAA 3933
hDMP1gamma mRNA   3961 AAAAAAAAAAAA 3972
```

II-B: Alignment of *fDMP1* to hDMP1 amino acid sequences of the spliced forms

```
hDMP1 gamma aa    1   MSTVEEDSDTVTVETVNSVTLTQDTEGNLILHCPQNEADEIDSEDSIEPPHKRLCLSS 58
hDMP1 beta aa     1   MSTVEEDSDTVTVETVNSVTLTQDTEGNLILHCPQNEADEIDSEDSIEPPHKRLCLSS 58
fDMP1 aa          1   MSTVEEDSDTVTVETVNSVTLTQDTDGNLILHCPQNEADEIDSEDSTEPPHKRLCLSS 58

hDMP1 gamma aa   59   EDDQSIDDSTPCISVVALPLSENDQSFEVTMTATTEVADDEVTEGTVTQIQILQNEQL 116
hDMP1 beta aa    59   EDDQSIDDSTPCISVVALPLSENDQSFEVTMTATTEVADDEVTEGTVTQIQILQNEQL 116
fDMP1 aa         59   EDDQSIDDSTPCISVVALPLSENDQSFEVTMTATTEVADDEITEGTVTQIQILQNEQL 116

hDMP1 gamma aa  117   DEISPLGNEEVSAVSQAWFTTKEDKDSLTNKGHKWKQGMWSKEEIDILMNNIERYLKA 174
hDMP1 beta aa   117   DEISPLGNEEVSAVSQAWFTTKEDKDSLTNKGHKWKQGMWSKEEIDILMNNIERYLKA 174
fDMP1 aa        117   DDISPLGNEEVSAVSQAWFTTKEDKDSLTNKGHKWKQGMWSKEEIDILMNNIERYLKA 174

hDMP1 gamma aa  175   RGIKDATEIIFEMSKDERKDFYRTIAWGLNRPLFAVYRRVLRMYDDRNHVGKYTPEEI 232
hDMP1 beta aa   175   RGIKDATEIIFEMSKDERKDFYRTIAWGLNRPLFAVYRRVLRMYDDRNHVGKYTPEEI 232
fDMP1 aa        175   RG                                                        176

hDMP1 gamma aa  233   EKLKEKKAIAACFFFTHRQLWTPKKGHTFKLWLSKYCCPQLPNQSNGKKKNEE 285
hDMP1 beta aa   233   EKLKE-------------QLWTPKKGHTFKLWLSKYCCPQLPNQSNGKKKNEE 272
```

II-C: Alignment of *fDMP1* to mDMP1 amino acid sequence

```
fDMP1 aa    1   MSTVEEDSDTVTVETVNSVTLTQDTDGNLILHCPQNEADEIDSEDSTEPPHKRLCLSSEDDQSI  64
mDMP1 aa    1   MSTVEEDSDTVTVETVNSVTFTQDTDGNLILHCPQNDPDEVDSEDSTEPPHKRLCLSSEDDQSI  64

fDMP1 aa   65   DDSTPCISVVALPLSENDQSFEVTMTATTEVADDEITEGTVTQIQILQNEQLDDISPLGNEEVS  128
mDMP1 aa   65   DDATPCISVVALPLSENDQSFEVTMTATTEVADDELSEGTVTQIQILQNDQLDEISPLGTEEVS  128

fDMP1 aa   129  AVSQAWFTTKEDKDSLTNKGHKWKQGMWSKEEIDILMNNIERYLKARG                  176
mDMP1 aa   129  AVSQAWFTTKEDKDSLTNKGHKWKQGMWSKEEIDILMNNIERYLKARGIKDATEIIFEMSKDER 192

mDMP1 aa   193  KDFYRTIAWGLNRPLFAVYRRVLRMYDDRNHVGKYTPEEIEKLKELRIKHGNDWATIGAALGRS 256

mDMP1 aa   257  ASSVKDRCRLMKDTCNTGKWTEEEEKRLAEVVHELTSTEPGDIVTQGVSWAAVAERVGTRSEKQ 320

mDMP1 aa   321  CRSKWLNYLNWKQSGGTEWTKEDEINLILRIAELDVADENDINWDLLAEGWSSVRSPQWLRSKW 384

mDMP1 aa   385  WTIKRQIANHKDVSFPVLIKGLKQLHENQKNNPVLLENKSGSGVPNSNCNSSVQHVQIRVARLE 448

mDMP1 aa   449  DNTAISPSPMAALQIPVQITHVSSTDSPAASADSETITLNSGTLQTFEILPSFPLQPTGTPGTY 512

mDMP1 aa   513  LLQTSSSQGLPLTLTTNPTLTLAAAAPASPEQIIVHALSPEHLLNTSDNVTVQCHTPRVIIQTV 576

mDMP1 aa   577  ATEDITSSLSQEELTVDSDLHSSDFPEPPDALEADTFPDEIPRPKMTIQPSFNNAHVSKFSDQN 640

mDMP1 aa   641  STELMNSVMVRTEEEIADTDLKQEEPPSDLASAYVTEDLESPTIVHQVHQTIDDETILIVPSPH 704

mDMP1 aa   705  GFIQASDVIDTESVLPLTTLTDPIFQHHQEASNIIGSSLGSPVSEDSKDVEDLVNCH 761
```

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