Hair follicle germinative epidermal cells: a molecular study

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"Hair Follicle Germinative Epidermal Cells: A Molecular Study"

by

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This thesis is entirely the result of my own work. It has not been accepted for any other degree and is not being submitted for any other degree,

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ABSTRACT

At the base of the hair follicle epidermal matrix is a population of germinative epidermal (GE) cells that is in close communication with the dermal papilla. These GE cells are at the core of activities that comprise the fundamental processes of cell signalling and differentiation in the hair follicle. Since it is in the germinative region that the signals that produce hair are being received and transcribed, identification of genes expressed in the GE cells will be important for our understanding of hair growth control and the molecular mechanisms operating at the site of epidermal proliferation and differentiation.

This study describes the production of a series of cDNA libraries, both by conventional means from rat vibrissa follicles and follicle end bulbs, and by PCR from the GE cells and the tissues of the upper end bulb. These libraries were then used for a variety of screening approaches to isolate cDNA clones, firstly for molecules which are known to be involved in the control of hair growth, and secondly for molecules which are differentially expressed in the follicular germinative epidermis. In order to identify such preferentially expressed genes, a dual labelling differential screen of the vibrissa follicle end bulb cDNA library was performed, using probes derived from the germinative epidermal and upper end bulb PCR generated libraries. Nine putative differentially expressed clones were isolated and sequenced. RNase protection analysis and non radioactive *in situ* hybridisation was then performed to confirm that these clones were expressed in the germinative epidermis of rat vibrissa follicles. Further characterisation by northern blotting revealed that several of the clones were expressed in multiple tissues. Nucleotide sequence analysis revealed that six of the clones had a concensus BC1 repeat sequence at the end of their 3'UTR. This has been implicated in post-transcriptional control of intracellular mRNA localisation. Three of these clones were related to genes implicated in induction and vesicle trafficking. These clones may therefore be involved in the signal transduction pathways operating in the germinative epidermis in response to primary signalling molecules received from the dermal papilla.

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Abbreviations

B	Bulge
BCIP	5-bromo-4-chloro-3-indolyl phosphate
bm	Basement membrane
BMP	Bone morphogenetic protein
bp	base pairs
BrdU	Bromodeoxyuridine
BSA	Bovine serum albumin
CAM	Cell adhesion molecule
CTAB	Cetyl timethylammonium bromide
DAB	Diamino benzidine
dd	Double distilled
DEPC	Diethyl pyrocarbonate
DIA	Differentiation inhibiting activity
DIG	Digoxygenin
DNA	Deoxyribonucleic acid
dNTP	deoxynucleotide triphosphate
DP	Dermal papilla
DS	Dermal sheath
EB	End bulb
EC	Embryonal carcinoma
ECM	Extracellular matrix
EDTA	Ethylene diamine tetra-acetic acid
EGF	Epidermal growth factor
EMBL	European Molecular Biology Laboratories
ER	Endoplasmic reticulum
ES	Embryonic stem
FBS	Fetal bovine serum
FGF	Fibroblast growth factor
FITC	Fluorescein isothiocyanate
GAG	Glycosaminoglycan
GE	Germinative epidermis
GAPDH	Glyceraldehye 3-phosphate dehydrogenase
HMG	High mobility group
HOX	Homeobox
HRP	Horse radish peroxidase
HSPG	Heparan sulphate proteoglycan
ICM	Inner cell mass
IF	Intermediate filament
IFAP	Intermediate filament associated protein
IGF	Insulin-like growth factor
IL	Interleukin
IPTG	β-D-isopropyl thiogalactopyranoside
IRS	Inner root sheath
ISH	In situ hybridisation

kb	Kilobase
K-CFC	Keratinocye colony forming cell
l	litre
LEF	Lymphoid enhancer factor
LIF	Leukemia inhibitory factor
m	milli-
M	Molar
MEM	Minimal essential medium
MIF	Macrophage migration inhibitory factor
MW	Molecular weight
n	nano-
NBT	Nitro blue tetrazolium
OD	Optical density
ORF	Open reading frame
ORS	Outer root sheath
p	pico-
PAGE	Polyacrylamide gel electrophoresis
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PDGF	Platelet derived growth factor
PEG	Polyethylene glycol
pfu	Plaque forming units
PGC	Primordial germ cell
PI	Phosphatidylinositol 3-kinase
RAR	Retinoic acid receptor
RNA	Ribonucleic acid
rpm	Revolutions per minute
SCID	Severe combined immunodeficiency
SCF	Stem cell factor
SDS	Sodium dodecyl sulphate
Shh	Sonic hedgehog
SSH	Suppression subtractive hybridisation
TAE	Tris-acetate-EDTA
Tag	T-antigen
TBE	Tris-borate-EDTA
TE	Tris-EDTA
TESPA	3-aminopropyl triethoxysilane
TGF	transforming growth factor
TNF	Tumor necrosis factor
U	units
	micro-
UTR	Untranslated region
X-gal	5-bromo-4-chloro-3-indolyl-galactopyranoside

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CHAPTER 1

INTRODUCTION

1: INTRODUCTION

As a skin appendage, the hair follicle embodies many of the problems common to skin biology. Hair follicles are composed of dermal and epidermal components which are both functionally and anatomically integrated (like skin and other skin appendages). Many skin appendages remain dynamic to some extent throughout maturity, and can therefore be used to investigate mechanisms of epithelial-mesenchymal interaction in adult tissues. However, in contrast to interfollicular skin, which exists in a state of continual self renewal, adult hair follicles display cyclical behaviour. This involves proliferation and differentiation of the follicular epidermis in a discontinuous fashion throughout the hair growth cycle. The hair follicle lends itself particularly well to the study of growth and development in general, as it represents an adult mammalian tissue exhibiting morphogenetic, inductive and regenerative capabilities (Oliver 1966b, 1967a, Ibrahim and Wright 1977, Horne *et al* 1986, 1989, Oliver 1980, Horne and Jahoda 1992, Reynolds and Jahoda 1992, Jahoda *et al* 1993). That the hair follicle can regress in one phase of its adult growth cycle and subsequently reform itself in response to the proper signal, is a phenomenon unique in adult mammalian systems. Furthermore, the clinical, social and psychological significance of disorders of hair growth, and the associated economic exploitation, are strong incentives to elucidate the mechanisms which control follicle development, growth and cycling.

Hair follicles are multifunctional organs involved in protection, insulation, sensation, cleansing and communication. Individual follicles are involved in the execution of one or several of these functions, displaying widely different morphological and functional characteristics depending on their body site. It is therefore essential that hair follicle growth and behaviour must be tightly regulated throughout life. The search for intrafollicular control mechanisms involves both the dermal and epidermal components of the follicle, since these are both spatially and functionally integrated throughout the life of the follicle, effecting hair growth by continuous dermal-epidermal interaction across the follicular basement membrane. It is already well established that hair growth can be influenced by a variety of systemic and environmental factors (reviewed in Johnson 1977, Ebling 1990). However, it seems that these

effects are largely extrinsic, merely modifying the underlying behaviour pattern acquired by the follicle during its development. Clearly, therefore, understanding the control of hair growth requires insight into the inherently determined mechanisms operating within hair follicles throughout embryonic development, growth and cycling.

Table 1.1: Examples of molecule families which have been shown to be important in the control of hair growth and behaviour.

MOLECULE FAMILY	EXAMPLES	IMPLICATED IN	REFERENCES
Epidermal growth factor (EGF) family	EGF, EGF-R, TGF-α, amphiregulin	Hair follicle morphogenesis, hair follicle cycling and differentiation.	Green and Couchman 1984, Holbrook *et al* 1993, Piepkorn *et al* 1995, Hansen *et al* 1997.
Transforming growth factor-β (TGF-β) superfamily	TGF-β1, TGF-β2, TGF-β3, TGF-βR	Hair follicle morphogenesis, hair follicle cycling and differentiation.	Millan *et al* 1991, Schmid *et al* 1991, Little *et al* 1993, Stones *et al* 1994, Higley *et al* 1995.
	BMP-2, BMP-4 Activin βA, Activin βB.	Hair follicle morphogenesis.	Lyons *et al* 1989, 1990, Jones *et al* 1991, Matzuk *et al* 1995, Bitgood and McMahon 1995.
Fibroblast growth factor (FGF) family	FGF-1 (aFGF), FGF-2 (bFGF), FGF-R1, FGF-R2, FGF-R3, FGF-R4, FGF-5, FGF-7	Hair follicle morphogenesis.	Du Cros 1993
		Hair follicle cycling and differentiation.	Peters *et al* 1992, Hébert *et al* 1994, Rosenquist and Martin 1996.
Homeobox (HOX) family	HOXa13, HOXb2, HOXc8, HOXd9, HOXd11, HOXd12, *msx*-1, *msx*-2	Hair follicle morphogenesis.	Bieberich *et al* 1991, Kanzler *et al* 1994, Noveen *et al* 1995, Reginelli *et al* 1995.
Platelet derived growth factor (PDGF) family	PDGF-A, PDGF-B, PDGF-Rα, PDGF-Rβ	Hair follicle morphogenesis, hair follicle cycling and differentiation.	Orr-Urtreger and Lonai 1992, Holbrook *et al* 1993, Ponten *et al* 1994, Akiyama *et al* 1996.

The control of hair follicle behaviour is thought to occur by means of cell-to-cell communication involving specific "message molecules", usually peptides or small proteins which may be soluble or membrane bound. The signalling mechanism involves both the "message molecule" or ligand, and its receptor, so that a particular process may be controlled through the restricted expression of either of these molecules. A particular instructive or

permissive signalling molecule may pass between adjacent cells, or it may be expressed by cells distant from the site of its action. However, the timing of the expression is crucial to the event, particularly as most signals occur as part of a highly complex and tightly co-ordinated signalling cascade between the epithelial and mesenchymal components of the follicle. The molecular mechanisms underpinning the growth of hair are still relatively poorly understood. However, it has been reported that a wide variety of molecules are involved in the normal development of hair and skin, and many may be important factors in the control of hair growth (Reviewed by Stenn *et al* 1996, and discussed further in chapter 2).

The development and behaviour of all integumental appendages follows a similar pattern, and often involves common families of molecules. However, the signals which determine organ type, growth and behaviour are by no means fully resolved, and specific molecules have been shown to be involved with specific appendages. Hair follicle development and behaviour is discussed and compared with the development and behaviour of other integumental appendages in section 1.1.

1:1 DEVELOPMENT AND BEHAVIOUR OF INTEGUMENTAL APPENDAGES

1:1.1 Patterning and Initiation

In the developing embryo, the determination of the site of appearance, particular spatial arrangement and the type of organ are crucial events, occurring before the first morphological indications of appendage development. The patterning of appendage growth must be established prior to morphogenesis, providing information which determines the subsequent growth and behaviour of each individual appendage. Currently, the molecular basis of organ initiation is not fully understood, although it appears increasingly likely that unique combinations of transcription factors and signalling molecules which are involved in establishment of the primary body plan may also be involved in patterning during early organ development. The most important components of hair follicle growth control are determined by

events occurring during follicle patterning. These are poorly understood, but they determine the position of each follicle in relation to the body pattern, the placement of a follicle in relation to its neighbours, the size of the follicle and the type of hair produced. Once the identity of a particular follicle is established, its characteristics are retained even if transplanted to an ectopic site (Johnson 1965).

Examples of molecules which have been implicated in the patterning and induction of hair follicles and other appendages include the transcription factors lymphoid enhancer factor-1 (LEF-1) (van Genderen *et al* 1994, Zhou *et al* 1995), *msx*-1 and *msx*-2 (Robert *et al* 1989, MacKenzie *et al* 1991a, 1991b, 1992, Jowett *et al* 1993, Noveen *et al* 1995), signalling molecules such as retinoic acid receptors α, β, γ (Hardy 1968, Dhouailly *et al* 1980, Hardy 1983, Chuong *et al* 1992, Viallet *et al* 1993, Viallet and Dhouailly 1994), sonic hedgehog (*Shh*) (Bitgood and McMahon 1995) and BMP-4 (Jones *et al* 1991, Bitgood and McMahon 1995), and extracellular matrix molecules such as syndecans (Thesleff *et al* 1987, Panaretto 1993), midkine (Mitsiadis *et al* 1995a, 1995b) and tenascin (Thesleff *et al* 1987, Jiang and Chuong 1992, Vainio and Thesleff 1992, Kaplan and Holbrook 1994, Noveen *et al* 1995). These molecules and others implicated in follicle patterning are listed in table 1.3A and discussed further in chapter 2.

It has been suggested that growth factors and receptors acting as inductive signals may be the targets of transcription factors regulating the patterning and form of organs. Evidence to support the role of growth factors as inductive signals in epithelial-mesenchymal interactions is provided by localisation of a growth factor and its receptor in adjacent interacting tissues (reviewed in Thesleff *et al* 1995).

1:1.2 Morphogenesis

Following induction, the development of all integumental appendages occurs through a process of epithelial-mesenchymal interaction. The effect of a particular signal between epithelium and mesenchyme is dependant on the organ and stage of development, and may act to up- or down- regulate the transcription of one or more other molecules in signalling

cascades and positive and negative feedback loops. The expression of signalling molecules and regulation of cellular proliferation and differentiation are closely co-ordinated throughout appendage morphogenesis. Initially, aggregation of dermal and thickening of epidermal tissue at the site of appendage formation occurs, followed by epithelial proliferation and invagination, giving rise to a dermal papillary structure surrounded by epithelial cells. These early processes follow a similar pattern in morphogenesis of all integumental appendages. An overview of hair, feather and tooth development is shown in figure 1.1.

1:1.2-1 Follicle Morphogenesis

The first appearance of follicle development in the rat and mouse embryo occurs in the mystacial pad. This involves the formation of a focal aggregation of dermal cells immediately below the epidermis. This marks the site of development of an individual follicle in an otherwise homogeneous skin structure. Development of pelage follicles over the rest of the body surface occurs later, with epidermal thickening occurring prior to dermal condensation. In the developing mouse embryo, vibrissa follicle morphogenesis begins at around E12, while pelage follicle morphogenesis is not initiated until E14 (Davidson and Hardy 1952).

The dermal aggregation, known as the papilla anlage, is made up of a specialised population of dermal fibroblasts which go on to form the dermal papilla. The epidermal aggregation is known as the placode. At the point of the papilla anlage, the basement membrane between the dermis and epidermis is lacking in glycosaminoglycans (GAGs), which are normally uniformly distributed along the dermal epidermal junction (Bernfield and Bannerjee 1982, Westgate *et al* 1984). The dermal cells of the papilla anlage then enter a stage of non-proliferation (Wessels and Roesner 1965) which is associated with a series of crucial dermal epidermal interactions which begin the process of follicle development.

The differentiation of mouse vibrissa and pelage follicles is essentially similar (described in figure 1.2). Initially, the epidermal placode grows down into the mesenchyme, forming an epidermal plug (stage 1). The cells of the mesenchymal condensation surround this to form the dermal sheath (stage 2), and invaginate to form a dermal papilla (stage 3).

Epidermal differentiation then begins to occur, with hardening of a cone of epidermal cells above the papilla anlage which will go on to form the inner root sheath (stage 4). As this differentiation extends upwards, a hair canal is formed throughout the length of the developing follicle, and cells appear which will form the sebaceous gland (stage 5). The epidermis then begins to undergo hair-type differentiation, with the appearance of a keratinised hair shaft inside the hair canal (stage 6). Differentiated sebaceous gland cells also appear at this stage. As the hair fibre grows, it pierces the cone of inner root sheath cells (stage 7) and emerges from the skin (stage 8).

Vibrissa follicle development differs slightly from pelage follicle development in that the epidermal plug is surrounded by a dermal sheath at stage 1, and thickening of the outer root sheath bulge is evident at stage 6. The timing of certain differentiation events is slightly different (summarised in table 1.2). The vibrissa follicle lower blood sinus begins to develop within a connective tissue sheath at stage 8, and both the upper and lower sinus and Ringwulst are completely differentiated by 3 days post-natal. These structures do not develop in pelage follicles. After birth, no new vibrissa follicles are initiated, but new pelage follicles continue to develop until about 8 days post-natal. Some of the molecules which have been shown to be expressed during the process of hair follicle morphogenesis are listed in table 1.3B. This is discussed in greater detail in chapter 2.

Table 1.2: Stages of morphogenesis of mouse vibrissae and pelage follicles (adapted from Davidson and Hardy 1952).

(Abbreviations - E - embryonic age, PN - post-natal)

STAGE	EVENT	AGE (DAYS) VIBRISSAE	PELAGE
0	Follicle patterning occurring	<E10	<E12
1	Downgrowth of follicle plugs	E12	E14
2	Appearance of dermal sheath surrounding follicle plugs	E12	E15
3	Formation of dermal papilla	E14	E16
4	Differentiation of inner root sheath	E14-15	E19
	Sebaceous gland initiation	E14-15	-
5	Formation of hair canals	E15	E20
	Sebaceous gland initiation	-	E20
6	Hair fibre differentiation	E15-17	E21
	Sebaceous gland differentiation	E15-16	-
	Outer root sheath thickening	E15-16	-
7	Hair fibre pierces inner root sheath cone	E17	1 PN
	Sebaceous gland differentiation	-	1 PN
8	Hairs emerge	E17-18	2-3 PN
	Development of lower blood sinus	E17-18	-
	Connective tissue sheath around developing blood sinuses	1 PN	-
	Complete differentiation of sheath, sinuses and Ringwulst	3 PN	-
	Development of secondary follicles	-	5-8 PN

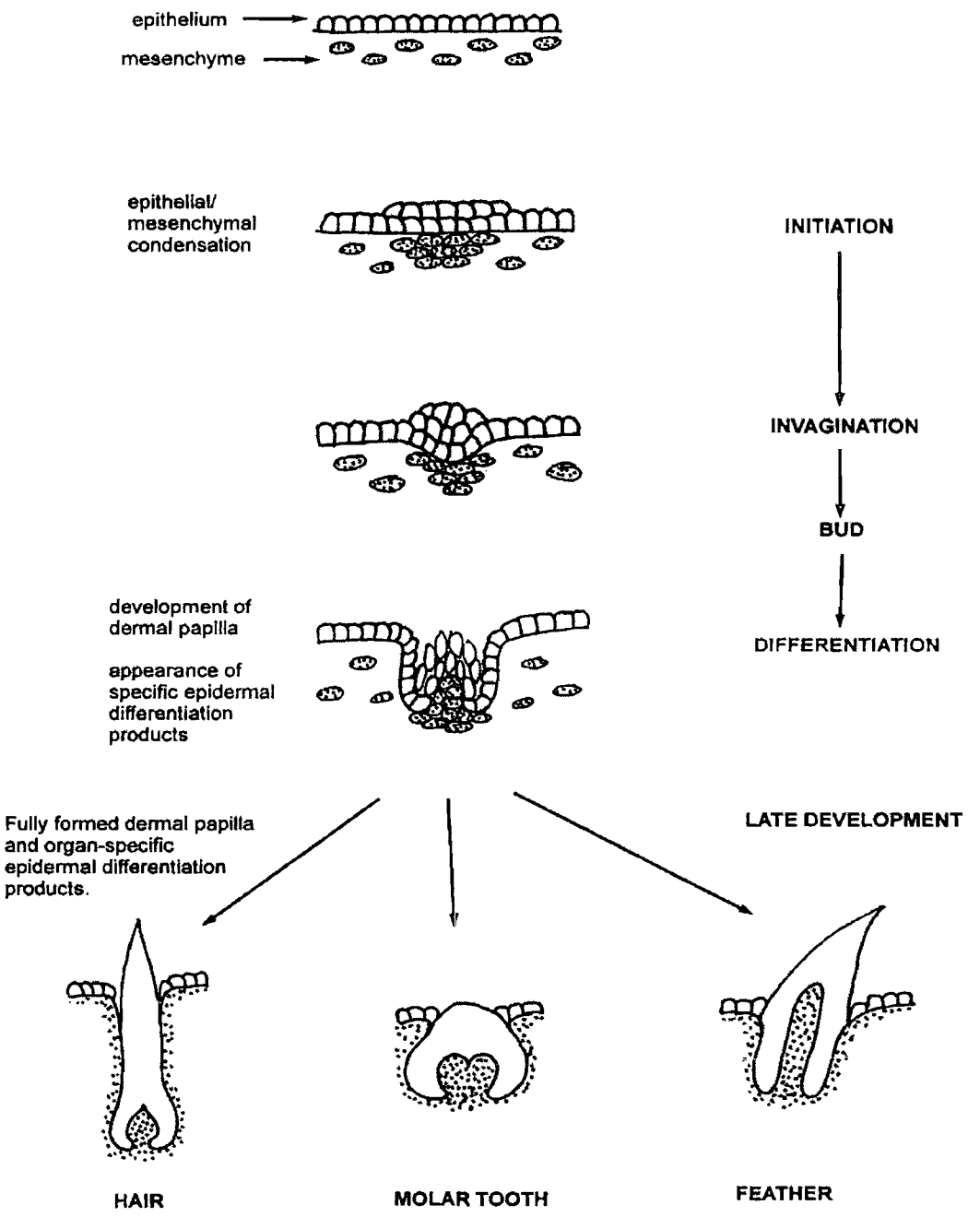


Figure 1.1: Schematic representation of hair follicle, tooth and feather development. Morphogenesis of these appendages is essentially similar, following a pattern of dermal and epidermal aggregation, epidermal proliferation and invagination, and development of a dermal papilla surrounded by differentiated epidermal tissue.

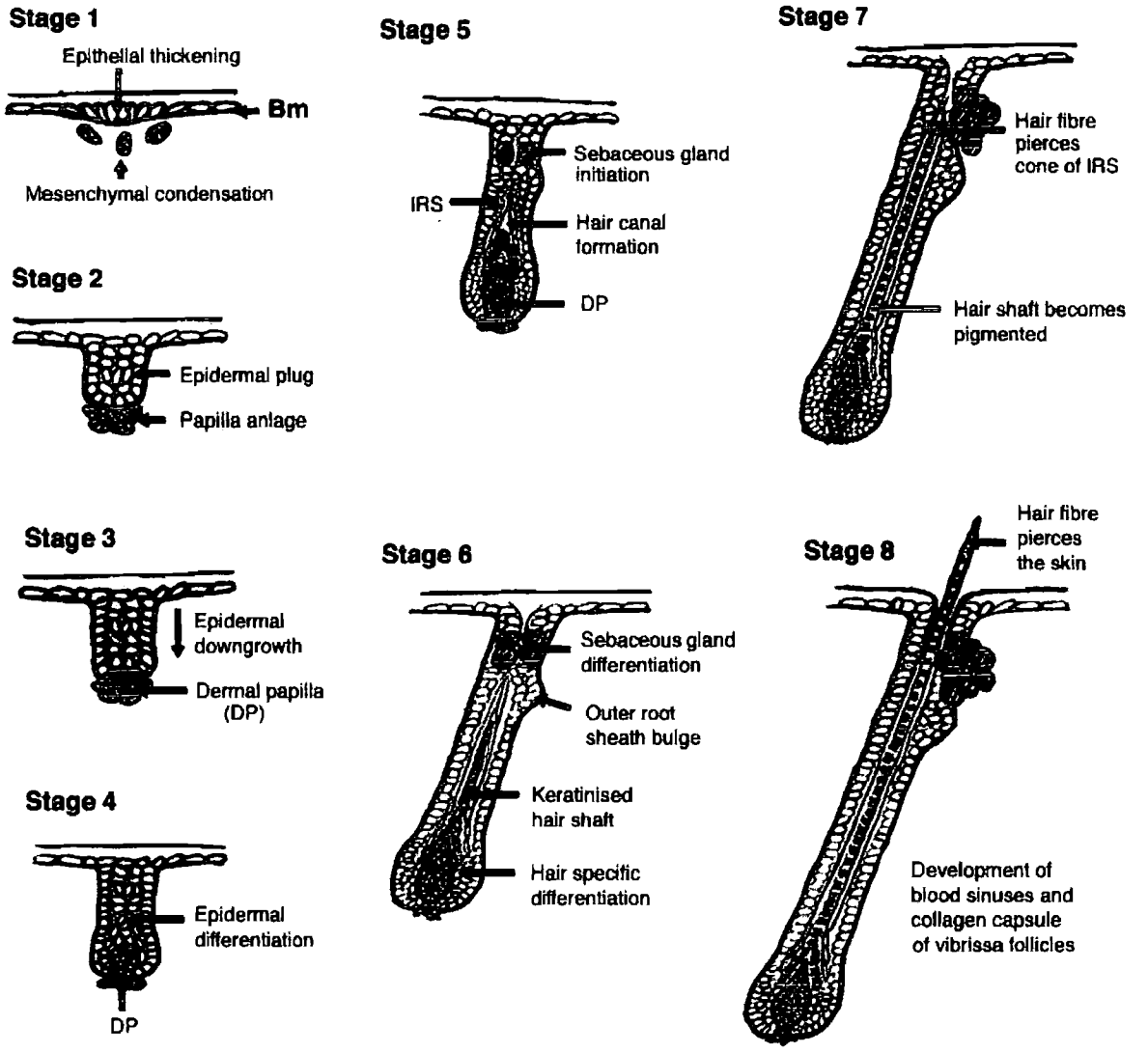


Figure 1.2: The development of the hair follicle. Initially, epithelial thickening and mesenchymal condensation occurs at the site of a developing follicle (stage 1). The epidermal plug then grows down into the mesenchyme (stage 2), and the cells of the mesenchymal condensation start to form the papilla anlage and dermal sheath. Epidermal downgrowth continues, and the cells of the papilla anlage invaginate into the epidermis to form the dermal papilla (DP) (stage 3). Epidermal differentiation then begins (stage 4), with hardening of a cone of epidermal cells above the DP which will form the inner root sheath (IRS). The hair canal then begins to develop, and cells appear which will form the sebaceous gland (stage 5). Hair type differentiation then begins in the epidermal matrix, and the outer root sheath bulge and a keratinised hair fibre appear (stage 6). The hair fibre continues to grow until it pierces the cone of IRS cells (stage 7). The hair fibre emerges through the skin (stage 8) and in the case of vibrissa follicles, the collagen capsule and blood sinuses develop during this stage.

1:1.2-2. Morphology of the Adult Follicle

At the base of the active follicle lies the end bulb, structurally the most dynamic part of the follicle. It consists of a pear shaped body of dermal cells - the dermal papilla (DP), surrounded by a cup of epithelial cells which make up the hair matrix. This consists of hair fibre precursor cells and several supportive epidermal layers. At the base of the matrix, lying in a ring around the basal stalk of the dermal papilla, are the germinative epidermal (GE) cells, which represent a population of epidermal progenitor cells (Reynolds and Jahoda 1991b), since it appears that the layers of the hair fibre and inner root sheath (IRS) grow up and differentiate from this original population. The basal stalk of the dermal papilla projects through the ring of germinative epidermis, and is continuous with the dermal sheath (DS), which forms a sleeve of dermal cells surrounding the follicular epidermis. Immediately inside the dermal sheath is the outermost layer of epidermis, the outer root sheath (ORS), separated from the dermis by the specialised follicular basement membrane (bm), which is known as the glassy membrane. About two thirds of the way up the follicle is a region of ORS known as the bulge region (B), thought to be the site of the follicular epidermal stem cells by some groups (Cotsarelis *et al* 1990, Lavker *et al* 1991), as this slow cycling population of cells does not appear to originate from the germinative epidermal cells, and there is some evidence to suggest that these cells may give rise to other epidermal cell types in the follicle (discussed further in section 1:3). However, it is interesting to note that not all follicle types have a morphologically distinct bulge region.

The morphology of the adult vibrissa follicle has been extensively studied, notably in the mouse (Davidson and Hardy 1952) and the rat (Young 1977), though studies have been carried out on other mammals. Since the vibrissa follicle is essentially a sensory organ, certain of its structural elements must be sensory in nature. Therefore, a hair, whether growing or not, is always present in all the vibrissa follicles of the mystacial pad, so that sensory signals may be received from all follicles at all times. However, the nerve itself appears not to affect the process of fibre production, since when the follicular nerve supply is experimentally severed,

there is no direct effect on whisker growth (Young 1977). This is in contrast to pelage follicles, in which hair fibres are not always present throughout adult life.

The essential components of the rat vibrissa follicle during its growth phase are shown in figure 1.3. Details of the muscular and nervous supply to the follicle are not shown. Other types of follicle from other species are essentially similar to this, although pelage follicles lack the collagen capsule and sinuses, and the structure of the lower portions of pelage follicles changes more radically during the hair cycle.

1:1.3 The Hair Cycle

Studies of vibrissa follicles have revealed that follicle structure is dynamic, changing as the hair follicle completes its growth cycle. The end bulb in particular undergoes profound changes in cellular synthetic activity and morphology (Young and Oliver 1976, Young 1980, Jahoda *et al* 1992a, 1992b) . The morphological sequence of events during the hair cycle is split into recognisable stages, using the following terminology (Dry 1926):

-Anagen: The active growth phase of the cycle, during which a hair fibre is produced.

-Catagen: The period of follicle regression and growth cessation.

-Telogen: The "resting" phase of the cycle.

Throughout the vibrissa follicle hair cycle, little or no mitotic activity is observed in the dermal papilla, with DNA synthesis observed only in vascular endothelium and migratory cells (Pierard and de la Brassine 1975). During anagen, the papilla is highly vascular, with a well defined basal stalk. The papilla extracellular matrix is rich in fibronectin and GAGs, and organelles associated with GAG and protein synthesis are particularly prominent in the cytoplasm. However, the papilla undergoes progressive reduction in cytoplasmic volume and extracellular matrix components during catagen, as GAG synthesis stops and the vascular network regresses. The dermal papilla persists as a condensed ball of cells throughout the telogen phase of the cycle, which is very short in vibrissa follicles.

During anagen, the epidermal matrix is highly active and dividing rapidly. Hair follicle epidermal cells are recognised as having one of the fastest rates of division in the adult body

(Van Scott *et al* 1963) and the rate of hair fibre growth may approach 1.5mm in 24 hours in the active rat vibrissa follicle (Jahoda 1982). Bromodeoxyuridine (BrdU) labelling studies of rat vibrissa follicle end bulbs show that most mitotic activity occurs during early anagen, with the germinative epidermis, lower matrix and outer root sheath all dividing rapidly. As anagen proceeds, cell division becomes limited to the germinative cells alone. As the follicle enters catagen, mitotic activity in the matrix is greatly reduced, and the lower region of the matrix disappears as it forms the club region of the hair. During telogen, there is little or no mitotic activity in the epidermal components of the end bulb, although some cell division may occur in the lower region of the club fibre as it lifts away from the dermal papilla (figure 1.4).

The start of the next anagen phase of the cycle, when hair growth begins again, is marked by an increase in DNA and RNA synthesis and mitotic activity in the epidermal components of the follicle (Silver and Chase 1970, 1977). The papilla enlarges and vascularises as GAG synthesis is upregulated, and the matrix at the base of the papilla proliferates, giving rise to a new hair fibre. The behaviour of pelage follicles throughout the hair cycle is similar to that of vibrissa follicles. However, the telogen phase of the pelage follicle cycle is much longer, and follicular regression during catagen and telogen is much more pronounced.

Many molecules have been implicated in control of adult hair follicle cycling. Some of these are listed in table 1.3C, and discussed in more detail in chapter 2.

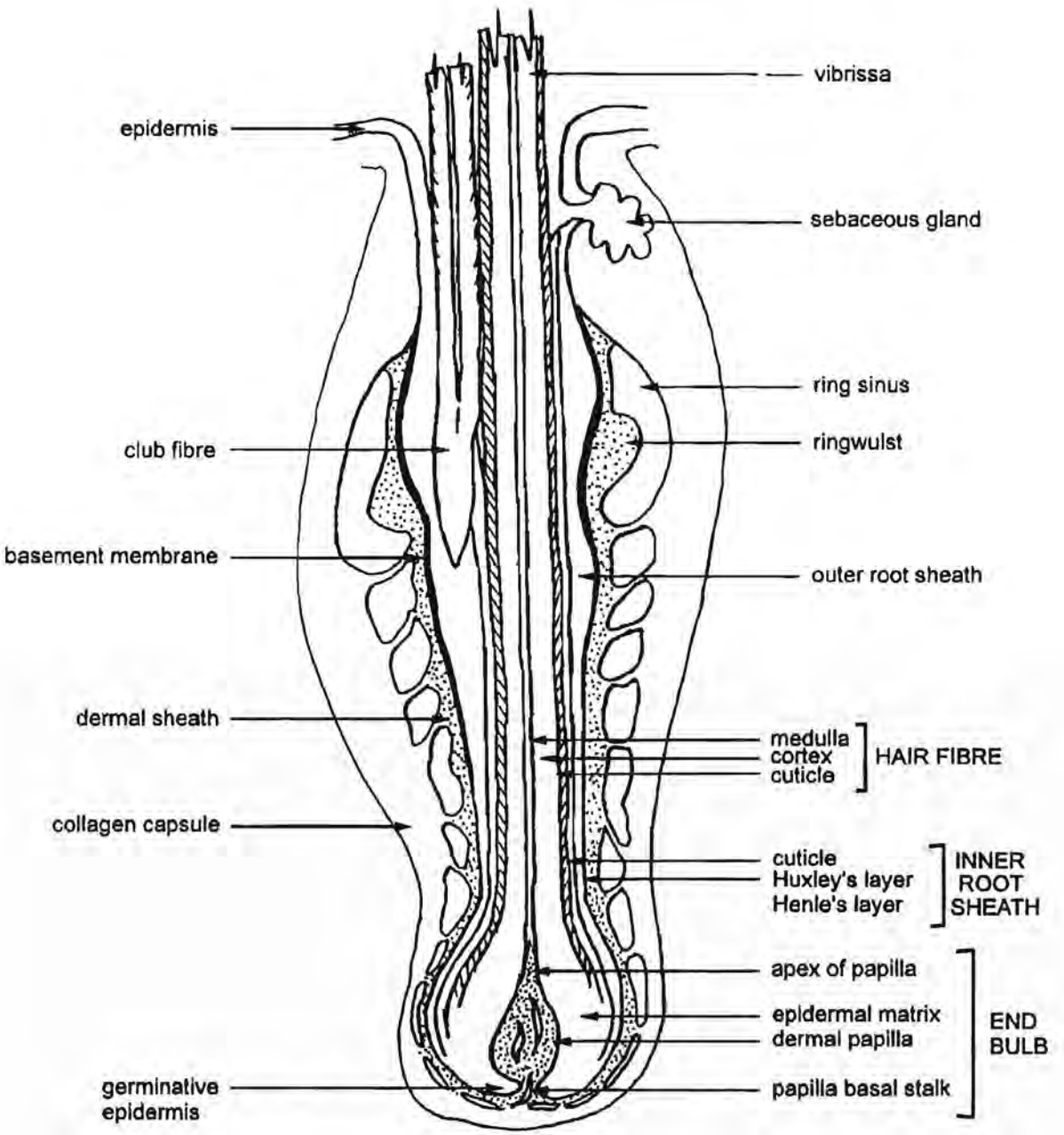


Figure 1.3: Schematic diagram of an anagen vibrissa follicle, showing the major components. Details of muscle attachments, blood supply and nerve attachments are omitted.

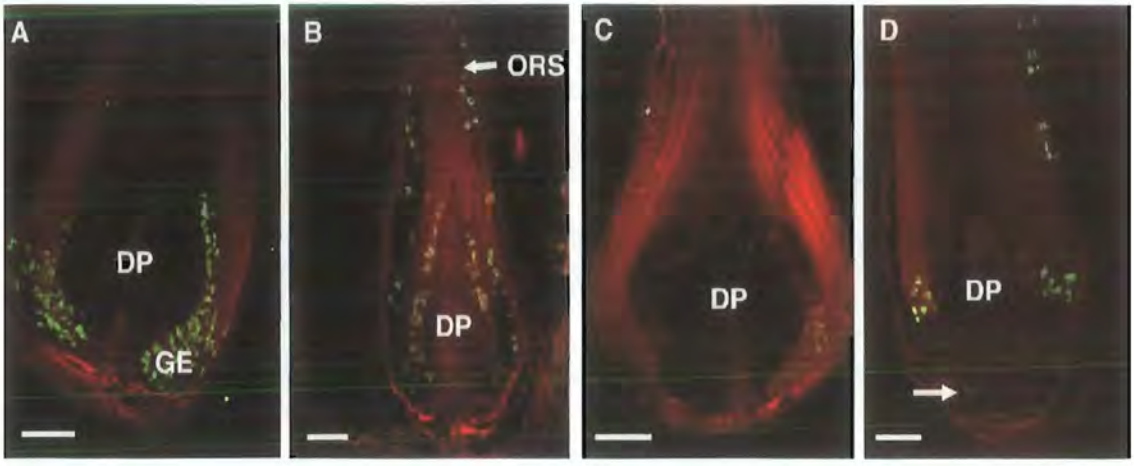


Figure 1.4: BrdU labelling of rat vibrissa follicle end bulbs throughout the hair growth cycle.

A During early anagen, cell division initially occurs only in the germinative epidermis (GE) and the lower matrix.

B As anagen proceeds, cell division occurs throughout the epidermal components of the end bulb, including the GE, matrix and outer root sheath (arrowed).

C As the follicle enters catagen, cell division again becomes restricted to the lower matrix cells.

D As the follicle enters telogen and the club hair begins to lift away from the base of the end bulb, a small amount of division occurs at the base of the club hair. The germinative epidermis is retained around the base of the dermal papilla (DP), and some cell division is also seen here (arrowed).

Photographs from Mr. M. Robinson. Bars = 100um.

Table 1.3: A list of some molecules implicated in the development, growth and cycling of hair follicles and other integumental appendages

A: Molecules implicated in patterning and induction of integumental appendages.

MOLECULE	REFERENCES
msx-1, *msx*-2	Robert *et al* 1989, MacKenzie *et al* 1991a, 1991b, 1992, Monaghan *et al* 1991, Noveen *et al* 1995.
Retinoic acid receptors (RAR) α, β, γ	Viallet and Dhouailly 1994
β–catenin	Gat *et al* 1998
Lymphoid enhancer factor -1 (LEF-1)	van Genderen *et al* 1994, Zhou *et al* 1995, Kratochwil *et al* 1996.
Sonic hedgehog (*Shh*)	Bitgood and McMahon 1995.
Bone morphogenetic protein (BMP) family	Jones *et al* 1991, Vainio *et al* 1993, Sleeman 1995.
Syndecan family	Thesleff *et al* 1987, Panaretto 1993, Thesleff *et al* 1995.
midkine	Mitsiadis *et al* 1995a, 1995b.
tenascin	Thesleff *et al* 1987, Jiang and Chuong 1992, Vainio and Thesleff 1992, Kaplan and Holbrook 1994, Noveen *et al* 1995.
integrin family	Holbrook 1991

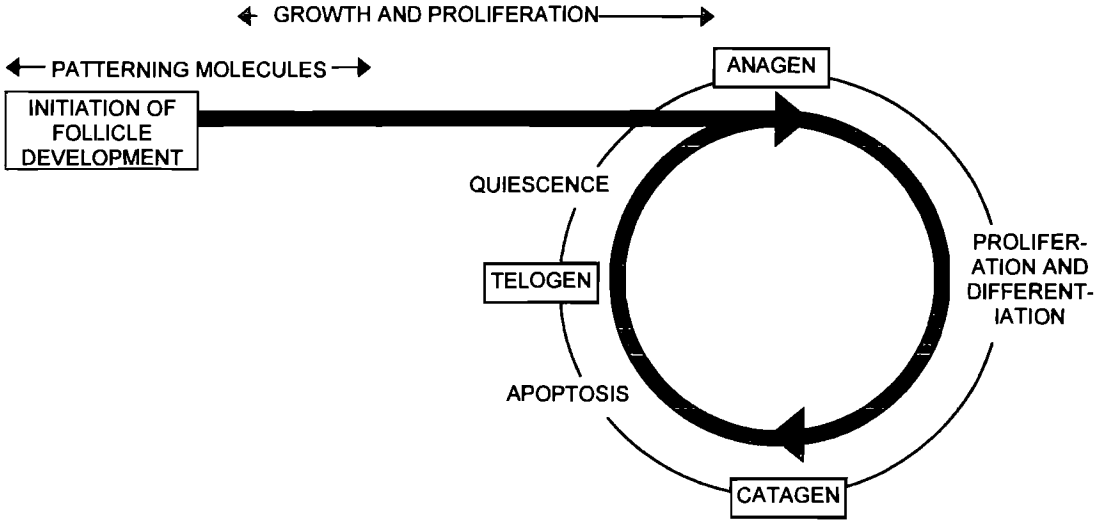
B: Molecules implicated in hair follicle morphogenesis and development

MOLECULE	REFERENCES
Activin βA, βB	Matzuk *et al* 1995
Epidermal growth factor receptor (EGF-R)	Holbrook *et al* 1993, Luetteke *et al* 1994, Hansen *et al* 1997.
Fibroblast growth factor (FGF) family	Du Cros 1993, Guo *et al* 1996
BMP family	Lyons *et al* 1989, 1990, Jones *et al* 1991, Sleeman 1995.
Insulin-like growth factor (IGF)-1, IGF-1 receptor	Liu *et al* 1993.
Transforming growth factor (TGF)-α	Luetteke *et al* 1993, Mann *et al* 1993
β-catenin	Gat *et al* 1998
LEF-1	van Genderen *et al* 1994
Homeobox (HOX) family *eg msx*-1, *msx*-2, HOXd9, 11, 12 *etc.*	Bieberich *et al* 1991, Kanzler *et al* 1994, Noveen *et al* 1995, Reginelli *et al* 1995.
tenascin	Kaplan and Holbrook 1994

C: Molecules implicated in control of follicle cycling and differentiation.

MOLECULE	REFERENCES
LEF-1	Zhou *et al* 1995
IGF-1 receptor	Hodak *et al* 1996
bcl-2	Stenn *et al* 1994
TGF-β	Seiberg *et al* 1995
EGF-R	Murillas *et al* 1995
FGF receptors 1-4, FGF-5, FGF-7	Peters *et al* 1992, Hébert *et al* 1994, Rosenquist and Martin 1996.
Clusterin	Seiberg and Marthinuss 1995
fibronectin	Jahoda *et al* 1992b.
hairless	Ahmad *et al* 1998

Figure 1.5: Schematic representation of hair follicle development and the hair growth cycle. Periods when patterning molecules and growth and differentiation controlling molecules are active are indicated (adapted from Stenn *et al* 1996).



1:2 MODEL SYSTEMS EMPLOYED IN HAIR GROWTH RESEARCH

The search for factors orchestrating hair follicle biology, based upon experimental results, is dependent on the existence of well defined laboratory models. Several model systems are currently employed in the study of hair growth control, including organ culture models, cell culture models and transgenic animal models. The advantages and disadvantages of each of these systems is outlined in table 1.4 and discussed further in section 1:2.1.

Table 1.4: Advantages and disadvantages of model systems currently employed in hair growth research.

MODEL SYSTEM	EXAMPLES	ADVANTAGES	DISADVANTAGES
Animal models	Mouse/rat	Easy to handle, inexpensive, well known genetics, can study all stages of the hair growth cycle.	No measurable androgen dependant sexual dimorphism of body hair.
	Macaque	Useful for studies of androgen effects on hair growth. Can study all stages of the hair growth cycle.	Expensive, difficult to handle, not readily available.
Organ culture models	Skin explants, individual follicles, follicle end bulbs, cultured in defined media.	Successfully used for a wide range of species including humans. Useful for study of intrinsic events. Easy to manipulate culture medium and introduce topical agents.	Currently can only be reliably used to study the anagen phase of the adult cycle. Limited penetration by topical agents. Cultures have very limited life span.
Cell culture models	Isolated follicle derived cell populations	Successfully used for a wide range of species including humans. Easy to study the effects of particular molecules on individual cell types.	Some follicular components very difficult to culture in isolation. Disruption of the cellular composition, spatial and structural organisation of the follicle gives rise to culture artefacts when studying single cell types or combinations. Cultures have limited life span.
	Culture in a nude mouse system.	Successfully used for a range of species including human cells. Useful for regeneration studies. Implanted cells/tissue survive longer than *in vitro*.	Relatively expensive and limited scale using current protocols.
Transgenic animal models	Gene knockouts, overexpression, ectopic expression	Useful for testing the function of specific gene products in follicle development and growth.	Tissue specific knockouts technically difficult. High levels of genetic redundancy masks phenotype of some knockouts. Early embryonic lethality in some cases. Some phenotypic effects may be artefacts of transgene construct insertion.
Naturally occurring mutants	Mouse *hairless*, *angora*, *rough*, *waved*, *balding*, *nude* etc.	Easy to handle, stable and non-artefactual	Genetic basis not always completely understood.

All model systems have their limitations, and it is necessary to select the system or combination of systems that is most suitable in order to address a particular question. For example, a follicle organ culture system developed by Philpott *et al* (1990) has successfully

been used for biochemical and molecular biological studies of anagen follicles (Harmon and Nevins 1993, Philpott and Kealey 1994, Philpott *et al* 1994), and systems based on the culture of dermal papilla cells in isolation (Jahoda *et al* 1984) have been used to determine some of the molecular mechanisms operating within the mesenchymal component of the follicle (Katsuoka *et al* 1987, Pisansarakit *et al* 1990, Randall *et al* 1992, Itami *et al* 1995, Randall *et al* 1995).

1:2.1 The Vibrissa Follicle As A Model System

The complexity and dynamism of the hair follicle means that it has immense potential as a model for fundamental cellular and developmental processes. The regenerative behaviour of the follicle and the cyclic growth of hair fibres indicates that hair follicles contain an active population of epidermal stem cells. In addition to addressing hair specific questions such as the mechanisms of early hair keratinisation and differentiation, an understanding of the mechanisms which control hair growth may also give greater understanding of mechanisms of dermal-epidermal interaction and the characterisation of adult epithelial stem cell subpopulations in general.

The establishment of a model system representing all aspects of natural hair follicle behaviour *in vivo* is important for analytical cellular and molecular studies. Many species and types of follicle have been utilised in hair growth studies with variable success. Some of the most successfully exploited systems involve the use of rodent vibrissa follicles or cell populations isolated from them. Rodent vibrissa follicles are ideal candidates both for *in vivo* studies and organ and cell culture models because they are large, and consequently easy to isolate and manipulate, and have a very regular and relatively short growth cycle (reviewed in Oliver 1980).

The rat vibrissa follicle has long been employed as an experimental model to study the inductive capability of the dermal components of the follicle (Cohen 1961). Isolation and recombination techniques similar to those used on adult feather follicles (Wang 1943, Lillie and Wang 1944) were adapted and extended for use on rat vibrissa follicles by Oliver (Oliver

1966a, 1967a, 1970, 1971), who showed that local dermal-epidermal interactions control hair growth, and that the dermal papilla has particular inductive capabilities, both within follicles and when associated with "foreign" epithelia (reviewed in Oliver 1980, and discussed further in section 1:2.1-1.2).

Vibrissae are highly adapted sensory hairs possessed by most mammals, man being one of few exceptions. They are commonly located in the facial and carpal regions, although they may occupy other functionally advantageous positions, for example, the ventral abdomen of the squirrel (Hyvarinen *et al* 1977). One of the earliest studies of vibrissa follicle anatomy and innervation was carried out by Vincent (1913) on the tactile hair of the white rat. Since then, the innervation of the whiskers of several other species has been described (southern elephant seal *Mirounga leonina*, (Ling 1966), Californian sea lion *Zalophus californianus* (Stephens *et al* 1973), Rhesus monkey (Halata and Munger 1980), Squirrel *Sciurus vulgaris* (Hyvarinen *et al* 1977), mink *Mustela vison*, polecat *Mustela putorius*, Meerkat *Suricata suricatta*, stoat *Mustela erminea* (Sleeman 1995), domestic cat (Schwarz *et al* 1997)). Several structural elements of the vibrissa, for example the capsule, sinuses and ringwulst, were suggested to be sensory adaptations (Stephens *et al* 1973). Since the fibre is growing almost continually, and the club hair is retained until the growing fibre is two thirds of its final length, there is a continuous sensory signal from every position on the mystacial pad.

The presence of a nerve supply is apparent in or around the vibrissa follicle from the earliest stages of development (Wessels and Roesner 1965, van Exan and Hardy 1980). However, a persistent nerve supply is not necessary for the normal development of the vibrissa follicle *in vitro* (Hardy 1951).

Two features in particular have encouraged hair growth investigators to utilise an adult facial vibrissa based model system. Firstly, their regular and constant distribution, observed and recorded by Danforth (1925), permits the recognition and repeated examination of individual follicle positions. As a consequence of the consistent follicle patterning on both sides of the face, each follicle has a more or less exact counterpart in the same animal for use as a convenient and reliable control for experimental follicles. Secondly, their large size enables quantitative measurements and experimental manipulations to be carried out in a

relatively simple manner. As a result, the growth and behaviour of vibrissa follicles has been well documented (Oliver 1965, 1966a, 1966b, Ibrahim and Wright 1975, Young and Oliver 1976, Young 1977, Jahoda and Oliver 1984a, 1984b).

It has been suggested that the behaviour exhibited by experimentally manipulated vibrissa follicles and vibrissa follicle components is not representative of tissues from other follicle types (Montagna 1980, 1984), as vibrissa follicles have specialised functions which do not apply to other types of follicle from other body sites. However, inductive capabilities identical to those exhibited by vibrissa follicle dermal papilla cells have been demonstrated for rat pelage dermal papilla cells (Reynolds and Jahoda 1992), indicating that the inductive capabilities of the dermal papilla are common to all types of hair follicle, reinforcing the validity of the vibrissa follicle as a model system for hair growth studies. Furthermore, vibrissa follicle behaviour is not modulated by hormonal or environmental factors, and is therefore ideal for studying the intrinsic behaviour of the follicle at a cell and molecular level.

When considering the molecular basis of the control of hair growth and cycling, the microsurgical approach clearly has limitations. However, microsurgical isolation of whole follicles and follicular tissues can be used as a starting point for a study of the molecules expressed in these tissues, both in combination and in isolation.

1:2.1-1 Animal Models

1:2.1-1.1 Experimental Manipulation Of Rodent Vibrissa Follicles

The rat vibrissa follicle has successfully been used as a model system for hair growth research *in vivo* since the pioneering work of Cohen in 1961. The comparatively large size of the vibrissa follicle structure enabled Cohen to isolate and manipulate the follicular components. It was shown that follicle end bulbs were capable of hair production when implanted into afollicular dermal sites. This experiment pioneered the use of microdissection techniques for use on the adult hair follicle.

Vibrissa follicles were examined after removal of the dermal papilla alone or the whole end bulb and varying lengths of the follicle. (Oliver 1966a, 1966b). In cases where the point of amputation was in the lower third of the follicle, a new end bulb containing a dermal papilla was regenerated. The regenerated papillae were often smaller then previously, and the resulting hairs were shorter. If the point of amputation was above the lower third of the follicle, no regeneration occurred. Similar results have been obtained for rat pelage follicles (Butcher 1965) and human axillary follicles (Inaba *et al* 1979). These experiments show that the dermal components of the follicle can be regenerated by conversion between cell types within the follicle or by recruitment from surrounding dermal populations. Such regenerative activity is relatively rare in adult mammalian biology.

The capacity of lower follicle dermal sheath to restore hair growth was tested by removing the lower halves of follicles and implanting the dermal sheath material from the follicle bases into the remaining follicle cavity. In over 80% of cases, hair growth was restored, with histological examination showing new dermal papillae below the original level of amputation (Horne and Jahoda 1992).

The sequence of events leading to the formation of a hair producing follicle after amputation of the end bulb has been detailed by immunohistological studies and electron microscopy (Jahoda *et al* 1992a). The initial response involves hyperproliferation of the follicle epidermis, which spreads below the level of amputation, associated with some downward movement of the upper follicle dermal sheath cells. Extracellular matrix proteins such as fibronectin, laminins and type IV collagen, may mediate dermal-epidermal interactions during such follicle regeneration.

1:2.1-1.2 Implantation Studies

Cohen implanted complete rat vibrissa end bulbs and papillae into ectopic sites such as the pinna. These implants formed follicles which grew short vibrissa type hairs. These findings stimulated a series of investigations designed to determine the role of the papilla in hair growth. An inductive role for the papilla in hair growth was demonstrated by implanting

whole dermal papillae into the bases of follicles after amputation of their lower portions (Oliver 1967a, Young 1977, Jahoda *et al* 1984). Provided that papilla-epidermal contact is maintained, the outer root sheath becomes organised around the papilla to form a matrix, hair growth begins and the follicle lengthens. This series of events occurs naturally during the early anagen phase of pelage follicle cycling.

In addition to their ability to induce pre-existing follicular material to grow hair, transplanted dermal papillae can interact with adult epidermis to form new hair follicles, demonstrating retention of their embryonic function. This was shown by recombining vibrissa papillae with sheets of epidermis obtained from the ear, hairless scrotal sac skin, or oral mucosa in an ectopic site in the pinna (Oliver 1970, 1973).

This finding has been confirmed by implanting whole papillae into wounds in the pinna. Where papilla-epidermal contact is maintained, the epidermis becomes organised around the papilla, forming matrix type structures with associated epidermal mitoses. Follicular structures then develop, with the appearance of inner and outer root sheath, followed by hair fibre emergence. Similar follicle induction has been observed following the implantation of a ball of cultured dermal papilla cells into wounds in the pinna, with the size of follicle produced depending on the papilla cell mass initially introduced into the wound (Jahoda *et al* 1993).

It has been consistently found that implanting vibrissa papillae into ectopic sites results in the growth of vibrissa type hairs. This suggests that it is the origin site of the dermal cells that determines the type of follicle produced. Transplanted papillae or papilla cells will remain as discrete entities for up to ten months after transplantation.

When cylindrical sleeves consisting of only dermal sheath and epidermal outer root sheath were inserted into wounds, as long as some of the material had come from the lower third of the follicle, papillae were able to regenerate with subsequent hair growth (Oliver 1967b). However, if such sleeves were slit longitudinally and implanted as sheets, regeneration did not occur. Therefore, the follicular components must be in the correct spatial orientation for follicle morphogenesis to occur.

A common problem with implantation studies such as those described here is the contribution made to induced follicles by tissues surrounding the site of implantation. It is difficult to determine to what extent the observed morphogenetic activity is due to the introduced tissue, and how much this activity depends on recruitment of cell types present at the implantation site. Some of these difficulties have been surmounted by using a nude mouse system (section 1:2.1-3.2), and a method described by Reynolds and Jahoda (1992) used an isolation chamber to eliminate the cellular and molecular influences from established hair follicles in the host implantation site. Briefly, cultured rat dermal papilla cells were recombined with footpad skin, which was then placed in a silicone chamber in a granulation tissue pocket under the dorsal skin of a host rat, effectively isolating the recombined tissue from the surrounding host follicles. This prevented cellular migration from the host skin, thus proving that hair fibres produced in the recombined tissue had arisen only from the implanted material.

1:2.1-2 Organ Culture Models

Dermal-epidermal recombination studies performed on mouse dorsal pelage and vibrissa follicles by Kollar in 1966 demonstrates that the dermal component of the follicle initially determines follicle type. Kollar found that vibrissa follicle development occurred both when snout dermis was combined with footpad epidermis, and when snout epidermis, containing vibrissa epidermal plugs formed under the influence of the vibrissa papilla anlage, was combined with dorsal dermis, thus showing that the dermal component initially controls hair follicle formation.

Many groups have reported systems for the culture of intact follicles to provide a model of hair growth *in vitro*. The first demonstration of post-embryonic hair growth *in vitro* was by Frater and Whitmore in 1973, who showed that development and growth could continue in explants of skin that were cultured in the presence of a serum supplemented medium containing an enzymatic digest of early mouse embryos. Several other short term studies have since been reported (Uzuka *et al* 1977, Rogers *et al* 1987, Buhl *et al* 1989,

Philpott *et al* 1990, 1992, 1994, Waldon *et al* 1993, Jindo *et al* 1994, Robinson *et al* 1997). Some of the most successful follicle culture studies were originally described by Philpott *et al* (1990), who demonstrated that human scalp follicles could routinely produce fibre for nine days in culture, with some follicles growing for up to 17 days. Although this work was a major breakthrough in follicle culture, the duration of growth observed in these experiments represents only a fraction of the total growing period of human scalp follicles *in vivo*. A system based on Philpotts work, recently described by Robinson *et al* (1997) using mouse vibrissa follicles, demonstrated that the stage of the hair growth cycle at which the follicles were isolated for culture was important in determining the subsequent behaviour *in vitro*. It was shown that vibrissa follicles isolated in early anagen could grow for up to 22 days, producing hair fibres up to 6.8mm long in culture, and clearly demonstrated that there is a significant difference between follicles isolated in early anagen and those isolated during later stages.

Organ culture studies like those described above are increasingly being used to investigate growth promoting and inhibiting molecules on hair follicle behaviour, e. g. minoxidil (Buhl *et al* 1989, Waldon *et al* 1993), hepatocyte growth factor (Jindo *et al* 1994), insulin and IGF-1 (Philpott *et al* 1994), protein kinase C (Harmon *et al* 1995) and retinoic acid (Williams *et al* 1996). Commonly, changes in hair growth are used as a measure of activity. However, it is clearly important that the cycle stage of follicles used in any such study is standardised to eliminate artefacts due to the different growth characteristics of follicles isolated at different stages of the cycle.

1:2.1-3 Cell Culture Models

1:2.1-3.1 Cell Culture *In Vitro*

The Dermal Components of the Follicle

Initial methods to establish cell cultures from isolated hair follicles relied on partial or complete disruption of amputated follicle end bulbs (Frater 1975, Uzuka *et al* 1977). This

produced a mixed cell population, the exact site of origin of which could not be specified. The first example of cell culture involving the dermal papilla alone was reported by Jahoda and Oliver (1981). When papilla cells are cultured, they show an initial reluctance to divide (Jahoda and Oliver 1981, Jahoda 1982, Messenger 1984). Their division rate is much slower and the cell number at confluency much lower than skin fibroblasts, and after each passage, the cells display a relatively lengthy lag before entering the rapid division phase. This is consistent with their behaviour *in vivo*, where division does not occur during the normal hair cycle. DP cells can be readily maintained in culture, and many groups have principally concentrated on this cell type as the dermal papilla is persistent throughout the life of the follicle, and cultured dermal papilla cells retain the inductive properties of freshly isolated papillae when implanted into afollicular sites (Jahoda *et al* 1984, Horne *et al* 1986, Jahoda *et al* 1993). Furthermore, it has been shown that cultured papilla cells induce type specific follicle neogenesis in several skin sites *in vivo* (Oliver and Jahoda 1989, Reynolds 1989, Reynolds and Jahoda 1991a, 1992, Jahoda *et al* 1993), confirming the embryonic developmental principles where it is the dermal component of the follicle that specifies follicle type.

Papilla cells have been shown to induce follicle formation and differentiation when combined with afollicular epidermis (Oliver 1970). This process involves increased mitotic activity and epidermal hyperplasia, suggesting that papilla cells may support epidermal proliferation when the two cell types are combined, whether this is within the hair follicle or not. To test this hypothesis, the capacity of various adult cell types to support skin basal epidermal cells *in vitro* was tested (Reynolds *et al* 1991), using adult rat vibrissa dermal papilla cells, skin fibroblasts and 3T3 cells as feeder layers. Each of these cell types was associated with newborn rat skin basal epidermal cells in the absence of epidermal growth promoting supplements to assess the epidermal sustaining capacity of each dermal support cell type. Analysis of epidermal cell counts and close photographic scrutiny revealed that low passage dermal papilla cells were most conducive to the successful attachment and subsequent proliferation of epidermal cell populations.

Cultured dermal papilla cells have been widely used in a variety of studies, particularly to compare DP cell behaviour with other cultured cell types, or with other DP cell populations

isolated from different body sites. For example, Randall *et al* (1992) used cultured human DP cells from androgen dependant follicles (e.g. beard) and non androgen dependant follicles (e.g. non-balding scalp) to confirm that, as expected, more androgen receptors were present in DP cells isolated from the androgen dependant areas, and it was also shown that beard DP cells secrete more SCF in culture when compared to DP cells from non-androgen dependant sites (Randall *et al* 1995). Katsuoka *et al* (1987) studied the effects of EGF, FGF, minoxidil and hydrocortisone on cultured DP and ORS cells, and Itami *et al* (1995) studied the effect of androgens on interaction of cultured DP cells and follicular epidermal cells. A molecular study by Sleeman (1995) used probes derived from cultured DP cells to screen a cDNA library for clones specific to a particular DP cell phenotype *in vitro*, and a study by Yu *et al* (1995) compared mRNA from cultured dermal papilla cells and cultured fibroblasts by differential display RT-PCR (DDRT-PCR) to isolate dermal papilla cell specific mRNA's. The characterisation of early passage dermal papilla cells in culture is therefore a useful "stepping stone" to characterisation of their behaviour *in vivo*.

Follicular dermal sheath (DS) cells are also readily maintained in culture. However, cultured DS cells show no inductive capabilities when implanted alone into ear wounds (Horne *et al* 1986, Reynolds 1989, Horne and Jahoda 1992). In this respect, they are similar to high passage dermal papilla cells, which "lose" their inductive abilities after 3 - 4 passages.

The Epidermal Components of the Follicle

Some of the epidermal components of hair follicles have also been grown in culture. Initially, ORS cells were grown from plucked or whole follicles (Wells 1982, Weterings *et al* 1982, Vermorken and Bloemendal 1986). Attempts have also been made to culture epidermal matrix cells from around the dermal papilla using plucked hair matrices (Jones *et al* 1988). However, this population appears to be morphologically and biochemically identical to cultured ORS cells. The hair matrix consists of some epidermal cells that are committed to hair type differentiation, and others that are apparently undifferentiated. These are known as germinative epidermal (GE) cells, and are a biochemically distinct population which can be

consistently isolated by microdissection (Reynolds and Jahoda 1991b). These cells have long been considered to be an epidermal stem cell population with a pluripotential nature, since they give rise to several cell lines as they differentiate, including hair medulla, cortex, cuticles, Huxley's and Henle's layers. However, these cells remain quiescent in culture, unlike cells from the outer root sheath bulge, the other putative epidermal stem cell population in the follicle.

Experiments performed in order to grow hair follicle germinative epidermal cells alone *in vitro* have been largely unsuccessful. Though the cells do not die, they remain small, round and undifferentiated, and are very reluctant to attach, even though a variety of substrates, growth media and supplements conducive to epidermal cell growth have been used (Reynolds 1989). However, when these cells are cultured in association with low passage dermal papilla cells, they are stimulated to proliferate and interact with the dermal cells to form organotypic structures (figure 1.6), having a distinct basal lamina at the papilla-epidermal cell junction (Reynolds and Jahoda 1991b). A similar study was also performed to investigate the behaviour of human germinative epidermal cells co-cultured with a human dermal papilla cell feeder layer (Reynolds *et al* 1993). These results provide evidence that hair follicle germinative epidermis behaves in a different way to other follicular epidermis, and may be fundamental to follicle induction and hair growth.

It is interesting to note that although cultured dermal sheath cells and high passage dermal papilla cells display no inductive capabilities when implanted into heterotypic skin, when these cell types are implanted with adult germinative epidermal cells, they regain their inductive capabilities and induce the neogenesis of large vibrissa type follicles (Reynolds and Jahoda 1996).

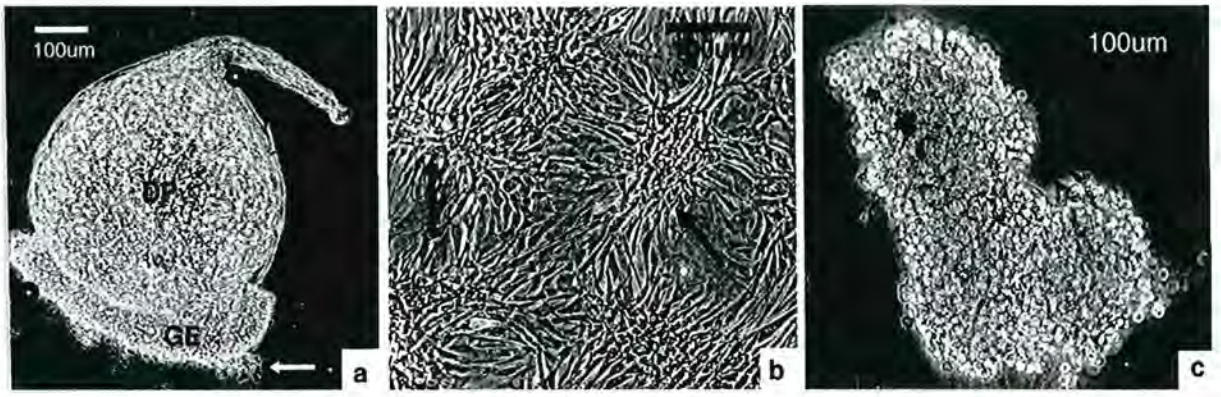


Figure 1.6: Culture of dermal papilla and germinative epidermal cells alone and in recombination

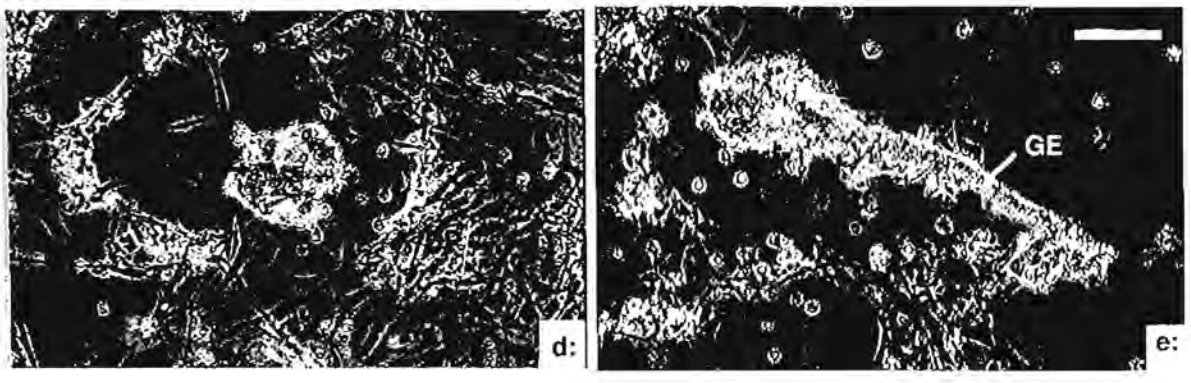
a: Freshly isolated rat vibrissa dermal papilla (DP), with ring of germinative epidermis (GE) adhering around the basal stalk of the DP. The GE cells are very sticky, and initially adhere very strongly to the substrate (arrowed).

b: Low passage dermal papilla cells beginning to form aggregations (arrowed) in culture.

c: Germinative epidermal cells after two weeks in culture. The cells remain small, round and undifferentiated, and cannot be stimulated to divide.

d: GE cells in coculture with a low passage DP cell layer at day 0.

e: The same GE/DP culture after 11 days. The DP cells are interacting with the GE cells and stimulating their proliferation with associated morphological changes.



(Bars = 50um except where shown)

1:2.1-3.2 The Nude Mouse System

Grafting of human skin onto athymic mice has frequently been used to study the development and behaviour of normal and abnormal skin exposed to a variety of topical agents (Lane *et al* 1994, Otulakowski *et al* 1994, Gilhar and Etzioni 1994, Limat *et al* 1996). Furthermore, several studies involving nude mouse grafts have been specifically used to examine hair growth and induction. Gilhar and Krueger (1987) studied hair growth in scalp skin from patients with alopecia areata grafted onto nude mice, Lichti *et al* (1993) grafted defined cell populations into a nude mouse system, Scandurro *et al* (1995) reported a method in which immortalised rat dermal papilla cell lines could give rise to hair development and fibre production when grafted onto nude mice, and Seki *et al* (1997) reported that human cultured dermal papilla cells could induce hair follicles in nude mouse grafts. A report by Jahoda *et al* (1996) demonstrated that human hair follicles from a variety of skin sites whose end bulbs had been removed could regenerate and grow hair when grafted into a nude mouse system, either as a skin graft or as subcutaneous implants of individual follicles. This confirms that the regenerative behaviour of the rat whisker follicle is a general phenomenon.

1:2.1-4 Transgenic Animal Models

Many strains of transgenic mice have been developed containing mutant genes which have been inactivated, or are overexpressed constitutively or in a tissue or time specific manner depending on the promoter used in the transgene construct. Alterations in follicle morphology and growth have often been produced using transgene constructs expressing genes which would not be expected to affect the hair follicle. Several gene knockouts have given valuable insights into molecules which are important in hair growth, and overexpression studies using transgene constructs containing keratin gene promoters have also been performed to observe the effects of targeted overexpression of particular molecules in the hair follicle. A brief summary of some of these is given in table 1.5, but these and others are discussed in detail in chapter 2.

1:2.1-5 Naturally Occurring Mutants

Naturally occurring mouse mutants showing altered hair phenotypes have been observed for many years. These include mutants with abnormal hair structure such as *waved-1* (Crew 1933) having a wavy coat and *angora* (Dickie 1963, Konyukhov and Berdaliev 1990) having extra long pelage hair, and hair colour mutations such as *Steel* (Sarvella and Russell 1956) which completely lacks pigmentation. A comprehensive description of all the natural mutant strains having hair defects is beyond the scope of this chapter. However, these mutants have proved particularly useful in combination with current molecular approaches to identify which genes are responsible for the phenotypes observed in the natural mutant strains.

Table 1.5: Important examples of transgenic mice displaying altered hair phenotype and/or behaviour.

TRANSGENIC	REFERENCES
Hair keratin overexpression	Stoler *et al* 1988, Abe and Oshima 1990, Bader and Franke 1990, de Mare *et al* 1990, Powell and Rogers 1990, Fuchs *et al* 1992, Baribault *et al* 1993, Byrne and Fuchs 1993, Thorey *et al* 1993, Takahashi *et al* 1994, Casanova *et al* 1995.
EGF-R knockout	Miettinen *et al* 1995, Murillas *et al* 1995, Threadgill *et al* 1995, Hansen *et al* 1996, 1997.
FGF-5 knockout	Hébert *et al* 1994.
FGF-7 disruption	Werner *et al* 1994, Guo *et al* 1996.
Integrin overexpression	Carroll *et al* 1995.
β-catenin overexpression	Gat *et al* 1998
LEF-1 knockout/ overexpression	van Genderen *et al* 1994, Zhou *et al* 1995,
TGF-α knockout/ overexpression	Vassar and Fuchs 1991, Luetteke *et al* 1993, 1994, Mann *et al* 1993.

1:2.2 Further Questions?

A great deal of information has been accumulated using combinations of all the models described above, firstly to describe the behaviour of the adult rodent vibrissa follicle, and subsequently the behaviour of other follicle types from other species. However, many questions remain to be addressed.

- What controls the initial stages of follicle patterning and initiation during development?
- What controls the timing of hair follicle cycling?
- Which follicular epidermal population constitutes the stem cell population from which all the other follicular epidermal cell types are derived?
- What signalling mechanism tells the follicle to "make hair"?
- Where is this signal expressed and where does it act?

This thesis aims to investigate the following questions in particular.

- Which population constitutes the follicular epidermal stem cells?

There is evidence to suggest that hair follicle epidermal stem cells are present in the bulge region of the outer root sheath (ORS). These cells have been shown to have stem cell like properties in that they are slow cycling, relatively undifferentiated, and can be stimulated to proliferate in response to wounding and certain growth stimuli (Cotsarelis *et al* 1990). However, the germinative epidermal (GE) cells in the end bulb have similar properties, and unlike the bulge cells, have no prekeratin filaments (Reynolds and Jahoda 1991b). Both populations remain in the follicle after plucking, and are therefore seen to be protected from the injury most common to hair follicles. It is important to determine whether both populations are epidermal stem cells, or if the GE cells are products of the bulge cells, acting as a transit amplifying cell compartment, since stem cells are ultimately responsible for epidermal maintenance and repair. This question is discussed further in section 1:3 and chapter 4.

- What signalling mechanism tells the follicle to "make hair"? Where is this signal expressed and where does it act?

The regulation and interaction of the molecular determinants of follicular epidermal proliferation and differentiation are very complex. The expression of any particular molecule during fibre production may be controlled or affected by the expression of one or several others, so it is difficult to speculate exactly what combination of molecules provides the signal to "make hair" or to "stop making hair". The "hair specific" signal is unlikely to be the result of a single gene product, instead resulting from the tightly controlled spatial and temporal expression patterns of a combination of molecules. Molecules which are already known to be involved in control of hair follicle behaviour are examined in chapter 2, and novel gene products whose expression is highest in potentially important follicular epidermal populations are investigated in chapter 3.

1:3 STEM CELLS

The cyclic growth of hair fibres throughout the hair cycle demonstrates that the epidermal component of the follicle is self renewing throughout life. All such self renewing systems in adult organisms depend on a population of stem cells for continued proliferation and activity.

Stem cells are characteristic of permanently renewing tissues in which the terminally differentiated cells are unable to divide and have a short life span. They tend to be located in sites in which they are protected from injury, and are slow cycling and undifferentiated. They can be stimulated to undergo asymmetric division in response to wounding and certain growth stimuli, maintaining the stem cell population and creating a pool of committed daughter cells. They are pluripotential, and give rise to terminally differentiated cells via transit-amplifying or committed progenitor cells having lower proliferative potential and a more restricted capacity for differentiation than stem cells (Lehman *et al* 1974, Fausto 1990, Sigal *et al* 1992). The existence of a transit-amplifying or progenitor cell compartment amplifies the effect of each

stem cell division, so that a large number of terminally differentiated cells with different phenotypes can be produced from relatively few rounds of stem cell division.

In certain mammalian tissues, such as nerve and skeletal muscle, the cells formed during development persist throughout adult life. However, many differentiated cell populations in the adult are not permanent, but are subject to renewal growth, the predominant type of cell division in adult mammals. Renewing stem cells in somatic tissues undergo continuous asymmetric divisions. One new daughter cell retains the division potential of the original stem cell, while the other differentiates into a functional constituent of the tissue. The differentiation pathway may be stochastic or deterministic or a combination of both, depending on the tissue in question.

An almost universal difficulty in studying adult stem cell populations is their correct identification and isolation. This has been hampered by a lack of good molecular markers specific to adult stem cell populations. Several adult stem cell populations have been described, including hematopoietic stem cells, various epithelial stem cell types (e. g. skin, mammary gland, intestinal), hepatic stem cells and putative hair follicle epidermal stem cells.

The plurality of function and complex differentiation of hair follicle epidermis suggests that this tissue may involve a similar system of stem and progenitor cell populations, or alternatively, it may have more than one stem cell location, from which are derived at least six different differentiation products.

1:3.1 Epithelial Stem Cells in the Skin

All external surfaces of the body are covered with a layer of epithelium, the stratified layers of which are derived from epithelial stem cell populations. However, the morphology of a particular epithelium, and the location of its stem cells, is dependent on tissue type and body site. For example, the morphology and biochemistry of epidermis at different skin sites is sufficiently different to suggest that epidermal keratinocytes at different sites are intrinsically divergent (Miller *et al* 1997).

Table 1.6: General characteristics of adult stem cells.

1	Must be capable of self replication to supply precursors for specialised cell types. Must have a regenerative or clonogenic capacity. Should be capable of unlimited cell renewal in the context of the lifetime of the organism.
2	Must have a long life expectancy. Likely to be firmly anchored at a particular point in the tissue to give maximal protection from environmental insult.
3	Must be the cells at the origin of any cell lineages or cell migration pathways in a specific tissue.
4	Should be distributed sparsely within the proliferative compartment.
5	Should have a long cell cycle time (long G_0) in relation to the majority of proliferative cells in a particular tissue.
6	May have evolved special mechanisms to conserve the integrity of their DNA.
7	Should be susceptible to the systemic or local factors determining the circadian rhythm.
8	Should have characteristic thymidine metabolism.
9	Are ultrastructurally unspecialised with few organelles and a large nuclear to cytoplasmic ratio.
10	May be pluripotent or unipotent. If a common ancestral stem cell gives rise to a number of terminally differentiated cell types (for example, hematopoietic stem cells) it is said to be pluripotent. If there is only one terminal differentiation pathway (as in the epidermis), the stem cells are termed unipotent.

Proliferation in normal adult epidermis occurs only in the basal layer of keratinocytes attached to the basement membrane separating the dermis and epidermis. These cells are the epidermal stem cells, and are ultimately responsible for epidermal maintenance and repair. Division of these stem cells gives rise to a transit-amplifying population of keratinocytes which detach from the basement membrane and move upward. However, in common with the morphological and biochemical differences characteristic of skin from different body sites, the spatial organisation of the stem cell, transit-amplifying and differentiated cell populations appears to vary at different body sites.

Distinct theories of epidermal stem cell organisation have developed to account for the apparent differences in the behaviour of stem cells and their progeny at different body sites. A theory based on evidence from ultrastructural studies was proposed by Potten in 1974. This theory described an "epidermal proliferative unit" (EPU), consisting of a hexagonal

region with a stem cell at the centre of the basal layer, surrounded and overlaid by a small population of transit-amplifying cells, which in turn were surrounded and overlaid by a population of committed cells (section 1:3.1-1, and reviewed in Potten 1981).

A second theory was proposed by Lavker and Sun (1982, 1983), based on a study of monkey palm epidermis, the basal layer of which is folded into a series of regular "deep" and "shallow" rete ridges. They suggested that cells present in the basal layer in the bottom of the "deep" ridges were the epithelial stem cells, and the basal cells at the bottom of the "shallow" ridges were transit amplifying cells involved with anchoring the skin layers together (section 1:3.1-2).

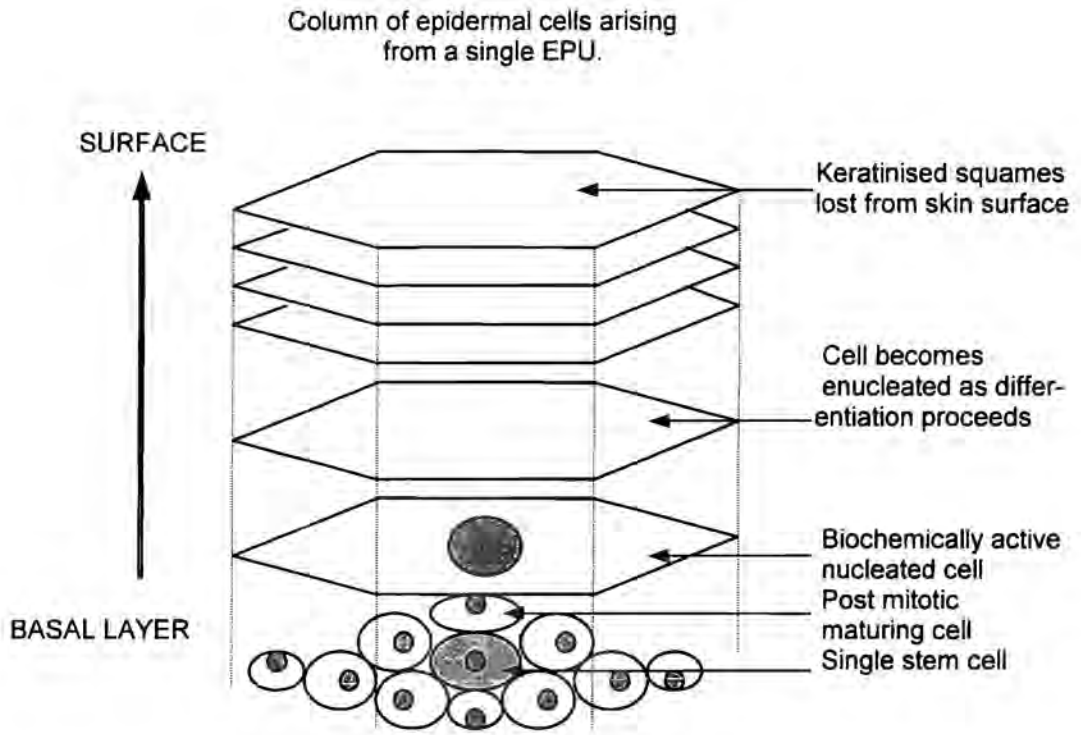
1:3.1-1 The Epidermal Proliferative Unit (EPU) Theory

The vast majority of basal cells in the skin (>85%) can be identified as keratinocytes. However, not all keratinocytes in the basal layer are proliferating. Some may be in a temporarily quiescent state, able to "rejoin" the proliferating compartment when required, for example, after wounding (Gelfant 1977, Potten and Major 1980), and others may have permanently lost the ability to divide. These are regarded as post mitotic maturing cells, and may be awaiting the correct signal to migrate away from the basal layer (Potten 1975a, Potten and Major 1980). Labelling experiments indicate that migration of cells away from the basal layer usually occurs 2-3 days after the last mitosis (Potten 1975a).

The organisation of cell layers in the skin is highly ordered. The lower layers are composed of biochemically active nucleated cells in various states of differentiation, and the upper layers are made up of thin, enucleated plates of keratin (squames) which are roughly hexagonal in shape with very little overlap between adjoining squames. This suggests that the cells are organised in columns with precise alignment of cell edges and a very regular pattern (MacKenzie 1969, 1970, 1975, Potten 1974, 1975a, 1975b, Potten and Allen 1975a, 1975b, Allen and Potten 1976a, 1976b). This arrangement appears to be restricted to areas where the epithelium is flat and thin, with a relatively low turnover. In order to maintain this organised structure, cell proliferation and migration must be a carefully programmed process. In mouse ear skin, the columnar organisation can be traced to the basal layer, with each column of suprabasal cells sitting over a patch of about ten basal cells. The epidermis can therefore be

regarded as a series of EPU's, made up of a few basal cells (including a single stem cell) and their maturing differentiating progeny (MacKenzie 1970, Potten 1974). This is shown in figure 1.7.

Figure 1.7: Structure and organisation of the epidermal proliferative unit (adapted from Potten 1981). A single stem cell in the basal layer undergoes renewal division to give rise to a small region of post mitotic maturing cells, which then migrate away from the basal layer and differentiate.



On average, each EPU has 10.6 basal cells, often arranged in a characteristic pattern with six or seven basal cells around the periphery of the EPU and a tightly associated central group of three to five cells (Potten 1974, reviewed in Potten 1981). Tritiated thymidine labelling studies indicate that the cells at the periphery of the EPU basal layer are cycling more rapidly than the central subgroup, except when the skin is wounded (Potten 1974, MacKenzie 1970, 1975).

1:3.1-2 Stem Cells in Other Skin Sites.

In some body sites, for example in palm and sole skin, the epidermis does not appear to be arranged in EPU's (Christophers 1971, Menton and Eisen 1971). The epidermal layer is much thicker in palm and sole skin, and the basal layer is folded into a series of ridges of varying depth. Biochemical differences can also be observed in such sites, for example, palm and sole skin express high levels of keratin-9, which is found in very small amounts elsewhere in the skin (Knapp *et al* 1986). Lavker and Sun (1982, 1983) proposed that the stem cells in monkey palm skin were located in the basal layer of the deepest epidermal rete ridges. Epidermal growth factor receptors have been localised to the same position (Misumi and Akiyoshi 1990, 1991). It appears that the arrangement of basal cells in other areas of human epidermis (arm, leg, back, abdomen and face) is similar (Lavker and Sun 1983). This is summarised in figure 1.8.

Figure 1.8: Arrangement of epidermal stem cells in palm and sole skin. Non-serrated stem cells are located at the bases of the deep rete ridges, and give rise to transit amplifying cells which migrate along the basal layer and into the suprabasal layers, where they become terminally differentiated.

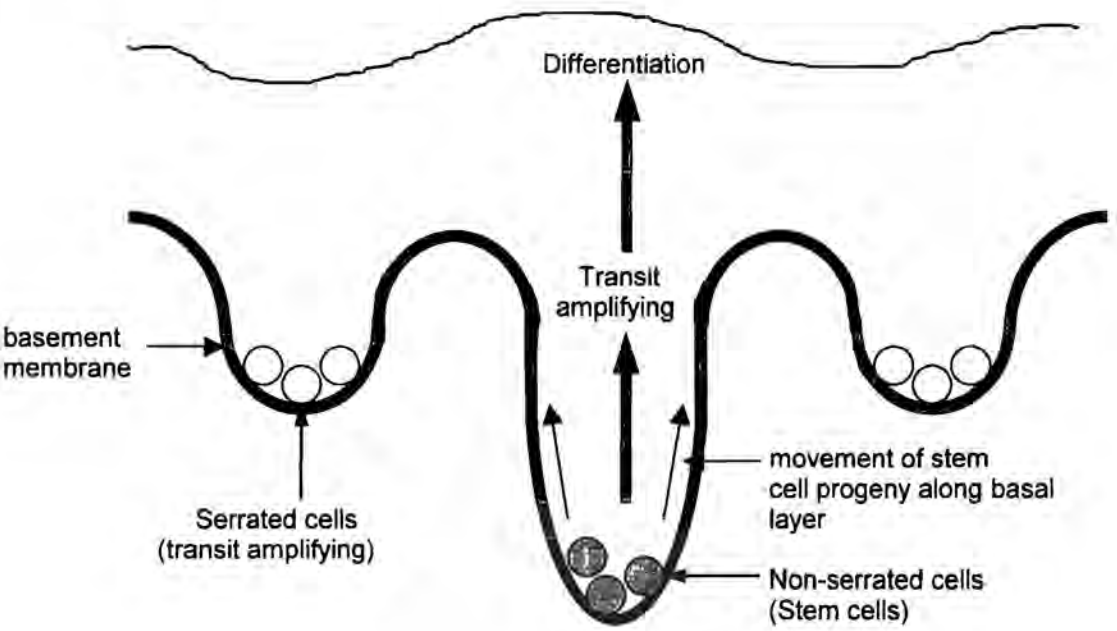


Table 1.7: A summary of early studies performed to characterise the epidermal stem cells.

Study...	Showing...	Reference
Ultrastructural studies of murine and hamster ear and trunk skin.	Hexagonal columnar arrangement of cells over an area of basal epidermis containing a single stem cell. (The EPU theory)	MacKenzie 1969, 1970, Christophers 1971, Potten 1974.
Injection of fluorescein dye into epidermal cells.	Vertical pattern of dye spreading resembling columnar arrangement of EPU's.	Kam *et al* 1986.
Studies of monkey palm skin and areas of human skin.	Arrangement of basal layer of epidermis in a series of ridges, with putative stem cells in the base of the deepest ridges.	Lavker and Sun 1982, 1983.
Growth factor receptor localisation studies.	Epidermal growth factor receptors localised at the bases of deep ridges, corresponding to putative stem cell location.	Misumi and Akiyoshi 1990, 1991.
Study of replicative capacity of skin basal epidermal cells.	About 28% of cells show high replicative potential, and may represent the stem cells.	Barrandon and Green 1987.
Tritiated thymidine labelling study of basal epidermal cells.	Only about 1% of cells show label retaining features characteristic of stem cells.	Morris *et al* 1985.
Morphological studies of putative stem cells.	Stem cells are small and undifferentiated, rich in ribosomes, with a high nucleus to cytoplasm ratio. Increasing cell size correlates with decreasing replicative potential, smallest cells with highest density show morphological and kinetic characteristics of stem cells.	Sun and Green 1976, Lavker and Sun 1982, 1983, Furstenburger *et al* 1986, Morris *et al* 1990, Pavlovitch *et al* 1991.
Studies of stem cell cycling using mitotic arrest and tritiated thymidine labelling *in vivo*.	Central cells of EPU's very slow cycling compared to surrounding cells in the basal layer. Basal cells in deep rete ridges slower cycling than cells in suprabasal layers.	MacKenzie 1970, Potten 1974, Lavker and Sun 1982, 1983, Morris *et al* 1985.
In vitro cell cycling studies.	Culture of label retaining cells isolated from epidermal basal layer (~1% of proliferating cells *in vivo*) produced small colonies of small, undifferentiated keratinocytes. Subpopulations of epidermal cells have different cycle times *in vitro*, with slowest cycling cells having other stem cell-like features.	Jensen *et al* 1985, Albers *et al* 1986, Morris *et al* 1990, Pavlovitch *et al* 1991.
Studies of the proliferative response of putative stem cells to stimuli.	Central cells of EPU's show a greater proliferative response to hair plucking than cells in the periphery. Phorbol ester stimulation produces a proliferative response in central basal cells of EPU's and a differentiation response in peripheral cells.	Potten 1974, Furstenburger *et al* 1986, Morris *et al* 1985, 1990.

Studies to locate transit amplifying cell populations.	Peripheral cells of EPU's proliferate rapidly, and are stimulated to differentiate by phorbol ester treatment. Suprabasal cells of palm skin are the most rapidly proliferating subpopulation.	Lavker and Sun 1982, 1983, Morris *et al* 1985.
Integrin expression studies.	High β1 integrin expression correlates with colony forming ability *in vitro*. However, high integrin expression appears to be characteristic of both stem cells and an early population of transit amplifying cells.	Jones and Watt 1993, Jones *et al* 1995.

1:3.1-3 Further Characterisation of Epidermal Stem Cells

Any population of adult stem cells should be sparsely distributed in the proliferative compartment, and slow cycling (see table 1.6). Several studies have been performed to determine the fraction of proliferating epidermal cells which are actually stem cells. Clonogenic studies and radiation repopulation studies indicate that 10-28% of basal keratinocytes may be stem cells on the basis of their high replicative capacity (Potten and Hendry 1973, Potten 1974, Barrandon and Green 1987). However, thymidine labelling and unit gravity centrifugation studies suggest that only 1% of the proliferating basal keratinocytes are stem cells (Morris *et al* 1985, Pavlovitch *et al* 1991). Morphologically, epidermal stem cells appear to be small and cuboidal, with a large nuclear to cytoplasmic ratio, few keratin filaments and large numbers of ribosomes (Lavker and Sun 1982, 1983). Increasing cell size correlates with decreased proliferative capacity, and small size and increased cell density correlates with stem cell characteristics (Sun and Green 1976, Furstenburger *et al* 1986, Morris *et al* 1990, Pavlovitch *et al* 1991). However, this suggests that stem cell populations may be heterogeneous. Table 1.7 describes some of the early studies performed to characterise epidermal stem cells.

The theory of stem cell heterogeneity proposes that a proportion of stem cells have a high probability of self renewal and others have a high probability of producing committed progeny. Schofield's "niche" hypothesis (1978) suggests that every stem cell exists in an optimal microenvironment or "niche". When the stem cell divides, one of its progeny will remain in the "niche" and the other will leave and become committed to terminal differentiation provided another "niche" is not available. Stem cell heterogeneity would result from

differences in the optimal microenvironment, where a stem cell at the periphery of such a microenvironment would tend to be more likely to produce committed progeny. This hypothesis embraces the concept of environmental influence on the stem cell population, where the optimal microenvironment would be dependent on neighbouring cell types, diffusible factors and extracellular matrix molecules.

The progression of stem cell - transit amplifying cell - terminally differentiated cell may potentially pose a problem, in that if the terminally differentiated cells are damaged, instability may result from the length of time spent in the transit amplifying population. This may be countered by feedback loops originating from the terminally differentiated cells which control the number of divisions occurring in the transit amplifying population or the stem cell compartment. In 1990, Potten and Loeffler described two models supporting this theory. In both models, a spiral is used to illustrate the cells moving down a pathway from the stem cell compartment to the mature functional compartment. The "abrupt differentiation-maturation spiral" model suggests that the transit amplifying population is able to divide like the stem cell population, but is unable to self-maintain. In this model, if the stem cell compartment is destroyed, the transit amplifying population will also disappear, suggesting that differentiation is unidirectional. In contrast, the "diminishing stemness spiral" model suggests that the transition from stem to transit amplifying cell is not abrupt, with a gradient of "stemness" existing across the stem-transit amplifying populations. This therefore suggests that the earliest transit populations retain a certain ability for self maintenance, which decreases as progeny pass along the spiral towards terminal differentiation. In consequence, if the stem cell compartment should be damaged, first generation transit amplifying cells can re-occupy the stem cell microenvironment and reconstitute the stem cell population. However, the biochemical differences between stem cells and first generation transit amplifying cells may be very slight.

1:3.1-4 Integrin Expression in Epidermal Stem Cells.

Stem cell division and daughter cell detachment from the basal layer is thought to involve inactivation of β_1 integrin receptors on the cell surface (Hotchin *et al* 1993). Studies of

epidermal stem cell properties have been hampered by the lack of molecular markers to correctly identify adult epidermal stem cell populations. However, Jones and Watt (1993) suggested that it is possible to separate epidermal stem cells from transit amplifying cells on the basis of their adhesive properties and integrin expression *in vitro*. Further studies suggested that it is also possible to use the differences in integrin expression between epidermal stem cells and transit amplifying cells to localise epidermal stem cell populations *in vivo* (Jones *et al* 1995).

Integrin receptors are involved in the adhesion of basal keratinocytes to the extracellular matrix. In the skin, stem cells follow a single differentiation pathway, producing one type of differentiated daughter cell (unlike the hair follicle epidermal stem cells, which produce at least six different daughter cell types). Basal epidermal keratinocytes plated on a 3T3 feeder layer form a multilayered structure, similar to the multilayered structure of normal skin *in vivo*. This indicates that the epidermal stem cells must persist in culture, at least for a few passages, as such cultured epidermis has been successfully used in skin grafting. However, when epidermal keratinocytes are cultured in suspension, they will all terminally differentiate within 24 hours. Addition of ECM proteins or β1 integrin blocking antibodies partially inhibits differentiation in suspension culture. Integrins therefore appear to be involved in regulation of differentiation, movement of cells out of the basal layer, and maintenance of stem cells and transit amplifying cells (Hotchin *et al* 1993, 1995, Jones *et al* 1995).

Staining of skin sections with anti-integrin antibodies shows that the expression of integrins is confined to the basal layer of the epidermis only. In the more differentiated layers above the basal layer, integrin expression is downregulated, so that the cells have less adhesiveness to the basement membrane components. Different combinations of integrin receptor subunits bind to different extracellular matrix molecules, as shown in table 1.8.

When epidermal keratinocytes are fractionated into populations on the basis of their level of integrin expression, the highest level of integrin expression correlates with the highest number of colonies formed in culture. Similarly, cells with the highest integrin expression appear to be most "sticky", attaching to the substrate most quickly. This appears to be dependent on the integrin β subunit. Stem cell characteristics in the epidermis therefore appear to include a high level of β1 integrin expression, and properties of rapid adhesion. *In*

vivo, there is a correlation between areas of high integrin expression in the basal epidermis and high γ-catenin expression, coupled with low expression of E-CAD and β-catenin. Catenins are known to mediate intercellular adhesion, linking cadherins with the intracellular actin cytoskeleton (Kemler 1993), and it has been shown that β-catenin is able to interact with LEF-1 (a HMG box protein essential for correct tooth and hair patterning and development *in vivo*, see chapter 2) to generate a functional transcription factor complex (Behrens *et al* 1996, van de Wetering *et al* 1997).

Table 1.8: Integrin receptor subunits and their ligands.

INTEGRIN RECEPTOR	LIGAND
α2β1	collagens
α3β1	laminins
α5β1	fibronectin

To investigate the role of integrins in skin, a transgenic mouse was produced in which human integrins were fused to the involucrin promoter (involucrin is a differentiation marker, being expressed in the more differentiated layers of skin.). This system was used to express functional human integrin subunits α2, α5 and β1 in the suprabasal epidermis of transgenic mice (Carroll *et al* 1995). Integrins normally only appear in the suprabasal layers of skin during wound healing and in psoriasis (Reviewed by Watt and Hertle 1994). Such expression is correlated with abnormal differentiation and keratinocyte hyperproliferation. In transgenic mice expressing the β1 integrin subunit suprabasally, a psoriatic phenotype was observed. This was thought to be due to the effect of the abnormal integrin expression on control of keratinocyte behaviour (Carroll *et al* 1995). The earliest changes observed in these mice were in the basal layer of the epidermis, where the transgene is not expressed. It may be that the presence of integrins in the differentiating cell layers stimulates proliferation in the basal layer, resulting in a hyperproliferative phenotype, with aberrant release of cytokines associated with this. Suprabasal integrin expression is now clearly established as a major cause of abnormal keratinocyte behaviour.

A study by Bagutti *et al* (1996) investigated the role of β1 integrins in the embryonic development of keratinocytes by studying the differentiation of wild type and β1-null ES cells in culture. Wild type ES cells were able to differentiate *in vitro* into epithelial-type cells expressing high levels of markers of simple and stratified epithelia, and low levels of keratinocyte differentiation markers (K10 and involucrin). The β1 null cells were able to express simple epithelial keratins, but keratinocyte differentiation markers were detected only occasionally and transiently. The assembly of ECM was also greatly reduced in the β1-null cultures. It would therefore appear that the capacity of β1-null cells to differentiate into keratinocytes *in vitro* is severely impaired. However, subcutaneous implantation of β1-null cells into wild type mice showed that the β1-null cells were able to express markers of stratified epithelia and keratinocyte differentiation, with normal levels of ECM components present in the cysts formed. It was therefore suggested that in the presence of a basement membrane *in vivo*, α6β4 integrin may be able to substitute for the β1 containing integrins in control of terminal differentiation. A requirement for functional integrins for ECM assembly has been established in other models (Wu *et al* 1995, Wennerberg *et al* 1996), and it is known that both dermal and epidermal cells contribute to the formation of the basement membrane. It was therefore suggested that the formation of a basement membrane in β1-null cysts *in vivo* may be due to the contribution of wild type cells from the surrounding tissue. This may suggest that the failure of differentiation *in vitro* is due to an absence of ECM, rather than being directly attributable to the absence of β1 integrin.

1:3.2 Hair Follicle Epidermal Stem Cells

Two competing theories exist regarding the location of the follicular epidermal stem cells. Bearing in mind the general characteristics of adult stem cells (table 1.6), two populations of follicular epidermal cells could conceivably represent stem cell populations. These are located at the base of the end bulb around the dermal papilla (the germinative epidermis), and in the upper outer root sheath (the bulge). Many arguments can be found in the literature supporting each of the putative follicular epidermal stem cell populations. Some of these are summarised in table 1.9.

Table 1.9: Some of the arguments "for" and "against" each of the putative follicular epidermal stem cell populations.

ORS bulge cells	End bulb GE cells
Present through all stages of the pelage follicle cycle, but no obvious bulge present in vibrissa follicles.	Present through all stages of the vibrissa follicle cycle, but not obvious during telogen of pelage follicles.
Cells remain in the hair follicle after plucking.	Cells remain in the hair follicle after plucking.
Cells relatively undifferentiated.	Cells very undifferentiated.
Cells shown to be slow cycling *in vivo*, with a burst of proliferative activity in early anagen.	Cells cycle at widely variable rate throughout the hair cycle *in vivo*, with most activity in early anagen.
Cells form adherent colonies when isolated and cultured *in vitro*.	Cells remain viable but do not form colonies when isolated and cultured *in vitro*.
Cells do not appear to form a basement membrane when cocultured with dermal papilla cells.	Cells form organotypic structures and a thick basement membrane when cocultured with dermal papilla cells.
Cells may interact with the dermal papilla during pelage follicle regression in telogen.	Cells interact with the dermal papilla during anagen and catagen in vibrissa and pelage follicles.
Regeneration of lower follicle and continued hair growth occurs after amputation of the end bulb.	Continued hair growth can occur from implanted end bulbs.

Clearly, both arguments have their merits, but it is currently impossible to give a definitive answer to the "stem cell" question. It is possible that both of these epidermal populations have differing functions depending on follicle type, or that there are two distinct epidermal stem cell types, each responsible for a different set of differentiation products. It is also possible that the two cell types represent the stem cell and a transit amplifying population.

1:3.2-1 Hair Follicle Epidermal Stem Cells 1 - The Germinative Epidermis

Originally, it was presumed that the follicular epidermal stem cells are present at the base of the end bulb (Van Scott *et al* 1963), since the matrix cells are known to differentiate into several different cell types, are highly proliferative during anagen, and are involved in

interactions with the dermal papilla which may regulate hair growth. The hair matrix consists of some epidermal cells that are committed to hair type differentiation, and others that are apparently undifferentiated. These are known as germinative epidermal (GE) cells, and are a biochemically distinct population which can be consistently isolated by microdissection (Reynolds and Jahoda 1991b). These cells have long been considered to be an epidermal stem cell population with a pluripotential nature, since they give rise to several cell lines as they differentiate, including hair medulla, cortex, cuticles, Huxley's and Henle's layers, and it has generally been assumed that GE cells play a central role in follicle growth and differentiation through their interactions with the dermal papilla.

The fact that GE cells appear to be replaced during the growth cycle of many follicles has led to the suggestion that they must have limited replicative potential (Cotsarelis *et al* 1990, Lavker *et al* 1991). However, they show immense capacity for division, with an estimated lifetime of 1000 divisions in human follicles (Lane *et al* 1991). In animals whose follicles grow continuously without an obvious pause, the GE cells may be immortal for the lifetime of the individual. Merino sheep, poodle dogs and angora rabbits are all believed to be cases in point. In catagen rat vibrissa follicles, a small but distinct group of epidermal cells persist at the base of the papilla when the rest of the matrix is lost (Reynolds and Jahoda 1993, and see figure 1.4), suggesting that the GE population persists throughout the life of the vibrissa follicle.

1:3.2-2 Hair Follicle Epidermal Stem Cells 2 - The Bulge Hypothesis

A study by Cotsarelis *et al* (1990) put forward evidence to suggest that a population of basal epidermal cells in the ORS bulge are the follicular epidermal stem cells. In mouse pelage follicles, the bulge region consists of a subpopulation of outer root sheath keratinocytes located in the midportion of the follicle at the arrector pili muscle attachment site.

Cotsarelis *et al* used newborn mice in a tritiated thymidine labelling study, to determine where label retaining cells were located in the hair follicle. During the first seven days of postnatal life, new hair follicles develop and the existing follicular cell population expands. It was therefore possible, by twice daily subcutaneous injection of tritiated thymidine

over the first seven days after birth, to radioactively label almost all nuclei in the epidermis and follicles. This was followed by a four week chase period, during which no further tritiated thymidine was injected. It was found that radioactive labelling disappeared from the matrix cells of the end bulb after as little as 1 week, showing that these cells must be rapidly dividing. Furthermore, it was also noted that a population of cells retaining radioactive labelling was present in the follicular outer root sheath, at the site of the bulge. This experiment was repeated using adult mice, and a similar result was obtained, although fewer follicles were labelled, possibly because the bulge cells of only a few adult follicles were replicating during the labelling period. This theory was tested by stimulating cell division in the skin and follicles by topical application of TPA, which is known to stimulate DNA synthesis in these cells (Bhisey *et al* 1982). This resulted in a higher frequency of radioactive labelling in the bulge, which retained the labelling for a long period after TPA stimulation was removed, suggesting that these cells returned to their normal slow-cycling state.

The idea of a multipotent stem cell population high up in the outer root sheath is further supported by studies of cytokeratin marking in this locality (Frater and Whitmore 1973), and evidence that in many cycling follicles at least some new matrix cells may derive from the cells of the outer root sheath bulge. Ultrastructural studies of bulge ORS cells also showed that they have characteristics typical of relatively undifferentiated or "primitive" cells (Cotsarelis *et al* 1990).

A study by Rochat *et al* (1994) examined the growth capacity of keratinocytes isolated from different regions of human scalp follicles. Anagen follicles were sectioned into four fragments. The lower fragment containing the end bulb was designated P1, the lower intermediate fragment P2, the upper intermediate fragment P3 and the upper fragment containing the sebaceous gland, P4. An epidermal suspension produced from these fragments was plated onto a feeder layer of irradiated 3T3 cells to assess their growth capability. Using this assay, it was found that 85% of the colony forming cells were present in the P3 fragment containing the bulge region. Further studies were then performed to divide the P3 fragment into a lower portion, and an upper portion containing the bulge region. Interestingly, it was found that most of the colony forming cells were present in the lower P3 fragment, away from the bulge. This was in contrast to a study performed by Kobayashi *et al*

(1993), which demonstrated that almost all of the colony forming cells present in the rat vibrissa follicle are segregated in the bulge.

2: SUMMARY

The germinative epidermal cells at the base of the hair follicle are very active during the anagen phase of the hair growth cycle, dividing rapidly to give rise to several differentiated cell types. They are both functionally and anatomically integrated with the follicular dermal papilla and dermal sheath, and have certain stem cell-like characteristics. The germinative cell population is at the core of activities involving fundamental processes of cell signalling and differentiation, where the signal to "make hair" is being received and transcribed. However, in spite of its potential importance, few morphological and biochemical studies have closely examined this cell population, and to date no molecular studies have been reported which focus specifically on genes expressed in the germinative epidermis. The aim of this study is therefore to begin the molecular characterisation of the germinative epidermal cells, and to identify genes which are highly expressed in these cells.

CHAPTER 2

PRODUCTION OF HAIR FOLLICLE cDNA LIBRARIES

1: INTRODUCTION

Currently, the molecular mechanisms underpinning the growth of hair are relatively poorly understood. However, it has been reported that a wide variety of cytokines and signalling molecules are involved in the normal development of hair and skin. Many of these molecules may be important factors in the control of hair growth (Stenn *et al* 1996). (discussed further in chapter 1 and sections 1:1 and 1:2).

One of the principal reasons for the increasing awareness of the molecular complexity of hair follicles stems, at least partly, from their position and visibility on the outside of the body, so that phenotypic abnormalities are readily apparent. A number of transgenic mice strains have been developed containing gene mutations which have given rise to marked effects on hair growth and fibre characteristics (discussed in section 1:2.1). Many of the phenotypic alterations observed were unexpected, but all have served to underline the complexity of hair follicle development and the fact that multiple families of molecules are associated with this process.

1:1 A STUDY OF SPECIFIC MOLECULES KNOWN TO BE INVOLVED IN HAIR FOLLICLE GROWTH CONTROL.

The hair follicle is a very complex structure whose behaviour must be tightly regulated throughout its life. Hair follicle development, in common with all integumental appendage development, is initiated by a series of embryonic dermal-epidermal interactions (Sengel 1976) (See chapter 1). It is therefore reasonable to expect that molecules known to be involved in embryonic dermal-epidermal interactions and in the development of other appendages would also be active in hair follicle development.

Similarly, molecules known to be involved in signal transduction and proliferation and differentiation control in other systems could be expected to play similar roles in the hair follicle, fulfilling similar functions during epidermal proliferation and differentiation through the hair growth cycle. Since the cellular components of the mature follicle repeat patterns of

activity established during embryonic development, it is reasonable to suppose that common signalling pathways are involved in control of both adult and embryonic follicle behaviour.

Several methods have been used to localise the expression of specific genes to regions of the hair follicle. These include immunolocalisation methods using specific antibodies on skin and follicle sections; radioactive and non-radioactive *in situ* hybridisation on whole follicles and follicle sections; analysis of mRNA expressed in cultured cells by northern blotting; and RT-PCR analysis of total RNA isolated from whole follicles. Few methods for localising the expression of specific genes to regions of the follicle at specific stages of cycling or development have been reported, due to the limited amount of tissue available. However, RT-PCR based methods have been shown to be useful in some cases, for example, Seiberg *et al* (1995) have used RT-PCR to study the expression of several genes in skin throughout the hair cycle, Yu *et al* (1995) have used a method based on RT-PCR to identify genes expressed in cultured papilla cells, and Stelnicki *et al* (1997) have used degenerate oligonucleotide primers to study the expression of *msx-1*, *msx-2* and *mox-1* in cDNA populations amplified from adult and fetal skin.

Construction of cDNA libraries from the individual tissues of the follicle is problematic, as it is difficult to isolate enough tissue to obtain sufficient mRNA for conventional protocols. However, it is possible to construct conventional cDNA libraries from whole follicles or portions of the follicle containing several tissues, for example, the follicle end bulb or the region containing the bulge. These can then be screened using probes generated by PCR from known mRNA sequences, or using cDNA clones isolated from other species or tissues. Probes used in library screening may be very specific; for example, a full length murine *BMP-4* cDNA clone has been used to isolate a rat *BMP-4* clone from a hair follicle end bulb cDNA library (Sleeman 1995), and a human hair keratin probe has been used to isolate the equivalent murine keratin and a novel related keratin gene from a mouse genomic library (Rogers *et al* 1996); or more redundant, for example, short PCR generated probes have been used to isolate cDNA clones encoding *msx*-homeobox containing clones from a follicle end bulb cDNA library (sections 2:4.1, 3:1.1 and 4:2) and a 32-mer oligonucleotide probe has been used to isolate a novel hair specific cDNA clone from a mouse skin cDNA library (Huh *et al* 1994, section 1:2.2). Short or redundant probes homologous to a conserved region common to several genes (either members of a gene family or otherwise unrelated genes sharing a

common domain) may be used to isolate related clones. Recently, several methods have been described for the construction of PCR generated cDNA libraries from very small amounts of tissue (Belyavsky *et al* 1989, Gurr and McPherson 1991) and commercial kits are now available for this purpose. This may allow the screening of tissue specific PCR generated libraries to become a useful tool in identifying genes expressed in particular tissues of the follicle. Another method which may also be useful with this aim in mind was described recently (Brady and Iscove 1993, Trumper *et al* 1993, Brady *et al* 1995). This involves a study of gene expression in single cells using a single-cell PCR based global amplification technique. This technique may therefore be used to distinguish between follicular epidermal cell types at different stages of differentiation, using absolutely minimal amounts of tissue.

1:1.1 Some Gene Families Expressed in the Adult and Embryonic Hair Follicle

Some of the molecules which have been localised to adult and embryonic hair follicles are briefly discussed below. Many of these molecules appear in other integumental appendages in similar patterns during embryonic development and adult life.

1:1.1-1 Structural Molecules

The structural molecules making up the hair follicle can be divided into two major groups; the hair specific keratins and their associated proteins, and the extracellular matrix (ECM) proteins. The hair specific keratins and associated proteins are expressed in the follicular epidermis and make up the hair fibre itself, with other keratins and associated proteins expressed in the other epidermal tissues of the follicle. The ECM proteins are associated with both the dermis and epidermis, and form a basement membrane separating the dermal and epidermal compartments. This may be involved in processes of cell signalling and control of gene expression, as well as providing adhesion (Panayotou *et al* 1989, Carter *et al* 1990, Stepp *et al* 1990, Hynes 1992, Vidal *et al* 1995, Xia *et al* 1996, Burgeson and Christiano 1997, Dogic *et al* 1998) (section 1:1.2). Several diseases have been shown to

result from disruption of basement membrane components, for example, epidermolysis bullosa, a heritable group of disorders characterised by blistering of the skin.

Several families of protein are expressed in the growth of hair, and an estimated 50-100 proteins belonging to ten distinct families constitute the final hair fibre (Powell *et al* 1991). The reason there are so many keratin proteins carrying out the same structural function is unclear, though it is possible that since keratin synthesis occurs only in the lower third of the follicle, a high rate of keratin gene expression is required in this region to produce sufficient protein to make up the hair fibre. If the rate of transcription from individual genes is limiting, transcription from several different genes will satisfy the requirement for abundant keratin mRNA.

The hair fibre itself is made up of intermediate filaments (IF) and intermediate filament associated proteins (IFAP). Historically, the intermediate filament proteins are referred to as low-sulphur proteins, and the intermediate filament associated proteins are made up of a high-sulphur, an ultra-high sulphur and a high-glycine/tyrosine group. Expression of these genes is carefully regulated. Intermediate filament mRNA expression first appears in the matrix cells of the upper bulb region, followed by the expression of the glycine/tyrosine rich IFAP's. Finally, the cysteine rich IFAP's are expressed, some of them very late in the differentiation of the cuticle cells. Presumably, temporally regulated expression ensures that the components of the hair fibre are available at the right times to be incorporated into the keratin superstructure. There is little information available on the detailed arrangement and bonding of the proteins making up the matrix between the intermediate filaments. However, several hair specific keratin intermediate filament proteins have been characterised, and all have relatively cysteine rich N- and C- terminal domains, allowing extensive cross linking to IFAP's (Bowden *et al* 1998). It is thought that glycine/tyrosine rich IFAPs are able to form glycine loops, structures consisting of random turns made up of a length of glycine residues, which can interact with similar structures on the intermediate filament chains. However, studies on keratin gene regulation and expression are still in their infancy (Rogers and Powell 1993, Emenet *et al* 1997, Bowden *et al* 1998, Mitsui *et al* 1998), although it is clear that genetic defects in keratin genes are responsible for a number of skin disorders (reviewed in Fuchs 1996).

Table 2.1 shows some of the important extracellular matrix proteins expressed during hair follicle initiation, morphogenesis and cycling.

Table 2.1 : Examples of some extracellular matrix molecules expressed in embryonic and adult hair follicles.

MOLECULE	EXPRESSED IN...	REFERENCES
syndecan family	-formation of dermal condensations in developing teeth and vibrissa. - cell-matrix interactions in adult tissues.	Thesleff *et al* 1987, Bernfield and Sanderson 1990, Panaretto 1993, Thesleff *et al* 1995.
midkine	- mesenchymal condensation in many organs including hair follicles and teeth.	Mitsiadis *et al* 1995a, 1995b.
tenascin	- hair follicle, tooth and feather initiation and development.	Thesleff *et al* 1987, Vainio and Thesleff 1992, Jiang and Chuong 1992, Kaplan and Holbrook 1994, Noveen *et al* 1995.
laminin family	- integumental appendage morphogenesis. - adult follicle basement membrane, dermal papilla and cultured DP cells.	Westgate *et al* 1984. Couchman 1986, Jahoda *et al* 1992a, 1992b, Rousselle *et al* 1997.
Fibronectin	-adult follicle basement membrane.	Jahoda *et al* 1992a, 1992b.
Catenins, e. g. β-catenin	- hair follicle initiation and morphogenesis.	Gat *et al* 1998.
Integrin family	- cell-matrix and cell-cell adhesion in skin and follicular basement membrane.	Carter *et al* 1990, Stepp *et al* 1990, Hynes 1992, Vidal *et al* 1995.
Cell adhesion molecules e.g. N-CAM	- mesodermal condensation and maintenance. - development of dermal papilla and dermal sheath in embryonic follicles.	Bard 1990 Kaplan and Holbrook 1994.

Extracellular materials have been implicated in a wide range of developmental activities, including the adult hair growth cycle (Couchman 1986, Couchman *et al* 1990). The roles that ECM proteins play in adult dermal-epidermal interactions is unclear, but there is evidence that the distribution of various ECM proteins changes during the hair cycle, and that ECM protein

synthesis occurs both in the epidermal and dermal components of the follicle (Yamane *et al* 1996).

1:1.1-2 Growth Factors And Growth Factor Receptors

Table 2.2 : Examples of growth factor, cytokine and receptor families expressed in embryonic and adult hair follicles.

MOLECULE FAMILY	EXPRESSED IN	REFERENCES
Fibroblast growth factors (FGF)	- fetal rodent epidermis during follicle induction and morphogenesis. - murine follicle development and cycling. -adult anagen follicles.	Gonzalez *et al* 1990, Pisansarakit *et al* 1990, 1991, Moore *et al* 1991, Du Cros 1993, Hébert *et al* 1994, Guo *et al* 1996.
FGF receptors		Peters *et al* 1992, Hébert *et al* 1994, Rosenquist and Martin 1996.
Bone morphogenetic proteins (BMPs)	- mesenchyme, prior to follicle induction. - sites of dermal epidermal interaction during organ development. - Adult follicle epidermis.	Jones *et al* 1991 Lyons *et al* 1990, Jones *et al* 1991, Vainio *et al* 1993, Takahashi and Ikeda 1996. Lyons *et al* 1989.
TGF-β and receptors	- adult follicle inner and outer root sheath, cycle stage dependent.	Wollina *et al* 1996, Paus *et al* 1997.
Insulin-like growth factors and related proteins	- adult follicle epidermis.	Liu *et al* 1993, Philpott *et al* 1994, Hodak *et al* 1996, Rudman *et al* 1997.
Retinoic acid receptors	-dermal epidermal interactions leading to initiation of appendage formation.	Hardy, 1968, 1983, Dhouailly *et al* 1980, Chuong *et al* 1992, Viallet *et al* 1993, Viallet and Dhouailly 1994.
epidermal growth factor family (EGF)	- hair follicle morphogenesis. - hair follicle cycling and differentiation.	Green and Couchman 1984, Du Cros 1993, Holbrook *et al* 1993, Piepkorn *et al* 1995, Hansen et al 1997.
Platelet derived growth factor family	- hair follicle morphogenesis. - hair follicle cycling and differentiation.	Orr-Urtreger and Lonai 1992, Holbrook *et al* 1993, Ponten *et al* 1994, Akiyama *et al* 1996.

Many growth factors and growth factor receptors which may be important in the control of hair growth have been localised in tissues of the hair follicle. For example, several members of the FGF receptor family have been localised to the hair follicle specifically during anagen (Rosenquist and Martin 1996, section 1:1.2-2); FGF-5 has been localised to the ORS in the anagen-catagen transition (Hébert *et al* 1994, section 1:1.2-2); Insulin-like growth factors have been shown to be important in hair follicle growth and cycling (section 1:1.2-2), transforming growth factor -β and its receptors have been localised to regions of the adult hair follicle during cycling (Wollina *et al* 1996, section 1:1.2-2), and several other factors have been localised to hair follicle epidermis during human follicle development (Akiyama *et al* 1996) and cycling (Mitsui *et al* 1997). Some of these molecules are listed in table 2.2, and discussed further in sections 1:1 and 1:2.

1:1.1-3 Transcription Factors and Signalling Molecules

Transcriptional regulatory proteins control many developmental processes. For example, DNA binding proteins expressed in specific cell lineages, or at specific stages of differentiation, participate in the developmental regulation of gene expression (examples in Gruss and Walther 1992, Voss and Rosenfeld 1992, Weintraub 1993). Although some transcriptional regulators are restricted to a single cell type, many are expressed by multiple lineages with unrelated phenotypes at different stages of development, suggesting that transcriptional regulators can serve multiple functions through combinations with other proteins in different cell types. There are many different classes of transcriptional regulator, grouped according to structural similarities. These include homeobox containing genes, HMG (high mobility group) box proteins, and other factors.

HMG proteins are transcriptional regulators containing a conserved 75 amino acid DNA binding sequence known as the HMG box. HMG boxes are highly structured and facilitate DNA/protein interactions, binding to both single and double stranded DNA. The classification and functional relationships of HMG box protein family members has recently been reviewed (Baxevanis and Landsman 1995).

Homeobox genes are transcription factors containing a 183bp conserved DNA sequence called the homeobox (reviewed in Krumlauf 1994). They were first discovered in *Drosophila*, where they were found to control pattern formation and segment identity during insect development (Nüsslein-Volhard and Wieschaus 1980). Vertebrate homeobox containing genes are categorised into different classes, among which are *hox* complexes and the *msx* family. The role of homeobox containing genes in skin is discussed further in sections 1:1 and 1:2 (reviewed by Scott and Goldsmith 1993).

Some of the regulatory molecules expressed in during follicle initiation, embryonic development and adult follicle cycling are listed in table 2.3 and discussed in greater detail in sections 1:1 and 1:2.

Table 2.3 : Some transcriptional regulators expressed in embryonic and adult hair follicles

MOLECULE	EXPRESSED IN	REFERENCES
Lymphoid enhancer factor -1 (LEF-1)	- patterning, initiation and morphogenesis of vibrissa follicles. - adult follicle germinative epidermis and matrix.	Van Genderen *et al* 1994, Zhou *et al* 1995. Zhou *et al* 1995.
Sonic Hedgehog (*shh*)	- epithelial thickening of the tooth and placode of hair at earliest stages of development. - possible role in angling of follicles.	Bitgood and McMahon 1995 Gat *et al* 1998
Homeobox family (*hox*)	- hair follicle morphogenesis	Bieberich *et al* 1991, Kanzler *et al* 1994, Noveen *et al* 1995, Reginelli *et al* 1995, Stelnicki *et al* 1998.
msx genes	- epithelial placode in embryonic feather and tooth development - other organs involving epithelial-mesenchymal interactions	Robert *et al* 1989, MacKenzie *et al* 1991a, 1991b, 1992, Monaghan *et al* 1991, Noveen *et al* 1995, Stelnicki *et al* 1997.
Winged-helix nude (*whn*)	- developing follicle epithelium	Brissette *et al* 1996
hairless (*hr*)	- hair follicle development and cycling	Cachon-Gonzalez *et al* 1994, Ahmad *et al* 1998, Panteleyev 1998a, 1998b.

1:1.2 Spatial and Temporal Expression Patterns of Specific Molecules Throughout Follicle Development and Cycling.

Many of the molecules expressed during embryonic hair follicle patterning, initiation and morphogenesis are common to the same processes in other integumental appendages. Furthermore, the spatial expression of any particular molecule may vary widely in a single organ at different stages of development and cycling. In order to gain a clearer understanding of which molecules are interacting at any particular time, it is useful to classify the "life" of a hair follicle into different stages, and follow the expression of particular molecules through these stages, rather than studying the expression of any particular molecule as a whole.

1:1.2-1 Embryonic Development

1:1.2-1.1 Follicle Patterning and Initiation

Molecules which are currently implicated in patterning and initiation of hair follicle development are almost all common to the development of other integumental appendages.

One of the first molecules to be expressed at the sites of future hair follicle development is the HMG box containing transcription factor, lymphoid enhancer factor - 1 (LEF-1). The *LEF*-1 gene was initially cloned as a pre-B and -T lymphoid specific gene encoding a DNA binding protein containing a HMG domain (Travis *et al* 1991, Waterman *et al* 1991). In the adult mouse, *LEF*-1 was thought to be expressed specifically in lymphoid tissues (Travis *et al* 1991, Waterman *et al* 1991). However, a wider pattern of *LEF-1* mRNA expression was detected during embryogenesis using *in situ* hybridisation analysis (Oosterwegel *et al* 1993). Van Genderen *et al* (1994) observed that *LEF*-1 expression occurred at sites of epithelial-mesenchymal interactions where appendages were forming. It was therefore concluded that *LEF*-1 expression is essential for the development of several structures and organs involving inductive tissue interactions. A study of *LEF-1* mRNA

expression using *in situ* hybridisation on embryonic mouse skin sections showed that *LEF-1* mRNA is first expressed in the epidermis overlying the future sites of mesenchymal condensation with specific timing and patterning (Zhou *et al* 1995). This up regulation and patterning of *LEF*-1 expression represents one of the earliest biochemical changes known to occur in the epidermis. However, a study by Kratochwil *et al* (1996) demonstrated that *LEF*-1 expression in the epidermis is activated by BMP-4 during tooth and hair development. LEF-1 has been shown to be essential for correct tooth and hair patterning and development *in vivo* (van Genderen *et al* 1994, Zhou *et al* 1995). This is discussed further in section 1:2.1-3. It has also been shown that LEF-1 can interact with β-catenin to generate a functional transcription factor complex (van de Wetering *et al* 1997), which may be involved in *Wnt* signalling (Clevers and van de Wetering 1997). This is particularly interesting in view of the recent report by Gat *et al* (1998) that β-catenin can induce *de novo* follicle morphogenesis. This is discussed further in section 1:2.1-1.2.

The homeobox containing gene family *msx* has been shown to be important in development of organs involving epithelial-mesenchymal interactions (Robert *et al* 1989, Monaghan *et al* 1991, MacKenzie *et al* 1991b, 1992, Vastardis *et al* 1996). A spatial and temporal study of *msx-1* expression in mouse craniofacial development suggested that it may be important in development of a number of craniofacial structures (MacKenzie *et al* 1991a). A study by Noveen *et al* (1995) using whole mount *in situ* hybridisation showed that *msx-1* and -2 are early markers of epithelial placode development in embryonic chicken skin, appearing as early as E 7, specifically in placode epithelium, prior to morphological changes. *Msx*-2 is expressed in the mesoderm of developing chick feather buds up to age 9dpc, and in the epidermis from day 10, suggesting that *msx* genes are among the very early molecules required during skin appendage induction. A similar study of human fetal skin cDNA populations showed *msx*-1 and -2 are expressed in developing human follicles (Stelnicki *et al* 1997), and a study by Vastardis *et al* (1996) demonstrated that msx-1 is important for normal human tooth development. However, in common with *LEF*-1, epithelial expression of *msx*-1 is activated by BMP-4 (Vainio *et al* 1993), suggesting that mesenchymal BMP-4 is crucial in control of follicle patterning.

It has also been suggested that *msx* gene transcription may be regulated by retinoic acid, either directly, or through the action of cellular retinoic acid receptors (Shen *et al* 1994, Brown *et al* 1997, Ahuja *et al* 1997). Retinoic acid receptors α, β and γ have long been implicated in control of cell fate during appendage development (Hardy 1968, 1983, Dhouailly *et al* 1980). Retinoids are known to be teratogenic for vertebrate embryos, with effects on many structures at several stages of development (Hale 1933, Shenefelt 1972). Ectopic retinoic acid (RA) treatment has been shown to alter cell fate, for example, induction of feathers on normally scaled chick foot skin; induction of glomerular glands instead of vibrissae in mouse upper lip skin (Viallet and Dhouailly 1994); ectopic expression of *Hox-c* genes and associated morphological defects in mouse embryos (Conlon and Rossant 1992). All vertebrate embryos contain endogenous RA and several retinoic acid receptors, which co-operate to transduce RA signals. A study by Shen *et al* (1994) demonstrated that *msx*-1 transcription was activated by ectopic RA in a time and dose dependant manner in cultured embryonal carcinoma cells. Conversely, Brown *et al* (1997) showed that application of ectopic retinoic acid to embryonic chick facial structures inhibited upper beak outgrowth and strongly downregulated expression of *msx*-1 and -2. These studies therefore suggest that the *msx* gene products mediate the effects of retinoids, which may either up- or down-regulate *msx* gene transcription through regulatory elements in the *msx* genes (Shen *et al* 1994).

A third early molecule whose epithelial expression overlies areas of *BMP*-4 expression in the mesenchyme is sonic hedgehog (Shh), a secreted peptide related to the *Drosophila* hedgehog (hh) protein. In *Drosophila*, hh regulates many aspects of embryonic and adult patterning (Perrimon 1995). The *Drosophila hh* gene encodes a protein which is cleaved to produce a 19kDa amino terminal peptide, and a 25kDa carboxy terminal peptide (Lee *et al* 1994), with all the signalling activity residing in the 19kDa fragment (Marti *et al* 1995, Porter *et al* 1995). Studies involving hh and shh suggest that this molecule is involved in both short- and long-range induction and patterning of developing organs such as the central nervous system (CNS), somites, limbs and digits (Riddle *et al* 1993, Roelink *et al* 1994, Marti *et al* 1995). Ectopic expression of *shh* in anterior limb mesenchyme leads to the induction of ectopic expression of *BMP*-2 (Laufer *et al* 1994), which is related to *Drosophila decapentaplegic* (*dpp*). In *Drosophila*, *dpp* expression is activated by hh, and can mediate

many of the patterning activities ascribed to hh (Ingham and Fietz 1995). This suggests that there may be a conserved role for hh and BMP/dpp interactions in embryonic patterning and development.

A study by Bitgood and McMahon (1995) showed that expression of *shh* in the epidermis of developing appendages including whiskers, hairs and teeth correlated with mesenchymal expression of *BMP*-4 in embryonic mice. Furthermore, in other organs such as gut, bladder, lung and cartilage, mouse hedgehog genes were coexpressed with BMP's -2, -4 and -6. The BMP's may therefore be general targets of hedgehog signalling in vertebrate development, and epithelial-mesenchymal interactions may be mediated in part by a signalling loop between hh and BMP's in adjacent cell populations. It has also been suggested that *Shh* may have a role in follicle angling, since disruption of the normal polarised *Shh* expression pattern in hair follicles expressing a mutant β-catenin resulted in misangling of those follicles (Gat *et al* 1998).

In mouse whisker follicle development, *BMP*-4 expression first occurs at 11.5dpc in the mesenchyme (Jones *et al* 1991). By 13.5dpc, its expression is notably highest in regions of condensing mesenchyme subjacent to the epithelial placode, the site of development of the whisker follicles. At this stage, *BMP*-2 expression is localised to the epidermal placode. *BMP*-3 and *BMP*-7 are also expressed in the developing hair follicle (Takahashi and Ikeda 1996). The function of BMP's within the developing whisker follicle is unclear, particularly since BMP's have a variety of functions depending on cell type. However, in a study by Vainio *et al* (1993), BMP-4 was shown to induce the expression of the transcription factors *Egr*-1, *msx*-1 and *msx*-2 in condensing dental mesenchyme, suggesting that BMP-4 acts as an inductive signal between tissue layers in developing skin appendages. Furthermore, a study by Kratochwil *et al* (1996) demonstrated that BMP-4 could induce *LEF*-1 expression.

The transcription factors which are expressed during the earliest stages of follicle initiation have been implicated in controlling the expression of various structural molecules involved in hair follicle induction. For example, the expression of tenascin in the developing follicle first appears at the dermal-epidermal interface prior to hair follicle initiation. Tenascin expression is common to the early stages of hair follicle (Kaplan and Holbrook 1994), tooth

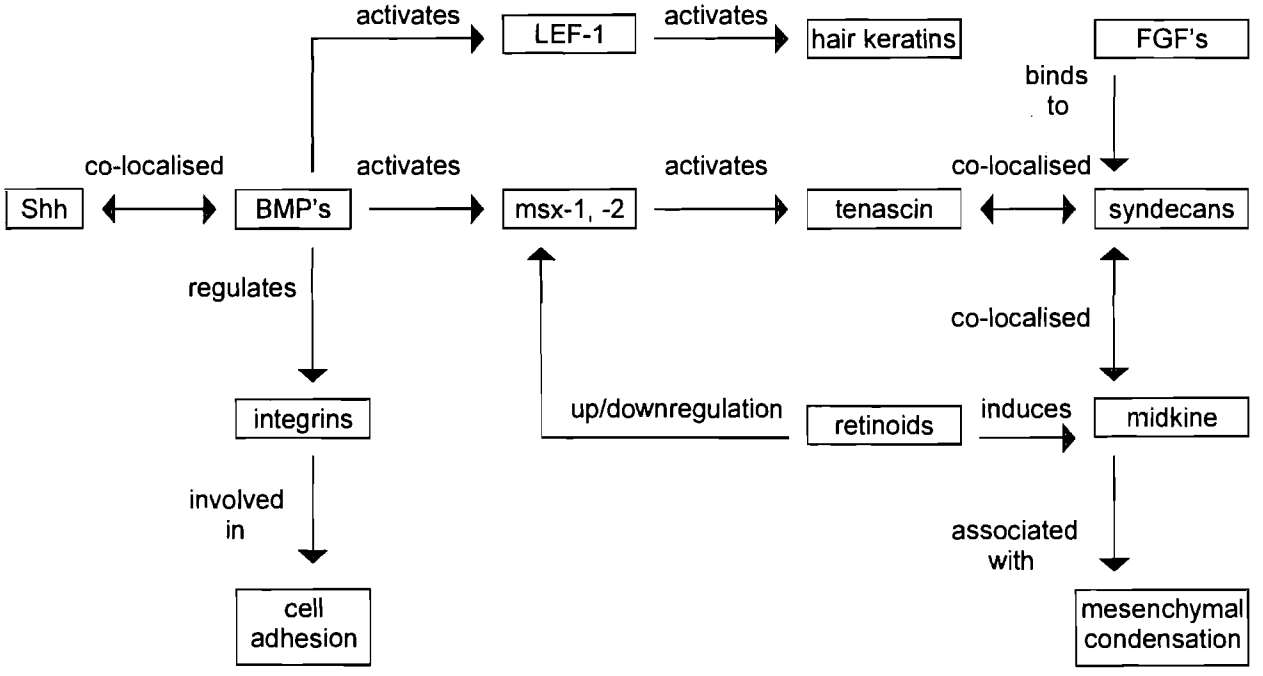
(Thesleff *et al* 1987, Vainio and Thesleff 1992), and feather (Jiang and Chuong 1992, Noveen *et al* 1995) development. In developing feathers, tenascin expression coincides so closely with *msx*-2 expression that it is likely that the expression of tenascin is regulated by msx-2. Syndecan-1 is co-expressed with tenascin at sites of vibrissa follicle development (Panaretto 1993), and is also localised in mesenchymal condensations of developing teeth (Thesleff *et al* 1987). Syndecan-3 has been shown to be transiently expressed in the mesenchyme of developing chick feather buds at around E12 (Gould *et al* 1995). Syndecans have also been shown to bind extracellular matrix molecules and growth factors including members of the fibroblast growth factor family (Kan *et al* 1993) through heparan sulfate groups bound to their extracellular domains, and are involved in cell-matrix interactions (Bernfield and Sanderson 1990). Syndecans may therefore function as low affinity receptors for members of the FGF family, and may be involved in maintenance of dermal condensations (reviewed in Thesleff *et al* 1995). Other proteoglycans (eg heparan- and chondroitin- sulfate proteoglycans) may also play a critical role in control of basement membrane permeability and tissue morphogenesis and differentiation (Couchman *et al* 1990). One such molecule is midkine, a heparin binding molecule induced by retinoic acid. Midkine is associated with mesenchymal condensation in many organs including hair follicles and teeth (Mitsiadis *et al* 1995a), and its expression is co-localised with syndecan-1 (Mitsiadis *et al* 1995b).

Fibroblast growth factor (FGF) and its receptors have long been implicated in the control of epidermal and mesenchymal cell function (Gonzalez *et al* 1990, Orr-Urtreger *et al* 1991, Moore *et al* 1991). It was originally recognised for its mitogenicity on epidermal and mesenchymal cells, but now also appears to be involved in cellular regulation of mature skin and its embryonic development. In fetal rodent skin, basic FGF (FGF-2) is primarily associated with the epidermis (Gonzalez *et al* 1990) and may be acting as a signalling molecule for cell migration and proliferation in the early stages of follicle induction (Moore *et al* 1991). Both acidic (FGF-1) and basic FGF (FGF-2) may act as autocrine and paracrine factors as their receptors are found on both epidermal and mesenchymal structures in skin. Follicle initiation is dependent on interactions between epidermal keratinocytes and dermal cells of the mesenchymal condensation. FGF-2 is expressed by keratinocytes and is known to be a potent mitogen for mesenchymal cells (Pisansarakit *et al* 1990, 1991). It could therefore be important

in the interactions initiating follicle morphogenesis. It is reasonable to speculate that saturation of FGF receptors in the mesenchymal condensations by exogenous application of FGF-2 may block cellular communication and interfere with cell migration and aggregation, thus delaying initiation.

The regulation and interaction of the molecular determinants of follicle patterning and initiation are clearly very complex. The expression of any particular molecule during this stage may be controlled or affected by the expression of one or several others (summarised in figure 2.1). Since all of the molecules described in this section appear in the development of a variety of other organs or appendages, it is difficult to speculate exactly what determines the characteristics of a developing appendage prior to its appearance and during the earliest stages of its morphogenesis. The "hair specific" signal is unlikely to be the result of a single gene product, instead resulting from the tightly controlled spatial and temporal expression patterns of the combination of molecules described here, molecules which have not yet been implicated in follicle patterning, and novel gene products.

Figure 2.1: The relationships between some of the molecules implicated in follicle patterning and initiation.



1:1.2-1.2 Follicle Morphogenesis

Following patterning and initiation, follicle morphogenesis proceeds, with invagination, proliferation and differentiation of the follicular epidermis. The morphological processes involved are described in chapter 1.

During the early stages of follicle morphogenesis, many of the molecules implicated in patterning and initiation continue to be expressed in particular compartments of the developing follicle. As differentiation proceeds, expression of hair specific keratins is initiated to produce the hair fibre. Zhou *et al* (1995) demonstrated that 13 out of 13 published hair keratin promoters contained consensus LEF-1 binding sites, suggesting that LEF-1 may be an important transcription factor involved in the expression of hair specific keratins. Van Genderen *et al* (1994) studied the expression of *LEF-1* mRNA in developing mouse embryos between E12.5 and E16.5 using *in situ* hybridisation. They found that the developing whisker follicles express high levels of *LEF-1* mRNA at E14.5, and at E16.5, when *LEF*-1 expression in other sites is markedly reduced and hair specific keratin synthesis is initiated.

As follicle development progresses, the expression pattern of BMP's becomes more restricted, for example, *BMP*-2 expression is confined to the epidermal matrix adjacent to the dermal papilla during later stages of follicle development.

Transforming growth factor-α (TGF-α), epidermal growth factor (EGF) and its receptor, platelet derived growth factor (PDGF) and its receptors and nerve growth factor receptor (p75) have all been localised in developing human hair follicles by Akiyama *et al* (1996). EGF, EGF-R and TGF-α were apparent in the developing bulge and inner root sheath, with TGF-α staining particularly apparent in the matrix cells of the follicle bulb. PDGF and p75 staining was observed in many follicular epidermal cell populations, particularly in the bulge.

The extracellular matrix components and adhesion molecules which first appeared at the dermal-epidermal junction during initiation are major basement membrane components during follicle development. Laminins, particularly laminin V, are omnipresent components of

the basement membrane of developing integumental appendages such as hairs (Westgate *et al* 1984). A study by Jahoda *et al* (1992b) showed that laminin was present in the follicular basement membrane using an immunolocalisation method.

Tenascin expression is apparent in the dermal sheath as well as the basement membrane during the later stages of follicle development (Kaplan and Holbrook 1994). Another glycoprotein appearing in the follicular basement membrane during development is clusterin. Clusterin is a sulphated glycoprotein that has been shown to be involved in many processes, including apoptosis (Buttyan *et al* 1989). Clusterin has been shown to be expressed in the embryonic whisker follicle from 14.5dpc (French *et al* 1993), and it has been proposed that expression of clusterin is involved in the morphogenesis and maintenance of the complex organised structure of the hair follicle, by maintaining cell-cell interactions between the IRS and both the hair shaft and ORS (Seiberg and Marthinuss 1995). Cell adhesion molecules are obvious candidates for maintenance of mesenchymal cell aggregations formed during follicle initiation. In hair development, the neural cell adhesion molecule (N-CAM) was localised to the dermal papilla and dermal sheath of developing follicles (Kaplan and Holbrook 1994). It was therefore proposed that cell adhesion molecules are a major influence in mesodermal condensation formation and maintenance (Bard 1990).

As follicle morphogenesis proceeds, epidermal differentiation products are synthesised to produce the hair fibre itself. Regulation of the expression of hair specific keratins is very complex (section 1:1.1-1), and is not discussed in detail here (reviewed by Rogers and Powell 1993).

1:1.2-2 The Adult Follicle Cycle

During the adult hair growth cycle, major changes occur in follicle morphology and biochemistry. The growth cycle is divided into three stages as described in chapter 1.

1:1.2-2.1 Anagen

During the active anagen phase of the hair growth cycle, structural proteins are highly expressed. Apart from the co-ordinated expression of the hair keratin genes, extracellular matrix proteins are also abundant in the basement membrane and especially in the ECM of the dermal papilla. A study by Jahoda *et al* (1992b) showed that laminin was present in the adult follicular basement membrane. However, the presence of laminin staining in the adult dermal papilla indicated that the dermal papilla cells were synthesising this basement membrane component. Cultured vibrissa papillae have been shown to produce laminin (Couchman 1986), and it has been evident for some time that mesenchymal cells may produce basement membrane components, but the level of laminin mRNA has been shown to be relatively low in cultured human skin fibroblasts compared with the level of expression of other basement membrane components (Olsen *et al* 1988). This suggests that the large amount of laminin present in anagen vibrissa papillae must have some functional significance. It has been shown that epidermal growth factor-like sequences in laminin can give rise to growth factor activity (Panayotou *et al* 1989), and it is possible that DP produced laminin may have a growth factor function in follicle activity. Laminin V in particular is produced by human epidermal keratinocytes, and acts to inhibit keratinocyte migration *in vitro* (O'Toole *et al* 1997). The attachment of keratinocytes to anchoring fibrils of type VII collagen via the laminin V network is mediated by integrins, particularly hemidesmosomal $\alpha 6\beta 4$ and interhemidesmosomal $\alpha 3\beta 1$ (Xia *et al* 1996, Rousselle *et al* 1997), and it appears that detachment of basal keratinocytes from the basement membrane signals initiation of the epidermal differentiation cascade in interfollicular skin. It has recently been suggested that tissue specific expression of laminin isoforms may modulate cell behaviour by the activation of distinct sets of integrins, and by the induction of distinct molecular assemblies in cell adhesion signalling complexes (Dogic *et al* 1998). Mutations in various subunits of laminin V have been discovered in various forms of epidermolysis bullosa (Pulkkinen *et al* 1994a, 1994b).

Jahoda *et al* (1992b) showed that changes in fibronectin distribution occurred during the rat vibrissa follicle hair growth cycle using an anti-fibronectin antibody. Levels of fibronectin around the basement membrane zone were shown to be highly heterogeneous, with strong

fibronectin labelling at the junction between the DP and follicular epidermis, and weak labelling at the DS-epidermal junction. This is the region of basement membrane surrounding the GE cells where much of the regulation of epithelial proliferation and differentiation takes place, and the cells within this area are under the influence of two distinct dermal populations; the dermal papilla and the dermal sheath. Fibronectin is associated with cell adhesion and migration, and has been shown to enhance skin epidermal cell attachment (Clark *et al* 1985).

Hemidesmosome complexes are known to play an important role in keratinocyte-ECM adherence in normal skin, and are involved in normal anchorage of keratin intermediate filaments to the basal keratinocyte membrane. The five hemidesmosomal plaque proteins which have so far been biochemically identified, termed HD1-HD5, are all products of the basal keratinocyte. HD1 is the molecule plectin, HD2 and HD4 are identical to bullous pemphigoid antigens (BPAG) 1 and 2, and HD3 and HD5 are the β4 and α6 integrin subunits (reviewed in Burgeson and Christiano 1997). Plectin is located at the inner hemidesmosomal plaque, and it plays an important role in hemidesmosome-intermediate filament attachment. It can also associate directly with the hemidesmosomal integrin α6β4 (Hieda *et al* 1992, McLean *et al* 1996, Uitto *et al* 1996). BPAG1 is a 230kDa protein also located at the inner hemidesmosomal plaque, and is involved in cell-matrix adhesion (Stanley *et al* 1988, Sawamura *et al* 1991, Guo *et al* 1995). BPAG2 is a 180kDa transmembrane protein with an extracellular collagenous domain. Since this protein consists of a series of collagen like repeats at its C-terminus, it has been classified as type XVII collagen (Giudice *et al* 1992). Mutations in this protein can cause abnormal tooth development (McGrath *et al* 1996), but the exact function of BPAG2 is not clear. Integrin α6β4 is located exclusively within hemidesmosomes and is critical for their assembly in adult epithelia (Carter *et al* 1990, Stepp *et al* 1990, Hynes 1992, Vidal *et al* 1995, Georges-Labouesse *et al* 1996, Van der Neut *et al* 1996).

Many of the growth factors and signalling molecules expressed in follicle development are also expressed during the adult cycle. Throughout adulthood, *BMP*-2 expression persists in the epidermal cortex and inner root sheath (Lyons *et al* 1989). Though the function of BMP-2 and BMP-4 within the developing and adult whisker follicle remains unclear, it is possible

that BMPs are involved in cell motility and/or extracellular matrix production and cell adhesion in adult follicles (section 1:1.2-1, section 4:4).

Zhou *et al* (1995) showed that *LEF-1* mRNA transcripts are present in total RNA isolated from cultured adult human skin keratinocytes and plucked adult hair follicles, and that both *LEF-1* mRNA and protein are present in the skin of new-born mice. A study of *LEF-1* mRNA expression using *in situ* hybridisation on adult skin sections showed that *LEF-1* mRNA is expressed in the GE and matrix cells of anagen follicles, prior to the expression of hair specific keratins.

Members of the FGF and FGF-receptor families have been localised to regions of the adult murine hair follicle by *in situ* hybridisation. FGF-5 was shown to be present only in the lower third of the outer root sheath and the germinative epidermal cells during the latest stages of anagen (Hébert *et al* 1994). FGF-receptors 1-4 were found during anagen, with FGF-R1 appearing in the dermal papilla, FGF-R2 in the epidermal matrix adjacent to the dermal papilla, FGF-R3 in pre-cuticle cells and FGF-R4 in the lower inner and outer root sheaths. FGF-3, -4, -6, -7, -8 and -9 expression was also studied, but only FGF-7 was found in the dermal papilla during anagen (Rosenquist and Martin 1996). Keratinocyte growth factor (FGF-7) has been shown to have an effect on migration and plasminogen activator activity of normal human keratinocytes *in vitro* (Tsuboi *et al* 1993). FGF-7 has a greater stimulatory effect on human keratinocytes than bFGF (FGF-2), probably because it contains a signal peptide and can be secreted readily.

Members of the FGF family have been shown to have strong affinities for heparin and heparan sulfate proteoglycan (HSPG), which may account for the localisation of bFGF to basement membrane, as this is rich in HSPG. Saturation of HSPG binding sites in FGF treated areas of the skin would therefore interfere with cell-cell or cell-matrix interactions, again inhibiting mesenchymal cell migration and aggregation at follicle initiation.

Another important family of molecules which have been shown to be involved in hair growth and cycling are the insulin-like growth factors (IGFs) and related proteins. The insulin-like growth factors 1 and 2 are structurally related hormones with distinct metabolic effects. IGF-I is a mitogenic peptide and a potent promoter of cell growth and survival, exerting its effects in multiple tissues through interaction with IGF-I receptor. IGF-I is essential for normal

hair growth and development, and may be important in regulation of the hair growth cycle (Liu *et al* 1993, Philpott *et al* 1994). IGF stimulates the proliferation of keratinocytes *in vitro* through synergistic interactions with EGF and FGF (Krane *et al* 1991). A study by Hodak *et al* (1996) using a monoclonal anti-IGF receptor I antibody localised IGF receptor I to the lower matrix cells of the end bulb and basal keratinocytes in the ORS during anagen, with an apparent correlation between cell surface IGF-I receptor expression and epidermal proliferation. A similar study by Rudman *et al* (1997) using immunocytochemistry and *in situ* hybridisation confirmed that follicular basal keratinocytes are IGF-I receptor positive and IGF-I negative. Since all known functions of IGF-I are mediated by its receptor, localisation of the receptor provides important clues for the role of IGF-I *in vivo*. Interestingly, Hodak *et al* (1996) noted that cell surface IGF-I receptor expression occurred principally in undifferentiated epithelial cells in both the epidermis and in skin appendages, and was strongly down-regulated in differentiated epithelial cells. IGF-I may therefore be acting as a morphogen as well as a mitogen in the hair follicle epidermis, since the peak of IGF receptor expression occurs in a zone between the proliferating and differentiated epidermal cells (Rudman *et al* 1997).

In many systems, IGF-I action is modulated by insulin-like growth factor binding proteins (IGFBP's). A study by Batch *et al* (1996) used *in situ* hybridisation analysis to localise mRNA expression for six IGFBP's in anagen hair follicles. *IGFBP* 3, 4 and 5 mRNA was identified in the dermal papilla, with *IGFBP* 4 mRNA particularly highly expressed in cells at the DP-epidermal matrix boundary, suggesting that it is expressed by both dermal and epidermal tissues. IGFBP 3 and 5 are known to be cell associated binding proteins which may bind to proteoglycans and matrix proteins, and it has been suggested that IGFBP 5 may potentiate the mitogenic effects of IGF-I through its association with the extracellular matrix (Jones and Watt 1993). It may be that interactions between IGFBP's 3 and 5 and matrix proteins in the dermal papilla modulate IGF-I action through the hair growth cycle, as the composition of the papilla ECM changes through the cycle.

Transforming growth factor-beta receptor type I and II expression has also been studied in adult follicles by Paus *et al* 1997. *TGF*-βRII expression levels were particularly high in the inner and outer root sheath during late anagen.

The *bcl-2* gene product is a 25kDa protein which has sequence similarity to a small group of proteins which appear to play a role in cell differentiation (Kozopas *et al* 1993). In experimental studies, it was found that overexpression of the *bcl-2* gene in lymphoid cells prevented them from undergoing apoptosis upon growth factor deprivation or glucocorticoid induction (Hockenberry *et al* 1990, Alnemri *et al* 1992). A study by Stenn *et al* (1994) to localise Bcl-2 protein in the cycling mouse pelage follicle showed that the only region of the follicle to remain Bcl-2 positive throughout the hair growth cycle was the dermal papilla. The epidermal regions of the follicle expressed *bcl-2* in the bulb, bulge and basal cells of the outer root sheath during anagen. It has been proposed that Bcl-2 could serve to maintain stem cell populations by protecting stem cells from apoptosis following post-mitotic differentiation, and it may have such a protective role in the dermal papilla.

Clusterin is a sulphated glycoprotein that has been shown to be involved in many processes, including apoptosis (Buttyan *et al* 1989). The association of clusterin expression with cell death has been demonstrated in several systems, for example, glucocorticoid induced apoptosis in thymocytes (Bettuzzi *et al* 1991), interdigital regression during development (Buttyan *et al* 1989), and in tissues whose survival is hormone dependant (Tenniswood *et al* 1992). However, lack of correlation between clusterin expression and apoptosis has been demonstrated in other systems (Garden *et al* 1991, Pearse *et al* 1992, Rosenberg *et al* 1993). A study by Seiberg and Marthinuss (1995) has localised clusterin expression in the adult follicle to the inner root sheath during anagen only. No correlation could be shown between clusterin expression and follicular regression, and clusterin could not be localised to morphologically apoptotic cells. Therefore, a role for clusterin in follicular apoptosis has been ruled out. Instead, it has been proposed that expression of clusterin within the IRS of the anagen follicle is involved in the morphogenesis and maintenance of the complex organised structure of the hair follicle, by maintaining cell-cell interactions between the IRS and both the hair shaft and ORS.

The transcription factors c-*myc*, c-*myb* and c-*jun* have previously been shown to be involved in the control of both cell proliferation and cell death. Seiberg *et al* (1995) used RT-PCR analysis of skin total RNA to demonstrate that these factors are expressed during

anagen. Similarly, retinoic acid binding proteins and retinoic acid receptors have been localised to the dermal papilla and dermal sheath of adult anagen follicles (Billoni *et al* 1997).

1:1.2-2.2 Catagen

During catagen, expression of structural molecules in the hair follicle changes dramatically. The dermal papilla is particularly rich in glycosaminoglycans (GAG's) at the end of anagen and into catagen, but the papilla ECM is progressively lost through catagen as the papilla condenses. Fibronectin, laminins and type IV collagen are present in the basement membrane of the follicle around the papilla at this stage (Jahoda *et al* 1992b). Expression of hair keratin genes stops as fibre production and epidermal proliferation are switched off.

A study by Little *et al* (1993) showed that IGF-I receptor mRNA levels decreased markedly at the onset of catagen, suggesting that IGF-I is involved in defining and restricting the location of cell proliferation in skin appendages. This is supported by the fact that follicles can be prevented from entering a catagen-like state *in vitro* by exogenous IGF-I (Philpott *et al* 1994). A study by Rudman *et al* (1997) demonstrated that IGF-I receptor expression in the dermal papilla is switched off during the transition from anagen to catagen, suggesting that IGF-I must have an important regulatory role in the hair growth cycle.

Seiberg *et al* (1995) used RT-PCR analysis of skin total RNA to demonstrate that the expression of c-*myc*, c-*myb* and c-*jun* decreases immediately before the onset of catagen. This suggests that these factors may be involved in the signalling or the initiation of catagen. TGF-β expression throughout the hair cycle was also studied by Seiberg *et al* (1995). They showed that TGF-β expression was restricted to very late anagen and early catagen, and was not found during late catagen. The timing suggests that TGF-β might play a role in the early steps of the commitment to programmed cell death, or in the induction of catagen. Overexpression of TGF-β in the epidermis of transgenic mice results in inhibition of both epidermal proliferation and hair follicle formation, suggesting that cells of the hair germ are negatively regulated by TGF-β. Studies of the expression of TGF-β and its receptor (Wollina *et al* 1996, Paus *et al* 1997) showed that TGFβ RI and RII expression was highest in late

anagen and early catagen, supporting the concept that TGF-β is involved in the process of catagen induction.

The expression of TNF-β in cycling hair follicles is increased during catagen (Seiberg *et al* 1995). The timing of its expression suggests that it could play an active role in the catagen pathway of programmed cell death. This study suggests that TNF-β exerts its effects in skin via binding to TNF receptor I, and that mRNA levels of the ligand might be important for follicular regression.

1:1.2-2.3 Telogen

Telogen is a quiescent state in which cell division and differentiation are switched off. Changes in the composition of the rat vibrissa papilla ECM and basement membrane during telogen were observed by Jahoda *et al* (1992b) using an immunolocalisation method. Type IV collagen and laminin staining was strongly apparent in the basement membrane surrounding the papilla right through telogen, but fibronectin staining was only present in the BM around the base of the papilla in early telogen, and almost completely absent prior to the induction of anagen. The structure of the basement membrane itself was also shown to be more amorphous and irregular around the papilla during telogen. These findings confirmed those observed by Couchman *et al* (1990) who demonstrated that fibronectin and chondroitin sulphate proteoglycan were lost from the papilla basement membrane during telogen in mouse pelage follicles.

Many molecular studies of telogen follicles demonstrate the absence of expression of the molecules found during anagen and catagen. For example, Stenn *et al* (1994) showed that the epidermal regions of the mouse pelage follicle did not express Bcl-2 at all during telogen, with expression tailing off during catagen. Since Bcl-2 appears not to be expressed an any of the follicular epidermal components during telogen, some other apoptosis-preventing molecule must be protecting the follicular epidermal stem cells. This is supported by the fact that *bcl-2* knockout mice have apparently normal hair (Veis *et al* 1993). A few of the molecules which are expressed during the other stages of the adult hair cycle are also expressed during

telogen; for example, BMP-4 expression has been localised to the outer root sheath of adult rat vibrissa follicles during telogen (Sleeman 1995).

The mechanisms controlling the switch from telogen into anagen are still poorly understood. However, it was suggested by Oh and Smart (1996) that an oestrogen receptor pathway may modulate the telogen-anagen transition. They showed that expression of an oestrogen receptor in the follicular dermal papilla was strongest during telogen, with expression concentrated around the nuclei of cells in the lower half of the papilla. The fact that oestrogen is expressed by the hair follicle itself (Schweikert *et al* 1975) and the oestrogen receptor is strongly expressed in the dermal papilla in a cycle specific pattern suggests that oestrogen may be an endogenous regulator of the hair follicle cycle. This is further supported by evidence provided from studies involving topical application of oestrogens and oestrogen receptor antagonists (Oh and Smart 1996, discussed in section 1:2.3).

1:2 IDENTIFICATION OF GENES EXPRESSED IN THE HAIR FOLLICLE ON THE BASIS OF EFFECT

Many genes whose expression in the hair follicle had not been studied or expected have been shown to be important in follicle growth and cycling, using both transgenic animals and techniques based on screening of mRNA populations or cDNA libraries. Several gene knockouts in particular have given valuable insights into molecules which are important in hair growth, (for example, the fibroblast growth factor-5 (FGF-5) knockout mouse (section 1:2.1-2.4), the epidermal growth factor receptor (EGFR) mutant mouse (section 1:2.1-2.1), the transforming growth factor-α (TGF-α) knockout mouse (section 1:2.1-2.2), the lymphoid enhancer factor-1 (LEF-1) knockout mouse (section 1:2.1-3.1) and the hox *c13* knockout mouse (section 1:2.1-3.2)). Overexpression studies using transgene constructs containing keratin gene promoters have also been performed to observe the effects of targeted overexpression of particular molecules in the hair follicle, (for example, hair keratin genes (section 1:2.1-1.1), β-catenin (section 1:2.1-1.2) and LEF-1 (section 1:2.1-3.1)). It is also possible to observe the effects on hair follicle growth and cycling of the exogenous application

of molecules by injection or topical application (section 1:2.3), (reviewed in Stenn *et al* 1996, Peus and Pittelkow 1996).

1:2.1 Transgenic Animals

Many strains of transgenic mice have been developed containing genes which have been inactivated, or are overexpressed constitutively or in a tissue or time specific manner depending on the promoter used in the transgene construct (see table 2.4 for examples). Alteration of the expression patterns of hair specific keratins will clearly have some effect on follicle development and hair growth. However, transgene constructs involving genes which would not be expected to have an effect on hair growth have often resulted in alterations in follicle morphology and hair growth. Some of these are discussed below.

1:2.1-1 Structural molecules

1:2.1-1.1 Hair Keratins

A number of transgenic mice strains have been generated carrying mutations in hair keratin genes, of which there are several families with many members (reviewed in Rogers and Powell 1993, Yu *et al* 1993). Often these have profound effects on hair follicle structure and behaviour. Many groups have used transgenic technology in order to study overexpression of particular keratins under the control of their own promoters, or expression of keratins under the control of promoters which give rise to ectopic expression of the gene in question, both in hair follicles and skin (Stoler *et al* 1988, Abe and Oshima 1990, Bader and Franke 1990, de Mare *et al* 1990, Powell and Rogers 1990, Fuchs *et al* 1992, Baribault *et al* 1993, Byrne and Fuchs 1993, Thorey *et al* 1993, Takahashi *et al* 1994, Casanova *et al* 1995).

Table 2.4 : Transgenics displaying effects on hair phenotype.

MOLECULE	TYPE OF TRANSGENIC	PHENOTYPE	REFERENCES
Activin βA Activin βB	knockout	reduced and abnormal follicle development	Matzuk *et al* 1995
Bcl-2	knockout	hypopigmented hair	Veis *et al* 1993
BMP-4	over-expression with K6 promoter	ectopic expression in ORS, balding after first hair growth cycle	Blessing *et al* 1993
EGF-R	knockout	Variable phenotype including curly hair, disoriented follicles, alopecia. Dependant on mouse strain	Miettinen *et al* 1995, Murillas *et al* 1995, Sibilia and Wagner 1995, Threadgill *et al* 1995
FGF-5	knockout	abnormally long hair due to prolonged anagen	Hébert *et al* 1994.
FGF-7	transgenic dominant negative	reduced number and abnormal follicles	Werner *et al* 1994
FGF-7	knockout	hair shaft abnormality	Guo *et al* 1996
IGF-1 IGF-1R	knockout	inadequate follicle morphogenesis	Liu *et al* 1993
Integrins α5, β1	overexpression	disorganised hair follicles, abnormal orientation, short curly whiskers	Carroll *et al* 1995
IL-6	overexpression with K14 promoter	retarded hair growth	Turksen *et al* 1992
IL-1α	overexpression with K14 promoter	increased hair loss with age	Groves *et al* 1995
LEF-1	knockout	hair shafts absent	van Genderen *et al* 1994
LEF-1	ectopic expression with K14 promoter	altered skin appendage patterning and fate	Zhou *et al* 1995
TGF-α	knockout	Wavy hair shafts Curved follicles	Luetteke *et al* 1993 Mann *et al* 1993
TGF-α	overexpression with K14 promoter	Stunted hair growth	Vassar and Fuchs 1991
β-catenin	expression of mutant protein with K14 promoter	Confers inductive capabilities on interfollicular epidermis	Gat *et al* 1998
TGF-β1	overexpression (K1 promoter)	Reduced follicle numbers	Selheyer *et al* 1993

1:2.1-1.2 β Catenin

A recent study by Gat *et al* (1998) investigated the effect of overexpression of a truncated β-catenin in the epidermis of transgenic mice. The K14 promoter was used to drive expression of N-terminally truncated human β-catenin in the basal layer of the epidermis and the follicle ORS of transgenic mice. These cells (which normally do not display inductive capabilities) were then able to induce *de novo* follicle morphogenesis postnatally. However, an overt phenotype did not become apparent until about 24 days postnatally, with enlarged hind paws and skin ridging around the face being the most obvious abnormalities. From around 18 days postnatal, epithelial invaginations began to appear in the interfollicular epidermis. These developed into structures similar to embryonic hair germs, and tended to be randomly arranged and clustered together. Multiple outgrowths also appeared originating from the upper regions of follicular ORS. Strong expression of the β-catenin transgene was required for such outgrowths to occur, but it appeared that high levels of transgene expression alone were not sufficient to promote initiation of new hair germs, since the transgene expression level was high from about E14, but no invaginations appeared until the first postnatal hair cycle was initiated. This suggested that some additional factor was required which was expressed in hairy skin at initiation of anagen. The hair germ-like structures induced in transgenic epithelium rapidly developed into epitheloid cysts with associated hair follicle-like structures, similar to human trichofolliculomas (TF), hair tumours composed of cysts containing densely packed hair follicle structures (Fitzpatrick *et al* 1993).

Many of the new follicles initiated postnatally in the transgenic mice displayed apolar expression of *Shh* and abnormal angling. In normal follicles, Shh is expressed in the hair bulb in a very restricted pattern, in a patch at the side of the matrix closest to the skin surface (Bitgood and McMahon 1995, Gat *et al* 1998). In many of the β-catenin generated hair germs, *Shh* was induced in the matrix cells in contact with the DP. However, in some cases, expression was randomly distributed on both sides of the matrix, becoming more aberrant as development progressed. Regardless of the behaviour of the *de novo* follicles, those established in embryonic development were able to retain normal *Shh* expression and polarity.

Based on this evidence, it was suggested that the transient stabilisation of β-catenin may be an important signal in induction of hair follicle morphogenesis. It is interesting that there are some phenotypic similarities between the β-catenin transgenic mice reported by Gat *et al* (1998) and the LEF-1 transgenic mice reported by Zhou *et al* (1995), in that epithelial invaginations developed postnatally, and follicles were present in irregular patterns and at inappropriate angles. However, the two transgenics displayed other phenotypic abnormalities which were non-overlapping. It has been shown that LEF-1 and β-catenin can interact to form a transcription factor complex which is active in the *Wnt/wingless* signalling pathway (Behrens *et al* 1996, Molenaar *et al* 1996), and in cells where β-catenin has been stabilised by mutation, it interacts constitutively with LEF/TCF proteins (Morin *et al* 1997, Rubinfeld *et al* 1997, Porfiri *et al* 1997). Furthermore, induction of β-catenin by wnt-1 promotes formation of LEF/β-catenin complexes in mammalian cells and drives activation of TCF/LEF reporter plasmids (Hsu *et al* 1998). When the truncated β-catenin described by Gat et al (1998) was coexpressed with a LEF-1 transgene in keratinocytes, expression of a reporter gene driven by an enhancer containing multiple LEF-1 binding sites was greatly upregulated. The LEF-1/β-catenin complex may therefore function to activate transcription of genes having LEF-1 binding sites in their promoters during hair follicle initiation.

1:2.1-2 Growth Factors And Growth Factor Receptors

1:2.1-2.1 Epidermal Growth Factor Receptor

Epidermal growth factor receptor (EGFR) is a key regulator of keratinocyte biology. To analyse the role of EGFR in skin, Murillas *et al* (1995) generated transgenic mice expressing an *EGFR* dominant negative mutant in the basal layer of epidermis and the ORS of hair follicles using a keratin 5 promoter. The mutant protein inactivated the wild type EGFR as mutant-wild type heterodimers were non-functional. New-born transgenic mice had curly whiskers and a wavy coat, similar to the phenotype observed in TGF-α knockout mice (section 1:2.1-2.2), but degeneration of hair rapidly occurred, resulting in an almost hairless phenotype after a few months. Histological examination of transgenic mouse skin showed that the

distribution and morphology of the follicles were altered, and progression through the hair cycle was also affected. The follicles of transgenic mice fail to enter into catagen and remain in an aberrant anagen state, showing that EGFR is essential for hair cycle progression. A transgenic mouse strain generated by Hansen *et al* (1997) having a targeted disruption of *EGFR*, display a severely disorganised hair follicle phenotype which is maintained in skin grafted onto nude mice. Hair follicles in grafted skin did not progress from anagen to telogen, and had wavy, flattened hair fibres with cuticular abnormalities. Analysis of hair keratin expression in null skin grafts revealed a pattern of premature differentiation in the null follicles. Since TGF-α is one of several ligands for EGFR, a similar phenotype would be expected in both knockouts. However, the TGF-α knockout phenotype is much less severe because other EGFR ligands are present, so that EGFR function is only partially blocked in TGF-α knockout mice.

1:2.1-2.2 TGF-α

Transforming growth factor -α (TGF-α), a member of the EGF family, is thought to be the major autocrine factor controlling growth in epidermal cells. TGF-α and EGF share a common receptor and are functionally and structurally homologous. Elevated levels of TGF-α have been associated with transformation of many cultured cell types (Derynck *et al* 1987), and overexpression of TGF-α in transgenic mice has been correlated with hyperproliferation in many organs (Matsui *et al* 1990, Sandgren *et al* 1990). To explore the role of TGF-α in growth and differentiation of epidermal cells, Vassar and Fuchs (1991) used the human keratin 14 promoter to target the expression of rat TGF-α to the skin of transgenic mice. They found that epidermis expressing the transgene was markedly thicker than normal, although terminal differentiation appeared normal. The most affected regions were those where hair follicle density is normally low, and hair follicles that were present appeared distorted, with stunted hair growth.

Disruption of the endogenous *TGF-α* gene (Luetteke *et al* 1993, Mann *et al* 1993) provided more information regarding the role of TGF-α in skin and hair follicles. Both groups used gene targeting in mouse embryonic stem cells to create lines of transgenic mice lacking

a biologically active TGF-α. They found that although the mice developed normally, their whiskers and pelage hairs were abnormally wavy, and the follicles were disorganised. The phenotype was found to be very similar to that of *waved*-1 homozygotes (Crew 1933). Crossing of *wa*-1 and *TGF*-α null mutants demonstrated that the two mutations are allelic, providing evidence that *wa*-1 is the result of a mutation in the *TGF*-α gene. This is supported by the presence of reduced levels of *TGF*-α mRNA in *wa*-1 mice.

1:2.1-2.3 Interleukin 6

Interleukin-6 (IL-6) is a cytokine that mediates a wide range of inflammatory and immune responses. Turksen *et al* (1992) studied the effect of overexpression of *IL*-6 in transgenic mouse skin using a transgene construct containing the human keratin 14 gene promoter fused to murine *IL*-6. They showed that transgenic mice had stunted hair growth and were underweight by an average of 30%. *In situ* hybridisation analysis was used to show where the gene was expressed in the skin, revealing a typical K14 distribution, present in the epidermis and the ORS of the hair follicle. However, the hair follicles and follicle density appeared similar to normal. Keratin expression is often used as a biochemical indicator to show whether the program of ORS and epidermal differentiation is normal (Stoler *et al* 1988). However, there appeared to be no difference in keratin expression in transgenic skin. An absence of *K6/K16* expression showed that the transgenic epidermis was not in a hyperproliferative state. However IL-6 overexpression in skin appeared to retard the hair growth cycle.

1:2.1-2.4 Fibroblast growth factors

Hébert *et al* (1994) produced a transgenic mouse strain homozygous for a null allele of *FGF*-5 by gene targeting in embryonic stem cells. These mice have abnormally long hair, a phenotype which appears identical to that of mice homozygous for the spontaneous *angora* mutation. The *angora* mutation has been defined by two recessive mutations (Dickie 1963, Konyukhov and Berdaliev 1990), and the follicles have no obvious structural abnormalities.

The increase in hair length appears to be due to prolongation of the anagen phase of the hair growth cycle by approximately three days (Pennycuik and Raphael 1984). Since *FGF*-5 expression only appears in normal hair follicles in late anagen, confined to the lower ORS and germinative epidermis (Hébert *et al* 1994), this suggests that FGF-5 may be responsible for regulating the transition from anagen to catagen, a process which is substantially delayed in *FGF*-5 null mice. Since catagen eventually does occur, even in the absence of FGF-5, it seems likely that there are other signalling molecules capable of inducing catagen acting in the hair follicle. Hébert *et al* showed that the *angora* mutation is a deletion of exon 1 and 2kb of upstream sequence of the *FGF*-5 gene, which prevents its expression. Sundberg *et al* (1997) demonstrated that the *angora* phenotype was maintained in skin grafted onto SCID mice with normal hair cycles, demonstrating that the mutation cannot be corrected by circulating FGF-5.

A transgenic mouse strain lacking FGF-7 was generated by Guo *et al* (1996) using embryonic stem cell technology. Over a period of time, these mice developed matted coats, with an appearance similar to the *rough* mutant, whose recessive mutation maps very near the KGF locus on chromosome 2. The hair defect in the FGF-7 knockout appeared to be restricted to the cells giving rise to the hair fibre, unlike the FGF-5 knockout which affected the outer root sheath cells and the progression of the hair growth cycle. The absence of FGF-7 in the transgenic mice did not give rise to wound healing or epidermal growth defects, even in double knockout mouse strains in which TGF-α was also lacking. There must therefore be a high level of redundancy in epidermal growth and wound healing pathways, probably because these functions are so vital to the life of the organism.

1:2.1-3 Transcription Factors

1:2.1-3.1 LEF-1

The HMG-box protein lymphoid enhancer factor-1 (LEF-1) is a transcriptional regulator which has been shown to have an effect on tooth and hair development in experimentally induced mutation studies. Normal expression of LEF-1 in hair follicles is

discussed in section 1:1.2. Mice carrying a homozygous germ line mutation in which the *LEF-1* gene has been inactivated are toothless and hairless (van Genderen *et al* 1994), lacking hair shafts although follicles appear to form, and mice in which *LEF-1* is overexpressed in a tissue specific fashion by using the keratin 14 promoter have altered skin appendage patterning and fate (Zhou *et al* 1995). The keratin 14 promoter is active in the basal layer of adult epidermis and the outer root sheath of the follicle (Byrne and Fuchs 1993), and led to ectopic expression of *LEF*-1 in adult transgenic mice, to a level greater than the endogenous level present in the matrix cells. A surprising feature of the *K14-LEF-1* transgenic mice was the appearance of large hairs erupting from the gums of several animals. It was shown that the developing lip furrow epithelium was a site of very high K14 expression, and therefore very high *LEF*-1 expression in the transgenic animals. It was therefore suggested that elevated LEF-1 in oral epithelium can trigger the formation of invaginations into the mesenchyme that are prerequisite to hair and tooth morphogenesis.

When the results of the LEF-1 knockout study are combined with the results of the LEF-1 overexpression study, it is clear that LEF-1 is important in both hair and tooth development. Overexpression of LEF-1 resulted in a disruption of the spatial organisation and orientation of hair follicles, suggesting that the patterning of LEF-1 in the early ectoderm is critical in influencing where, how and at what angle the follicles will form. Subsequently, expression of LEF-1 in the developing dermal papilla shows a similar pattern to BMP2 and msx2 expression, which also switch from epidermal to mesenchymal expression during follicle development, suggesting that these three factors could be involved in similar pathways. It is intriguing that LEF-1 may function to activate chromatin for subsequent expression of the structural hair keratin genes - this could explain why LEF-1 knockout mice are hairless, as hair keratin gene expression is not initiated.

1:2.1-3.2 *Hox c13*

A recent study by Godwin and Capecchi (1998) described the generation by gene targeting of a transgenic mouse strain carrying mutant alleles of *hox* c13. Normally, *hox* c13 expression is observed in the vibrissae, pelage follicles, nails and in the filiform papillae of the

tongue. Homozygous mutant mice have defects in all of these areas, but one of the most striking defects is brittle hair resulting in alopecia. It is also interesting to note that *hox*c13 is expressed in hair follicles all over the body, and does not conform to the normal anterior-posterior patterning of *hox* gene expression.

1:2.1-4 Other Molecules

1:2.1-4.1 Plasminogen Activator

Plasminogen activator enzymes have been implicated in the regulation of growth, migration and differentiation of normal skin and follicular epidermis. Studies using mice transgenic for human plasminogen activator inhibitor 1 have implicated plasminogen activator activity in the regulation of epidermal shedding and follicular neogenesis. (Lyons-Giordano and Lazarus 1995). Transgenic mice overexpressing human plasminogen activator inhibitor I showed delayed hair growth up to 2 weeks post birth, and *in situ* hybridisation showed that the transgene was expressed most highly in the bulge region of the ORS. Plasminogen activator activity may stimulate keratinocyte growth and dermal invasion during the first anagen, thereby contributing to follicular neogenesis.

1:2.1-4.2 SV40-T antigen

Directed expression of SV40 large T antigen (Tag) in transgenic mice can induce tissue specific tumour formation. In order to produce an immortalised mouse hair follicle cortical cell line for the study of hair keratin gene expression and control, Keough *et al* (1995) targeted SV40 T antigen expression to the hair follicles of transgenic mice using a sheep keratin gene promoter. Mice expressing the transgene were initially identifiable by the presence of wavy vibrissae, noticeable 2-3 days after birth. Pelage hairs were also affected, producing phenotypes varying from a ruffled coat appearance in moderate cases to stubble in severely affected mice. Scaling and wrinkling of the skin was also apparent. The transgenic hairs appeared to be weakened at the base, leading to premature hair loss and regions of

nudity. It appeared that SV40 *Tag* expression altered the keratin structuring in the cortical cells, so that the IF proteins appeared as a structureless mass. Amino acid analysis showed that the level of many amino acids was altered, notably cysteine, glycine and glutamine. This suggests that expression of the transgene alters the normal protein composition of the cortical cells. It was not known how Tag itself was effecting the changes in hair protein composition, though it may be due to binding by Tag of transcription factors such as AP-2 which may be involved in the expression of hair keratin genes (Powell *et al* 1991). Keough *et al* (1995) found that they could not produce an immortalised cell line from the cortical cells of the transgenic mice. *In situ* hybridisation studies demonstrated that cell proliferation had ceased prior to expression of the transgene construct, and could not be re-established in the cortical cells. *Tag* expression has been used to produce immortalised cell lines from post-mitotic cells (Suri *et al* 1993), but the cells must first be competent to undergo cell division so that Tag can stimulate re-establishment of proliferation. In order to produce a follicle specific immortalised cell line through *Tag* expression, it must first be targeted to a site in the epidermis where cell division is still possible.

1:2.2 mRNA/cDNA Screening Methods

Methods based on screening of follicle mRNA or cDNA populations to identify genes which may be important in follicle growth controls are now widely used (discussed further in chapter 3). Two such studies are briefly described below.

A hair follicle specific gene was isolated incidentally from a mouse skin cDNA library by Huh *et al* (1994), using a short oligonucleotide probe derived from the methyl acceptor site of human O^6-methylguanine DNA methyltransferase. Thirty eight clones were isolated from the library screen, of which 24 were shown to be derived from the same gene. This gene, designated *Hacl-1*, was localised by *in situ* hybridisation to the cortical cells of the lower hair shaft of hair follicles. *Hacl-1* expression is closely associated with differentiation of matrix cells into cortical cells, and several lines of evidence suggest that *Hacl-1* may be a novel member of the hair specific IFAP gene family.

A study performed by Yu *et al* (1995) involving screening of mRNA populations from cultured dermal papilla cells and cultured fibroblasts by differential display RT-PCR (DDRT-PCR) isolated several candidate dermal papilla cell specific mRNA's, including *nexin-1,* a potent protease inhibitor known to inactivate a number of serine proteases. This is discussed further in chapter 3.

1:2.3 The Effect of Exogenous/Topical Application

Many studies have combined the methods described in the previous sections with examination of the effects of topical application or injection of various proteins on hair follicle behaviour. Some of these studies are described below.

A study of the effect of bFGF (FGF-2) injection on murine follicle development and cycling (Du Cros 1993) demonstrated that exogenous bFGF inhibited follicle initiation and development. When bFGF was administered postnatally for a period of seven days, initiation of new follicles and development of follicles initiated before birth was inhibited until the exogenous bFGF was removed. This may be due to the saturation of FGF receptors by the exogenous protein, which has been shown to delay follicle initiation (section 1:1.2-1.1).

Another molecule whose exogenous application has been shown to inhibit hair growth is TGF-α. Daily injections of TGF-α from birth for eight days leads to retardation of hair growth in a dose dependent manner. Interestingly, this treatment has the opposite effect on tooth development, resulting in premature tooth eruption (Tam 1985).

Mann *et al* (1993) studied the effect of exogenous application of EGF on TGF-α null mutants using two methods. Firstly, new-born mutants were given daily subcutaneous injections of EGF, and secondly, EGF was applied topically to plucked areas of flank skin of five week old mice. Although both treatments failed to correct the TGF-α null phenotype, hair regrowth in the five week old animals was markedly accelerated by the topical EGF treatment. This demonstrates that EGF cannot functionally substitute TGF-α in this case. Adelson *et al* (1997) studied the effects of subcutaneous injection of mouse EGF on the development of hair follicles in the Northern brown bandicoot (*Isoodon macrourus*). Hair follicle initiation in this marsupial occurs postnatally, so it was possible to study the effect of exogenous EGF during

hair follicle initiation and development. It was found that murine EGF injection inhibited follicle initiation and perturbed the initiation patterning, suggesting that EGF and its ligands may play a role in pattern formation in the skin.

A study by Oh and Smart (1996) on the topical application of oestrogen and oestrogen receptor antagonists to mouse dorsal skin demonstrated that 17-β-estradiol application potently blocks hair growth and arrests hair follicles in the telogen phase of the cycle, while application of the receptor antagonist ICI 182,780 causes the hair follicle to exit telogen and enter anagen. However, continued application of ICI 182,780 had no effect on the length of the anagen phase. That these effects are not systemic was confirmed by intraperitoneal injection of ICI 182,780 during the synchronised second telogen phase of 6-9 week old mice. This treatment did not induce anagen, demonstrating that the oestrogen/receptor effects were localised to the skin. This data provides evidence that an oestrogen receptor pathway within the follicle may regulate the telogen-anagen transition. However, this work has recently been questioned by Stenn *et al* (1998), who reported that topical oestrogen receptor agonists and antagonists failed to alter murine follicle cycling in their hands.

1:3 A STUDY OF MOLECULES EXPRESSED IN THE RAT VIBRISSA FOLLICLE END BULB

As the basis of a study of gene expression in the adult rat hair follicle, cDNA libraries were constructed from skin, whole vibrissa follicles and vibrissa follicle end bulbs from all stages of the follicle cycle. These libraries could then be used both for screening for known genes and as the basis of a study to isolate genes which are preferentially expressed in particular regions of the follicle end bulb by differential hybridisation or cDNA library subtraction (see chapter 3).

The end bulb cDNA library was used as the basis of this study in order to further characterise the molecular basis of activity at the site of follicular epidermal proliferation. This library has been used in this and other studies, both to isolate rat homologues of genes known to be expressed in the hair follicle (such as *BMP-2* and *BMP-4* (Sleeman 1995)), to identify genes whose expression had not previously been shown in the hair follicle (for example, *Tcl-*

30, *MIF* (Sleeman 1995), *hox d1* (J. Huckle, unpublished)), and to identify genes specific to particular regions of the end bulb (Sleeman 1995).

The construction and testing of follicle cDNA libraries, the screening of the end bulb library for specific genes, and further studies to localise the expression of specific clones to regions of the hair follicle, will be described in this chapter.

2: MATERIALS AND METHODS

2:1 MATERIALS

Unless stated in the text, buffers used for molecular biology were prepared as described in Sambrook *et al* (1989).

E. coli growth media (Luria-Beltrami (LB) medium, NZY broth, superbroth, 2xYT medium) were prepared as described in Stratagene's ZAP-cDNA synthesis protocol and Sambrook *et al* (1989). All recipes are given in appendix A.

Unless stated in the text, restriction and modifying enzymes were obtained from Promega, tissue culture media and supplements were obtained from Gibco, and other chemicals were obtained from Sigma. Plastics were obtained from Greiner. Silica fines were a gift from Prof. N. J. Robinson, University of Newcastle upon Tyne.

Bacterial strains used were as follows

XL1-Blue: *recA1, endA1, gyrA96, thi-1, hsdR17, supE44, relA1, lac*, [F', *proAB*, $lacI^qZ\Delta M15$, Tn*10*(tet^r)]

SOLR™ Strain: e14$^-$*(mcrA), Δ(mcrCB-hsdSMR-mrr)171, sbcC, recB, recJ, umuC::Tn5(kan^r), uvrC, lac, gyrA96, relA1, thi-1, endA1,* λ^R, [F' *proAB*, $lacI^qZ\Delta M15$], Su- (non-suppressing).

SURE™ Strain: e14-*(mcrA), Δ(mcrCB-hsdSMR-mrr)171, sbcC, recB, recJ, umuC::Tn10 (tetr), uvrC, supE44, lac, gyrA96, relA1, thi-1, endA1*, [F' *proAB*, $lacI^qZ\Delta M15$]. All strains were obtained from Stratagene.

Vectors used were as follows: UniZAP-XR™ (λZAPII®); pBluescript (Stratagene);

The human cDNA clones for ribosomal protein L3A and GAPDH (in pGEM4Z) were a gift from Dr. I. H. Still, Durham University. The partial cDNA clone for rat MIF (in pBluescript) was a gift from Dr. M. A. Sleeman, Durham University.

2:2 PRODUCTION OF TISSUE SPECIFIC cDNA LIBRARIES

Libraries were constructed using a UNI-ZAP XR cDNA library kit (Stratagene, cat. no. 200401) using mRNA extracted from rat vibrissa follicle end bulbs, whole vibrissa follicles and dorsal skin in which all of the pelage follicles were in anagen. The methods used are briefly summarised below.

2:2.1 Dissection of Tissue for the Construction of cDNA Libraries

Follicles were dissected from the mystacial pads of Wistar rats (Durham University) using methods similar to those pioneered by Cohen (1961). Whole follicles in all stages of the hair growth cycle were transferred to a dish of E-MEM (Gibco) on ice, and the emergent hair fibres were cut off. Whole follicles taken from ten rats were snap frozen in liquid nitrogen in batches of ~50. Follicle end bulbs removed from the vibrissa follicles of a further twenty rats were also snap frozen in batches of ~100. The tissue obtained was then stored at -80°C until required. Skin from two rats was obtained from areas showing regrowth of dorsal pelage hair, seven days after shaving of the dorsal region. This indicates that all the follicles in these areas were in anagen. The tissue obtained was finely chopped with scissors, frozen in liquid nitrogen and stored at -80°C.

2:2.2 Extraction of Total RNA

Total RNA was extracted from each stored tissue sample using a guanidine hydrochloride extraction buffer as described by Chomczynski and Sacchi (1987). The frozen tissue was transferred to a pre-flamed, cooled mortar and pestle containing liquid nitrogen. 1ml of guanidine extraction buffer (8M guanidine hydrochloride, 20mM MES, 20mM EDTA, 50mM β-mercaptoethanol) was added dropwise, and the mixture finely ground. The powdered tissue was then transferred to baked glass 15ml Corex tubes and allowed to thaw.

Proteins and cell debris were removed by two extractions with phenol:chloroform (1:1) pH7.5. After the second extraction, the aqueous layer was transferred to a clean Corex tube, mixed with 0.4ml 1M acetic acid and 3ml cold absolute ethanol, and incubated at -20°C overnight. Nucleic acids were then pelleted by centrifugation at 9000g for 30 minutes at 4°C. The pellet was then washed with 2ml ice cold 70% ethanol, air dried, resuspended in 500µl DEPC treated H_2O and transferred to an RNase free microcentrifuge tube.

2:2.3 Purification of mRNA

Polyadenylated mRNA was extracted from the three samples of total RNA using the Pharmacia QuickPrep™ Micro mRNA purification kit, according to the manufacturers protocol. Briefly, each total RNA sample was added to 1ml oligo-dT cellulose slurry, and mixed for 3 minutes to allow binding of polyadenylated RNA to the cellulose support. The cellulose was then pelleted by centrifugation and the supernatant removed and discarded. The pellet was washed five times with 1ml high salt buffer (10mM Tris-HCl pH 7.4, 1mM EDTA, 0.5M NaCl), followed by two washes with 1ml low salt buffer (10mM Tris-HCl pH 7.4, 1mM EDTA, 0.1M NaCl). A further 0.3ml low salt buffer was used to resuspend the pellet, and the slurry was transferred to a microspin column. The column was washed a further three times with 0.5ml low salt buffer, and the polyadenylated RNA was eluted with two 0.2ml aliquots of prewarmed (65°C) elution buffer (10mM Tris-HCl pH7.4, 1mM EDTA).

The mRNA was precipitated by the addition of 10µl glycogen solution ($10mgml^{-1}$ in DEPC treated H_2O), 40µl 2.5M potassium acetate pH 5.0 and 1ml 100% ethanol, followed by incubation at -20°C overnight. The precipitated mRNA was collected by centrifugation at 10,000g for ten minutes at 4°C. The supernatant was removed and the pellet washed with 1ml 70% ethanol. The pellet was then dried under vacuum, resuspended in 5µl DEPC treated H_2O, and quantified by ethidium bromide plate assay (appendix A).

2:2.4 cDNA Library Production

The mRNA samples from the three tissue types were then used to make cDNA libraries as described below.

2:2.4-1 cDNA Synthesis

First strand cDNA was synthesised from mRNA using 50U M-MuLV reverse transcriptase and 2.8μg polyT oligo (5'$(GA)_{(10)}$ACTAGTCTCGAGT$_{(18)}$) in 1xRT buffer (50mM Tris-HCl, pH 8.3, 75mM KCl, 3mM $MgCl_2$), 10mM DTT, with 0.6mM dATP, dTTP, dGTP and 0.3mM 5-methyl dCTP in a total volume of 50μl. cDNA was synthesised from 6μg mRNA for the end bulb and skin libraries, and 4.5μg mRNA for the follicle library. For each reaction, 5μl of the reaction mixture was added to 0.5μl α^{32}P-dATP (800Cimmol^{-1}) in a separate tube to allow analysis of the quality and quantity of the first strand synthesis. All reactions were then incubated at 37°C for one hour and placed on ice. The radioactive control samples were stored at -20°C for subsequent analysis.

Second strand cDNA synthesis was performed using 100U DNA polymerase I in a 400μl reaction volume, containing 1x second strand buffer (50mM Tris-HCl, pH 8.0, 5mM $MgCl_2$, 5mM DTT, 200mM HEPES, pH 6.6,), 5U RNase H and 50μCi α^{32}P-dATP (800Cimmol^{-1}). The reaction was incubated at 16°C for 2.5 hours, and then extracted once with 400μl phenol:chloroform (1:1) pH 8.0 and once with chloroform. The aqueous layer was transferred to a fresh tube and 33.3μl 3M sodium acetate and 879μl 100% ethanol were then added, and the cDNA allowed to precipitate overnight at -20°C. The cDNA was then collected by centrifugation at 12,000g for 60 minutes at 4°C, washed with 1ml 80% ethanol, dried and resuspended in 43.5μl H_2O. An aliquot (4.5μl) was then removed for analysis of second strand synthesis.

The radioactive first and second strand synthesis control reactions for each RNA sample were electrophoresed on a 1.0% alkaline agarose gel (appendix A) to determine the size and quantity of cDNA produced. The gel was wrapped in cling film and autoradiographed,

showing that cDNA's in excess of 5kb were present after second strand synthesis for each of the initial RNA samples. The peak of radioactivity for first and second strand synthesis for each of the cDNA samples was present in the size range 1.5-2kb.

2:2.4-2 Preparation of cDNA for Ligation into Vector Arms.

Following second strand synthesis, the cDNA samples were blunt ended using 10U T4 DNA polymerase in a 100µl reaction volume with 2.5mM dNTP's in 1x T4 buffer (33mM Tris-acetate pH 8.0, 66mM potassium acetate, 10mM magnesium acetate, 0.5mM DTT, 0.1mgml^{-1} BSA). The reaction was incubated at 37°C for 30 minutes, followed by addition of 10µl 3M sodium acetate and 300µl ethanol. The reaction was then incubated at -20°C for 3 hours to precipitate the blunted cDNA. The precipitate was collected by centrifugation at 12,000g for 30 minutes at 4°C, washed with 1ml 70% ethanol and dried under vacuum. The cDNA was then resuspended in 3.5µl H_2O, and *Eco*RI adaptors were ligated onto the blunt ended cDNA using 4U T4 DNA ligase in a 5µl reaction volume containing 1x ligase buffer (30mM Tris-HCl, pH 7.8, 10mM MgCl2, 10mM DTT, 0.5mM ATP). The reaction was then incubated at 8°C for 48 hours, and the ligase inactivated by heating to 70°C for 30 minutes. Following inactivation of the ligase, the sticky ends of the adaptors were kinased with 10U T4 polynucleotide kinase in T4PNK buffer (50mM Tris-HCl pH 8.2, 10mM MgCl2, 0.1mM EDTA, 5mM DTT, 0.1mM Spermidine), and the cDNA samples digested with *Xho*I to produce cDNA's with 5' *Eco*RI and 3' *Xho*I sticky ends.

Free *Eco*RI adaptors and fragments of cDNA produced by *Xho*I digestion were removed by Sephacryl S-400 column chromatography. Five 60µl fractions were taken for each cDNA sample, and the size of the cDNA present in each fraction determined by electrophoresis and autoradiography of an aliquot of each fraction. Fractions containing cDNA greater than 500bp were pooled. The cDNA was then extracted with phenol:chloroform to remove any remaining kinase, and precipitated by the addition of 2 volumes of 100% ethanol followed by overnight incubation at -20°C. The cDNA was collected by centrifugation at 12,000g for 60 minutes at 4°C, washed with 200µl 80% ethanol and dried under vacuum. The

pellet was then resuspended in 10μl H_2O, and the concentration of cDNA determined by ethidium bromide plate quantitation (appendix A).

2:2.4-3 Ligation Into Vector, Packaging And Titering

An aliquot (100ng) of each cDNA sample was then ligated into 1μg of precut UNI-ZAP XR vector arms using 2U T4 DNA ligase in 1x ligase buffer (30mM Tris-HCl, pH 7.8, 10mM MgCl2, 10mM DTT, 0.5mM ATP). The reactions were incubated at 4°C for 48 hours. Each reaction was then split into five 1μl aliquots, and packaged using five Gigapack®II packaging extracts (Stratagene) according to the manufacturers instructions. The titer of the library was then determined by plating serial dilutions of the pooled packaging reactions in SM buffer (100mM NaCl, 50mM Tris-HCl, pH 7.5, 10mM $MgSO_4$, 0.01% gelatin) as follows.

LB broth supplemented with 0.2% maltose (w/v) and 10mM $MgSO_4$ was inoculated with a single colony of SURE cells, and incubated at 30°C overnight with vigorous shaking. Cells were then collected by centrifugation for ten minutes at 2000g. The supernatant was carefully removed, and the cells resuspended in 10mM $MgSO_4$ to an $O.D._{600}$ of 0.5. Aliquots (200μl) of cells were then infected with 1μl aliquots of library (dilution factor 10^0-10^{-8}) by incubation at 37°C for fifteen minutes. Each aliquot of bacteriophage and host cells was then mixed with 3ml top agar (NZY broth containing 0.8% agarose) at 48°C and plated onto 90mm NZY agar (NZY broth with 1.5% agar) plates. The plates were incubated at 39°C for eight to sixteen hours to allow plaques to grow. The titer of each library (plaque forming units (pfu) per ml) could then be calculated from the number of plaques per plate, taking into account the dilution factor and the volume of library plated.

2:2.4-4 Amplification

At least $6x10^5$ plaques of each library were amplified to produce large stable stocks. A single round of amplification was performed to prevent a disproportionate increase in the representation of abundantly expressed clones and a corresponding decrease in the relative abundance of rare clones.

An aliquot of *E. coli* SURE cells (600µl, $O.D._{600}$ = 0.5) in 10mM $MgSO_4$ was infected with $1x10^5$ pfu of each library per plate as described in section 2:2.4-3, and plated onto 20cm square NZY agar plates. The plates were incubated at 39°C for 16 hours, and then flooded with 50-100ml SM buffer and incubated with shaking at 4°C overnight. The SM buffer was then carefully decanted off into a sterile 500ml bottle, and each plate washed with a further 20ml SM buffer, which was then added to the stock. Chloroform (20ml) was then added, and the amplified libraries were then stored at 4°C. Since the titer of phage libraries drops on prolonged storage at 4°C, to obtain long term stable stocks, aliquots of newly amplified library were mixed with glycerol to a final concentration of 30% , frozen in liquid nitrogen and stored at -80°C. Before each use, the library titer was checked by test plating a serial dilution of 1µl on 90mm NZY agar plates.

2:2.5 Determination of the Quality of the cDNA Libraries

Following amplification, it was necessary to determine the titer of the libraries, the proportion of non-recombinant phage, and the overall quality in terms of insert length and abundance of highly expressed genes. Tests performed are described below. The test results for each of the libraries are summarised in table 2.5.

2:2.5-1 Determination of Background and Titer of Amplified Stocks

Serial dilutions of each library were made in SM buffer, and 1µl of each dilution plated with 200µl SURE cells ($O.D._{600}$ = 0.5) in top agar containing 2.5mM IPTG and $4mgml^{-1}$ X-gal on 90mm NZY agar plates. The plates were incubated at 39°C for 8 hours to allow plaques to grow and colour selection to occur. Plates with 50-100 plaques were selected and the number of blue and white plaques were counted. The ratio of recombinant:non-recombinant plaques (white:blue) could then be determined. In all three libraries, non-recombinant plaques made up less than 8% of the total. The titer of each of the amplified libraries used for this assay was in the region of 10^9 pfu/ml of library.

2:2.5-2 Determination of Mean Insert Size

Twenty recombinant plaques from each of the libraries were selected at random. Plugs of agar containing single plaques were transferred to 1.5ml microcentrifuge tubes containing 1ml SM buffer and 25μl chloroform. These were vortexed and incubated at 4°C overnight. The tubes were then briefly centrifuged to sediment debris and 1μl of the supernatant from each tube was used as the template in a PCR reaction containing 1U *Taq* DNA polymerase (Promega) and 0.5μg of each primer (complementary to regions of the polylinker adjacent to the site of cDNA insertion, having sequences as follows; 5' CAGGAAACAGCTATGAC 3' and 5' GTTTTCCCAGTCACGAC 3') in a 25μl reaction volume containing 1.5mM dNTP's, 1.5mM $MgCl_2$, 10mM Tris-HCl pH 8.8, 50mM KCl and 0.08% Nonidet-P40.

Thirty-five cycles were performed of denaturation at 94°C for 1 minute, primer annealing at 45°C for 1 minute and primer extension at 72°C for 5 minutes. An aliquot (10μl) of each reaction was then analysed by electrophoresis on a 2.0% agarose/TAE gel containing 0.5mgml^{-1} ethidium bromide. The products were visualised by exposure to UV.

2:2.5-3 Determination of Abundance of Highly Expressed Genes

A volume of cDNA library corresponding to 400,000 pfu was plated on NZY agar plates at 100,000 pfu per plate as described in section 2:2.4-4. Duplicate lifts onto nitrocellulose membranes (Schleicher & Schuell) were carried out as follows. Nitrocellulose filters (20cm square) were carefully placed onto the surface of the plates for 1 minute, and the orientation marked. The filter was then carefully peeled off, and a second filter was placed onto the surface of the plate for five minutes. The orientation was marked as for the first filter. The filters were then denatured in 1.5M NaCl, 0.5M NaOH for 1.5 minutes, and neutralised for 5 minutes by immersion in 1.5M NaCl, 0.5M Tris-HCl, pH 7.5. They were then soaked in 3xSSC for 3 minutes, air dried for 30 minutes, and the DNA was immobilised on the filters by baking in a vacuum oven at 80°C for 1-2 hours. The subsequent hybridisation reactions were carried out in heat sealed polythene bags which were contained in plastic boxes.

The filters were then prehybridised for two hours in prehybridisation buffer (6x SSC, 5x Denhardts reagent, 0.5% SDS, 100μgml^{-1} denatured herring sperm DNA, 0.1% sodium pyrophosphate) at 65°C, as described by Sambrook *et al* (1989). The prehybridisation buffer was then replaced with hybridisation buffer (6x SSC, 5x Denhardts reagent, 0.5% SDS, 2mM EDTA), and denatured ^{32}P labelled cDNA probes (human GAPDH or human ribosomal L3A) were then added, and the filters allowed to hybridise at 65°C for sixteen hours. Radiolabelling of double stranded cDNA was performed as described in section 2:3. The hybridisation solution was then removed, and the filters sequentially washed for 20 minutes each in 2x SSC, 0.1% SDS and 1x SSC, 0.1% SDS at 65°C. Each pair of filters was then mounted on Whatman 3MM paper so that both filters were in the same orientation, wrapped in cling film and exposed to X-ray film (Fuji) in autoradiography cassettes with intensifying screens, which were incubated at -80°C for the required exposure time. The film was then developed, and the number of duplicated spots determined.

2:3 GENERATION OF RADIOLABELLED DNA PROBES

Double stranded DNA was routinely labelled with α-^{32}PdCTP using random primers and Klenow DNA polymerase, based on a method described by Feinberg and Vogelstein (1983).

Template DNA (200ng-1μg) was denatured by heating to 95°C for five minutes, and snap cooled on ice. Labelling reactions were then performed in 1x labelling buffer (Promega) (50mM Tris-HCl, pH 8.0, 5mM $MgCl_2$, 2mM DTT, 200mM HEPES, pH 6.6, random hexadeoxyribonucleotides), with 1.5mM each dATP, dGTP and dTTP, and 50μCi α-^{32}PdCTP. The reaction was incubated at room temperature for one hour, and unincorporated nucleotides were removed by Sephadex G-50 gel permeation chromatography using 3ml (total volume) columns.

2:4 SCREENING OF THE END BULB cDNA LIBRARY FOR KNOWN GENES

In order to confirm that the end bulb cDNA library contained genes known to be expressed in the adult hair follicle, it was screened using a variety of methods. The rat homologues of known genes isolated from the library are shown in table 2.6 (genes isolated as a result of this study are given in bold type).

2:4.1 Screening For Msx Genes

2:4.1-1 Generation Of A Radiolabelled Msx-Homeobox Specific Probe

A probe to screen the end bulb cDNA library for clones containing *msx*-homeobox sequences was generated by PCR from rat genomic DNA. Degenerate primers were designed from the murine *msx-1* and *msx-2* homeobox sequences (figure 2.2, Monaghan *et al* 1991). Rat genomic DNA (20ng) was then used as the template in a PCR reaction, using 20pM *msx* primer 1 (5' CAG(T/C)TCCGCCTC(T/C)TGCAGTCT3') and *msx* primer 2 (5'CACAAGACCAACCG(G/C)AAGCCC3'), 1.5mM dNTP's, 1.5mM $MgCl_2$, 10mM Tris-HCl pH 8.8, 50mM KCl, 0.08% Nonidet-P40, and 1U Taq DNA polymerase (ImmunoGen International) in a 50µl reaction volume. Thirty five cycles of denaturation for 1 minute at 94°C, annealing for 1 minute at 50°C, and primer extension for 2 minutes at 72°C were then performed. PCR products were recovered following electrophoresis on a 1.5% agarose/TAE gel using the method described in section 2:8.1, and labelled with ^{32}P dCTP as described in section 2:3.

2:4.1-2 Screening The End Bulb cDNA Library For Clones Containing *Msx*-Homeobox Sequences

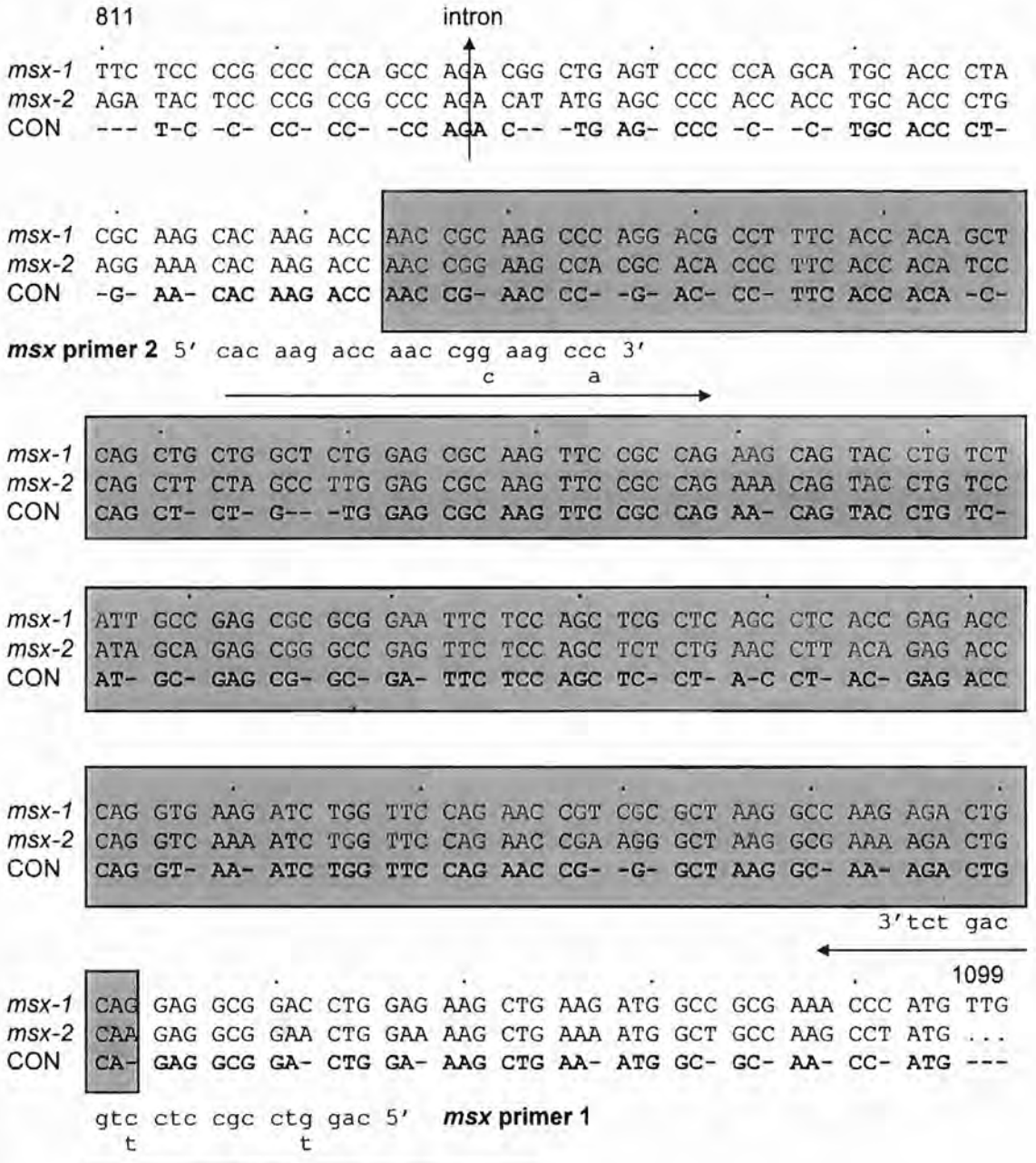
Primary screens of the end bulb library with the *msx*-homeobox probe were carried out as described in section 2:2.5-3. Following autoradiography of the filters, the number and

position of duplicated spots was determined. The corresponding plaques were then cored from the library plates and incubated in 1ml SM buffer with 20µl chloroform at 4°C overnight to allow diffusion of the phage particles into the buffer. Serial dilutions of the supernatant containing the phage from the primary core were made in SM buffer, and 1µl of each dilution plated with 200µl SURE cells ($O.D._{600}$ = 0.5) in top agar on 90mm NZY agar plates. The plates were incubated at 39°C for 8 hours to allow plaques to grow. Plates with 20-30 plaques were selected for secondary screening, and duplicate lifts onto 90mm nitrocellulose discs were performed, marking the orientation of each filter on the plate. The filters were denatured, neutralised and baked, and probed with msx-homeobox probe as described in section 2:2.5-3. The filters were then autoradiographed, and the position of duplicated spots determined. The plaques corresponding to the duplicated spots were then cored from the plate.

2:5 *IN VIVO* EXCISION OF pBLUESCRIPT PHAGEMID CLONES

Single plaques cored from secondary screens were transferred to sterile microcentrifuge tubes containing 500µl SM buffer and 20µl chloroform. The tubes were vortexed and incubated at 4°C overnight to allow release of the phage particles into the buffer. An aliquot (100µl) of phage stock was then added to 200µl *E. coli* XL1-Blue cells ($O.D_{600}$=1.0) and 1µl ExAssist helper phage (Stratagene) in a 10ml snap-cap tube (Sarstedt). The mixture was incubated at 37°C for 15 minutes to allow infection, 3ml of 2xYT medium was then added, and the culture was incubated at 37°C for 2.5 hours with shaking. The culture was then heated to 70°C for 20 minutes, and bacterial debris pelleted by centrifugation at 4000g for 15 minutes at 4°C. The supernatant, containing the plasmid packaged as a filamentous phage particle, was then decanted into a sterile tube and stored at 4°C. Rescued phagemid were plated by incubation of 50µl phagemid stock with 200µl *E. coli* SOLR cells ($O.D_{600}$=1.0) at 37°C for 15 minutes. Aliquots (50-150µl) of this was then plated on LB agar plates containing 100µgml^{-1} ampicillin and incubated overnight at 37°C.

Figure 2.2: Design of degenerate primers for amplification of the msx-homeobox. The murine msx-1 and msx-2 sequences (Monaghan *et al* 1991) were aligned and primers designed to span the homeobox in the most conserved regions. These were then used to amplify the homeobox containing region from rat genomic DNA. The primers are indicated by the arrows, and the intron position is shown. The homeobox is indicated by the shaded box.



2:6 PREPARATION OF PLASMID DNA

Plasmid DNA was routinely prepared from bacterial cultures using an alkaline lysis method based on that described by Birboim and Doly (1979). However, for applications involving subsequent direct automated sequencing, a commercial kit obtained from Promega was used. These two methods are described below.

2:6.1 Plasmid minipreps by alkaline lysis

A single colony of *E. coli* containing the plasmid of interest was grown in 10ml LB broth for 16 hours at 37°C with appropriate antibiotic selection (dependent on the plasmid concerned). An aliquot (1.5 ml) was removed to a microfuge tube and the cells collected by microcentrifugation at 3000g for 10 minutes. The supernatant was then removed, and the cells were resuspended in 100µl of an ice cold solution of 50 mM glucose, 10 mM EDTA, 25 mM Tris-HCl (pH 8.0). After a 5 minute incubation at room temperature, 200µl of a solution of 0.2 N NaOH, 1 % SDS was added and the contents of the tube were mixed by inversion. This was then incubated on ice for 10 minutes, followed by addition of 150µl of an ice cold solution of potassium acetate (5 M potassium acetate 60ml, glacial acetic acid 11.5ml, H_2O 28.5ml). The tube was incubated on ice for a further 10 minutes. Following centrifugation at 12,000g for 5 minutes, the supernatant was transferred to a fresh tube, RNase A was added to a final concentration of 20µgml^{-1} and the tube was incubated at 37°C for 30 minutes. An equal volume (~500µl) of phenol/chloroform (1 : 1) was then added and the contents vortexed thoroughly. The phases were then separated by centrifugation at high speed, and the aqueous phase was transferred to a fresh tube. 2.5 volumes of 100 % ethanol were added and the tube was incubated at -80°C for 10 minutes. Plasmid DNA was then collected by centrifugation at 12,000g for 5 minutes. The pellet of plasmid DNA was then washed in 1ml ice cold 70 % ethanol, dried briefly under vacuum, and resuspended in 16µl of water. To precipitate the DNA, 4µl of 4 M NaCl and 20µl of 13 % polyethylene glycol (MW 8000) were then added, and the tube was incubated on ice for 30 minutes. The purified plasmid DNA was

recovered by centrifugation, washed with 1ml 70% ethanol, dried under vacuum and resuspended in 20μl of water.

2:6.2 Plasmid minipreps using the Wizard™ Magic Minipreps kit

In order to obtain plasmid DNA for direct sequencing, a modification of the Magic Minipreps DNA purification system protocol described by Promega (Technical bulletin 117) was used. A single colony of *E. coli* containing the plasmid of interest was cultured in 10ml LB broth containing the desired antibiotic selective agent for 16 hours at 37°C with shaking. The cells were then pelleted by centrifugation at 2000g for 15 minutes. The pellet was resuspended in 200μl resuspension buffer (50mM Tris-HCl, pH 7.5, 10mM EDTA, 100μgml^{-1} RNase A), and transferred to a microcentrifuge tube. Following the addition of 300μl of cell lysis buffer (0.2M NaOH, 1% SDS), the solution was mixed by inversion until the bacterial cells were lysed. 300μl neutralisation buffer (2.55M Potassium acetate, pH 4.8) was then added, and precipitated chromosomal DNA and proteins were then removed by centrifugation at 12,000g for 5 minutes. The supernatant was carefully transferred into two microcentrifuge tubes, and 500μl Magic Miniprep DNA purification resin was added. The plasmid DNA was allowed to bind to the resin for 5 minutes at room temperature. The suspension of resin/DNA was then pipetted into a 2ml syringe barrel. A minicolumn was attached to the barrel, and the resin/DNA mix pushed into the column. The resin was then washed with 3-4ml of column wash buffer (100mM NaCl, 10mM Tris-HCl, pH 7.5, 2.5mM EDTA, 50% ethanol), and dried by centrifugation at 12,000g for 1 minute. The minicolumn was then transferred to a fresh microcentrifuge tube, and 50μl sterile ddH_2O heated to 70°C was added. After a one minute incubation at room temperature, the plasmid DNA was collected by centrifugation at 12,000g for 1 minute. The concentration of plasmid DNA was determined by U.V. spectrophotometry, and adjusted to approximately 200ngμl^{-1}. Direct sequencing was then performed as described below.

2:7 DIRECT SEQUENCING OF PLASMID DNA

Direct sequencing of plasmid DNA prepared as described in section 2:6.2 was performed by the dideoxy-sequencing method described by Sanger *et al* (1977), using fluorescent dye-linked universal M13 primers and an ABI377 automated DNA sequencer. Reactions were prepared according to protocols provided by the manufacturer. Sequence analysis was performed using DNA Strider and searching of the GenBank and EMBL databases.

2:8 RECOVERY OF DNA FROM GELS

2:8.1 Recovery of DNA from gel slices using NaI and Silica Fines

This method was routinely used to purify DNA fragments >1kb.

NaI solution:

90.8 g NaI and 1.5 g Na_2S0_3 were dissolved in 100 ml distilled water (the final volume is greater than 100 ml). The solution was then filter sterilised and a further 0.5 g Na_2SO_3 added. The resulting saturated solution was stored in the dark at 4°C.

The gel slice containing the DNA to be recovered was placed in a microcentrifuge tube, and 1 ml of NaI solution was added. The tube was then incubated at 60°C until the gel slice had completely melted. Silica fines (5 µl) was then added, followed by incubation at room temperature for 15 minutes to allow the DNA to bind. The fines were collected by centrifugation at 12,000g for 1 minute, followed by the aspiration of the supernatant. The pellet of fines was then resuspended in 1 ml 70% ethanol and centrifuged for 30 seconds. Following a second wash in 70% ethanol, the supernatant was removed and the fines were resuspended in 50 µl water. The DNA was then eluted by incubation at 37°C for 15 minutes. The fines were removed from the eluate by centrifugation for 30 seconds in a microcentrifuge,

and the supernatant containing the DNA was transferred to a clean microcentrifuge tube and stored at -20°C until required.

2:8.2 Recovery of DNA from gel slices using the Wizard™PCR-preps kit

This method was routinely used to purify DNA fragments <1kb, using low-melting point agarose gels (Sigma). The gel slice containing the DNA to be recovered was transferred to a microcentrifuge tube and incubated at 70°C until the gel slice had completely melted. The melted agarose was then quickly mixed with 1ml Wizard™ PCR-preps resin, and the DNA allowed to bind for 20 seconds at room temperature. The DNA/resin slurry was then pipetted into the barrel of a 2ml syringe attached to a minicolumn, and pushed into the column using the syringe plunger. The minicolumn was then washed with 2ml 80% isopropanol, and dried by centrifugation in a microcentrifuge at 12,000g for 1 minute. The DNA was eluted from the column by incubation for one minute with 20µl water heated to 70°C. The eluate was then collected by centrifugation, and stored at -20°C until required.

2:9 NON RADIOACTIVE WHOLE MOUNT *IN SITU* HYBRIDISATION

Non radioactive whole mount *in situ* hybridisation was used to localise expression of *msx*-2, *BMP*-2 and *BMP*-4 in rat pelage follicles. The *msx*-2 clone was isolated as described in section 2:4.1. The rat *BMP*-2 and *BMP*-4 cDNA clones in pBluescript were a gift from Dr. M. A. Sleeman, Durham University (Sleeman 1995). The method described below is adapted from that of Wilkinson (1992).

2:9.1 Riboprobe Construction

Anti-sense riboprobes were transcribed *in vitro* from plasmid DNA containing the cDNA insert of interest, prepared as described in section 2:6.1. Plasmid DNA was linearised

by restriction with an enzyme which cut the plasmid at the 5' end of the cDNA insert (*Eco*RI for *msx*-2 and *BMP*-4 and *Pst*I for *BMP*-2), and purified using silica fines as described in section 2:8.1. The linearised plasmid DNA (1μg) was then used in a transcription reaction with 15 units T7 RNA polymerase (Promega) in 1x transcription buffer (40mM Tris-HCl pH 7.5, 6mM $MgCl_2$, 2mM spermidine-HCl, 10mM NaCl, 10mM DTT) with 1mM ATP, 1mM CTP, 1mM GTP, 0.65mM UTP and 0.35mM DIG-UTP (Boehringer). The reactions were incubated at 37°C for 2 hours, and the efficiency of the reaction was determined by electrophoresis of a 1μl aliquot of each reaction on a 1.0% agarose/TAE gel containing 0.5μgml^{-1} ethidium bromide. The template DNA was then removed from the reaction by incubation with 2U RNase free DNase (Promega) at 37°C for 15 minutes. The RNA probe was then precipitated with 0.1 volume 4M LiCl and 2.5 volumes 100% ethanol at -20°C for 2 hours, and collected by centrifugation at 12000g for ten minutes at 4°C. The pellet was washed with 1ml 70% ethanol, air dried and resuspended in 50μl H_2O. If the probe length obtained by *in vitro* transcription was greater than 250 bases, the probe was partially hydrolysed as follows: The pH was adjusted to pH10 by adding 30μl freshly made DEPC treated 0.2M Na_2CO_3 and 20μl 0.2M $NaHCO_3$. The reaction was then incubated at 60°C for **t** minutes to give a mean probe length **x** of 250 bases (formula for calculating incubation time to give desired probe length given below). The hydrolysis was stopped by adding 3μl freshly made DEPC treated 3M sodium acetate, pH 6.0 and 5μl 10% glacial acetic acid. The hydrolysed probe was precipitated by addition of 330μl absolute ethanol, followed by incubation at -20°C for 2 hours. The precipitate was collected by centrifugation at 12000g for ten minutes at 4°C. The pellet was washed with 1ml 70% DEPC treated ethanol, vacuum dried and resuspended in 100μl DEPC treated ddH_2O to give a probe concentration of approximately 0.1μgμl^{-1}.

Calculation of time t for alkaline hydrolysis of long probes to give a mean probe length of x kb. **t (min)** = $\frac{\text{(unhydrolysed probe length kb) - (required probe length } \mathbf{x} \text{ kb)}}{\text{(0.11)(unhydrolysed probe length kb)(required probe length } \mathbf{x} \text{ kb)}}$

The *msx2* clone selected for *in vitro* transcription of DIG labelled probe for *in situ* hybridisation had a cDNA insert of approximately 1kb. The probe was therefore hydrolysed for

25 minutes. The *BMP*-2 and *BMP*-4 clones used in this study had cDNA inserts of approximately 1.7kb, and probes synthesised from them were therefore hydrolysed for 30 minutes.

2:9.2 Preparation Of Rat Pelage Follicles For Whole-Mount *In Situ* Hybridisation

Pelage follicles were dissected from the skin of adult Wistar rats, taken from multiple sites. Thin strips of skin were taken from freshly killed animals and transferred to DEPC treated PBS on ice. Individual follicles and small groups of follicles were then teased free from surrounding connective tissue, taking care not to damage the follicles themselves. Individual pelage follicles and groups of 2-6 follicles were then transferred to 35mm dishes, washed in fresh DEPC treated PBS, and fixed in 4% paraformaldehyde in DEPC treated PBS for 3 hours at 4°C. The paraformaldehyde was then very carefully removed using a glass Pasteur pipette, taking care not to remove any of the follicles from the dish, and replaced with 2ml PBT (PBS + 0.1% Tween-20). The follicles were then dehydrated through a methanol series in PBT (25% - 100% methanol), taking care not to remove any of the follicles from the dish. The follicles could then be stored until required in 100% methanol at -20°C.

Prior to hybridisation, the follicles were rehydrated through a methanol series (100% - 25%) to PBT. Bleaching treatments were omitted as the tissue was not pigmented. The follicles were permeabilised by incubation with $10\mu gml^{-1}$ proteinase K for 25 minutes at 37°C. The proteinase K treatment was stopped by washing for 5 minutes in PBT containing $2mgml^{-1}$ glycine, followed by a further two five minute washes in PBT. The follicles were then briefly refixed in 0.2% glutaraldehyde/4% paraformaldehyde for 15 minutes, and washed twice in PBT.

2:9.3 *In Situ* Hybridisation And Washing

Follicles were prehybridised for 1 hour at 70°C in 50% formamide, 5x SSC (pH 5.0), 50μgml^{-1} yeast tRNA, 1% SDS and 50μgml^{-1} heparin. The solution was then replaced with 2ml fresh solution containing 1μgml^{-1} DIG labelled antisense RNA probe, and hybridised overnight at 70°C with gentle rocking. The hybridisation solution was then carefully removed and the follicles washed twice for 30 minutes at 50°C in solution 1 (50% formamide, 5x SSC (pH 5.0), 1% SDS). The follicles were then washed once in solution 2 (25% formamide, 2.5x SSC (pH 5.0), 0.5% SDS, 0.25M NaCl, 5mM Tris HCl (pH 7.5), 0.1% Tween-20) for 30 minutes at 50°C, and three times in solution 3 (0.5M NaCl, 10mM Tris-HCl (pH 7.5), 0.1% Tween-20) for 15 minutes each at 50°C. The specimens were then treated with 100μgml^{-1} RNase A in solution 3 for 30 minutes at 37°C to remove unbound probe, and washed for 15 minutes at 37°C in solution 3. They were then washed twice in solution 4 (50% formamide, 2x SSC (pH 5.0)) for 30 minutes at 50°C and once in TBST (0.14M NaCl, 2.7M KCl, 0.025mM Tris-HCl (pH 7.5), 0.1% Tween-20) at room temperature for 20 minutes. The follicles were then incubated for 1.5 hours at room temperature in 10% heat inactivated normal sheep serum in TBST.

2:9.4 Preparation Of Anti-DIG Antibody

Anti-digoxygenin antibody (Boehringer) was prepared for use in the *in situ* hybridisation protocol as follows. The antibody was diluted 1:2000 in 1% heat inactivated sheep serum in TBST containing 1.5mgml^{-1} rat follicle acetone powder (for preparation of rat follicle acetone powder, see appendix A), and incubated at room temperature for 1.5 hours to prevent non-specific binding of the antibody to follicle proteins. The blocked antibody was then separated from the rat follicle acetone powder by centrifugation at 3000g for 15 minutes. The supernatant was then carefully removed, transferred to a fresh tube and stored at 4°C until required.

2:9.5 Immunological Detection Of DIG Labelled Probe

After blocking, the follicles were incubated overnight at 4°C with preabsorbed anti-DIG antibody (prepared as described in section 2:9.4). The follicles were then washed once for 5 minutes at room temperature in TBST, and three times for 5 minutes at room temperature in NTMT (100mM NaCl, 100mM Tris-HCl pH 9.5, 50mM $MgCl_2$, 0.1% Tween-20). The antibody was then revealed by incubation in the dark at room temperature with 75μgml^{-1} NBT and 50μgml^{-1} BCIP in NTMT. When the colour had developed, the reaction was stopped by washing in NTMT. The stained follicles were then stored in PBT at 4°C.

2:10 NORTHERN BLOTTING

RNA for northern blotting was extracted from vibrissa follicle end bulbs, flank skin and liver obtained from freshly killed adult animals. Total RNA was extracted from whole vibrissa follicles, skin and liver as described in section 2:2.2. Alternatively, total RNA was extracted from whole skin as follows. Hair was removed from an area of flank skin ~2cm x 2cm using depilatory cream ("Immac", Whitehall laboratories). A 1cm square of skin was then removed and transferred to a dish of sterile diethylpyrocarbonate (DEPC) treated PBS on ice. The tissue was finely chopped with sharp scissors, frozen in liquid nitrogen, and transferred to a flamed, cooled mortar and pestle. The tissue was then finely ground and transferred to RNase free microcentrifuge tubes with a flamed, cooled spatula. After addition of 1ml TRIzol (GIBCO), the tissue was allowed to thaw. The tubes were then vortexed thoroughly, and incubated at room temperature for 15 minutes. Chloroform (200μl) was then added, and the tube vortexed for 15 seconds. The phases were separated by centrifugation at 12000g for 15 minutes at 4°C, and the aqueous phase carefully transferred to a clean tube. The RNA was precipitated by the addition of 750μl isopropanol, followed by incubation at room temperature for ten minutes. The precipitate was collected by centrifugation at 12000g for 15 minutes at 4°C, washed with 1ml 70% ethanol, air dried and resuspended in DEPC treated H_2O. Total RNA was extracted from liver and whole follicles using a similar method.

The protocol was modified slightly for extraction of total RNA from vibrissa follicle end bulbs. Vibrissa follicles were dissected from the mystacial pads of adult rats and placed into a dish of sterile DEPC treated PBS on ice. End bulbs were removed from follicles using a sharp scalpel and washed in ice cold DEPC treated PBS. The end bulbs were then transferred to a baked glass homogeniser containing 1ml TRIzol. The tissue was then homogenised on ice for ten minutes, and the homogenate transferred to a clean 1.5ml microcentrifuge tube. Chloroform (200μl) was then added, and the RNA extracted as described above.

The total RNA (10μg/lane) was then subjected to denaturing agarose/formaldehyde gel electrophoresis on a 1.4% agarose gel, and blotted onto nylon membrane as described by Sambrook *et al* (1989), using 20xSSC as the transfer buffer. Ribosomal RNA bands were used as size markers for these gels. The blots were then probed with ^{32}P-labelled inserts of the cDNA clones of interest and autoradiographed.

2:11 RACE-PCR

2:11.1 Primer Design For RACE-PCR

In order to obtain the 5' ends of truncated clones isolated from the end bulb cDNA library, clone specific antisense oligonucleotide primers were designed from 5' sequence data for each clone. Thirty base-pair segments of the sequence were analysed by comparison with sequences in the GenBank database to determine which regions had least homology to other known sequences in the database (RACE primer sequences given in appendix B). Primers were then designed so that an overlap of 30-300bp of known sequence would be produced. These primers were then used for 5'RACE-PCR.

2:11.2 5'RACE-PCR To Obtain Full Length cDNA

Total RNA was extracted from whole vibrissa follicles and vibrissa follicle end bulbs using TRIzol as described in section 2:10.

Total RNA (1μg) was denatured by heating to 70°C and reverse transcribed using 2.5μM poly-T primer (5' ATC CTC GAG CCT TTT TTT TTT TTT TTT 3'), or 2.5μM clone specific primer (for primer sequences, see appendix B), 1.5mM dNTP's and 5 units *Tth* DNA polymerase (Bioline) in 25mM $MnCl_2$, 16mM $(NH_4)_2SO_4$, 67mM Tris-HCl pH 8.8, 0.01% Tween-20. The reaction was incubated at 65°C for 20 minutes, and stopped by adding 1μl 0.5M EDTA, pH 8.0. 0.5μg polyI.polyC was added to the reaction mixture, followed by 3μl 10% CTAB (cetyltrimethyl-ammonium bromide). The cDNA/RNA hybrids were allowed to precipitate at room temperature for 5 minutes, then pelleted by centrifugation at room temperature for 20 minutes at 13000g. The supernatant was then carefully removed, and the pellet resuspended in 14μl 1M NaCl. The volume was made up to 39μl with ddH_2O, and 1μl 10% CTAB was then added, and the nucleic acid pelleted once more by centrifugation at room temperature for 20 minutes at 13000g. The pellet was then resuspended in 10μl 1M NaCl and precipitated with 27μl ethanol overnight at -20°C. The nucleic acid was then collected by centrifugation at 13000g for 20 minutes at 4°C, washed in 500μl 70% ethanol and vacuum dried.

A tailing reaction was then performed in order to provide the annealing site for the 5' amplimer during the PCR reaction. This involved the addition of several G residues to the 3' end of the first strand cDNA using terminal transferase. The concentration of dGTP and enzyme and reaction temperature were controlled to give tail lengths of 17-20 bases.

A reaction mixture was prepared containing 1x tailing buffer (200mM potassium cacodylate, 25mM Tris-HCl pH 6.6, 5mM CoCl2, 0.25mgml-1 BSA) and 5μM dGTP. The pellet of nucleic acid was resuspended in 7μl ddH_2O, and added to the reaction mixture. Twenty-five units of terminal transferase (Promega) was then added to give a final reaction volume of 20μl. This was then incubated at 37°C for 20 minutes. The reaction was stopped by the addition of 4μl 100mM EDTA and 2μl 1M NaCl. Unincorporated dGTP was removed by the

addition of 1μl 10% CTAB, followed by centrifugation at 13000g for 20 minutes at 4°C. The supernatant was carefully removed and the pellet of nucleic acid resuspended in 10μl 1M NaCl. Glycogen (0.5μl, 0.5mgml^{-1}) was then added as a carrier, and the nucleic acid precipitated overnight at -20°C by the addition of 30μl cold absolute ethanol. The precipitate was collected by centrifugation at 13000g for 20 minutes at 4°C and the pellet washed in 70% ethanol and dried under vacuum.

The RNA template was removed from the tailed first strand cDNA by alkaline hydrolysis. Briefly, the pellet of nucleic acid was resuspended in 20μl 50mM NaOH, 2mM EDTA, and incubated at 65°C for 1 hour. 3μl 3M sodium acetate, pH 5.2, and 70μl ethanol was then added, and the cDNA allowed to precipitate overnight at -20°C. The precipitate was collected by centrifugation at 13000g for 20 minutes at 4°C, and the pellet washed in 70% ethanol and vacuum dried. The cDNA was then resuspended in 20μl ddH_2O. An aliquot (1μl) of this was then used as a template in a PCR reaction, using 20pM clone specific primer (sequences in appendix III) 20pM oligo-dC primer (5' ATC GAA TTC CCC CCC CCC CCC CCC 3'), 1.5mM dNTP's, 1.5mM $MgCl_2$, 10mM Tris-HCl pH 8.8, 50mM KCl, 0.08% Nonidet-P40, and 1U Taq DNA polymerase (ImmunoGen International) in a 25μl reaction volume. In cases where clone specific primers had been used to synthesise the first strand cDNA, nested clone specific primers were used for the PCR amplification (sequences in appendix III). Forty cycles of denaturation for 1 minute at 94°C, annealing for 1 minute at 60°C, and primer extension for 1 minute at 72°C were performed, and a 10μl aliquot of the PCR reaction was analysed by electrophoresis (2.0% agarose/TAE gel containing 0.5μgml^{-1} Ethidium bromide). PCR products were recovered directly from the PCR reaction mixture using the Wizard^{TM} PCR Prep kit (Promega), according to the manufacturers protocol, or from the gel when multiple PCR products were produced (section 2:8.2).

2:11.3 Cloning of RACE-PCR Products

PCR products were blunt ended by incubation with 2U Klenow DNA polymerase (Promega) in a 20μl reaction volume containing 1mM dNTP's. Blunt ended cDNA was

precipitated with absolute ethanol at -20°C, collected by centrifugation, washed with 1ml 70% ethanol, dried and resuspended in 10µl ddH_2O. Blunt ended pGEM4Z for cloning was prepared by digesting 1µg pGEM4Z with 24U *Sma*I at 25°C overnight. The plasmid was then precipitated and resuspended in 10µl ddH_2O. An aliquot of the blunt ended PCR product was then cloned into the linearised pGEM4Z using 2U T4 DNA ligase in 30mM Tris-HCl, pH 7.8, 10mM MgCl2, 10mM DTT, 0.5mM ATP at 4°C overnight. The resulting plasmids were then transformed into competent *E. coli* (section 2:12, below). Plasmid DNA was then prepared using a Wizard™Magic minipreps kit (Promega) as described in section 2:6.2, and sequencing was performed using an ABI377 automated DNA sequencer as described in section 2:7.

2:12 PREPARATION AND TRANSFORMATION OF HIGH EFFICIENCY COMPETENT *E. COLI* CELLS.

The method to prepare high efficiency competent cells was essentially as described by Alexander *et al* (1984).

A 5 ml culture of *E. coli* cells was grown in 2 XL (recipe in appendix A) overnight at 30°C. An aliquot (1 ml) was used to inoculate 100 ml 2 XL prewarmed to 30 °C in a 500 ml flask. This was cultured (with shaking) at 30 °C until the OD_{600} = approximately 0.2, then sterile 2 M $MgCl_2$ was added to a final concentration of 20 mM. Growth was allowed to continue until OD600 = 0.5 (0.45-0.55). The cells were then incubated in ice-water for 2 hours. Fifty ml aliquots were spun down in sterile 50ml polypropylene tubes (Falcon) at 3000 g for 5 minutes in the bench-top centrifuge, and the supernatant was aspirated off. The resulting pellets were resuspended gently in one half of the original volume of ice-cold Ca/Mn medium (100 mM $CaCl_2$, 70 mM $MnCl_2$, 40 mM sodium acetate, pH 5.5). This solution was prepared fresh and filter sterilised (start with a stock NaAc solution of about pH 7, the Ca^{2+} and Mn^{2+} salts will drop the pH considerably and the pH must be adjusted down). The cells were then incubated on ice for 1 hour and collected by centrifugation at 3000 g in the bench top centrifuge for 5 minutes. The resulting pellet was resuspended (very gently) in 1/20 the original culture volume of Ca/Mn solution containing 15% (v/v) glycerol. Aliquots (0.2 ml) of

cells were collected in 1.5 ml microcentrifuge tubes, frozen in liquid nitrogen and stored at -80 °C until required.

The cells were thawed on ice and transformed immediately by the addition of DNA in 100 µl TE and incubation on ice for 30 minutes. The transformation sample was subjected to heat shock (37 °C for 5 minutes), diluted to 4 ml with 2 XL broth (pre-warmed to 37 °C) and grown with shaking for 1.5 hours at 37 °C. The transformed cells were then plated out onto LB agar containing the desired selective agent.

2:13 WESTERN BLOTTING

Embryonic rat skin protein extracts were prepared from skins dissected from 14.5dpc, 16dpc and 18dpc embryos. The back skin of 2-5 embryos was carefully removed and washed several times in PBS to remove traces of blood from the surface of the tissue. Total protein from the embryonic skin samples, rat vibrissa follicles, human scalp follicles and human balding and non-bald scalp skin (surgically removed from a normal subject displaying androgenic alopecia) was isolated using TRIzol™ (Gibco), following the manufacturer's protocol. The protein concentration of the extracts was then determined using a dye-binding microassay (Bradford 1976). Equal amounts of total protein were then treated with 2x SDS loading buffer (125mM Tris-HCl, pH 6.8, 1M β-mercaptoethanol, 4% SDS, 20% glycerol, 0.01% bromophenol blue), and electrophoresed on a single concentration SDS-polyacrylamide gel (The percentage of polyacrylamide in the gel was dependant on the size of the protein under study. For this study, a total acrylamide concentration of 15% was used). Blotting onto 0.45µm nitrocellulose (Biorad) using a Biorad semi-dry blotter was carried out at 15V/150mA according to the manufacturers' instructions. Non-specific binding to the blot was blocked by incubation in 5% dried milk (Marvel™) in PBS overnight. The filter was then washed in PBS and incubated for 4 hours with primary antibody diluted in PBS. Following extensive washing in PBS containing 0.1% Triton X-100 (Sigma) to remove unbound antibody, the blot was incubated for 2 hours with a labelled secondary antibody diluted in PBS. Unbound antibody was removed by washing in PBS, and the blot was equilibrated in either PBS (for HRP conjugated antibodies) or alkaline phosphate buffer (100mM Tris-HCl pH 9.5, 10mM

NaCl, 5mM $MgCl_2$)(for alkaline phosphatase conjugated antibodies). The conjugate was then detected by incubation with the appropriate chromogenic substrate (DAB for HRP conjugated antibodies and NBT/BCIP for alkaline phosphatase conjugated antibodies).

2:14 LOCALISATION OF MIF BY IMMUNOFLUORESCENCE

Single follicles were dissected from human scalp skin, embedded in Tissue-Tek (AgarAids) and snap frozen in liquid nitrogen. Sections (5mm thick) were then cut using a Bright cryostat and adhered onto poly-L-lysine coated slides. The sections were allowed to dry at room temperature, and then fixed in acetone at -20°C for fifteen minutes. The slides were then washed three times in PBS for five minutes and excess PBS drained off. The sections were covered with 200μl FBS and incubated for one hour at room temperature in a humidified chamber. The FBS was then removed, and the slides washed 3 x 5 minutes in PBS. The sections were then covered with primary antibody diluted in PBS, and incubated for one hour at room temperature in a humidified chamber. Following a further three five minute washes in PBS, FITC-conjugated secondary antibody diluted in PBS was added, and incubated for one hour in a humidified chamber.

Unbound secondary antibody was removed by washing three times in PBS for five minutes. The slides were then carefully dried, and the sections were mounted in vectashield mounting medium (Vector) for visualisation.

3: RESULTS

3:1 SCREENING OF THE END BULB LIBRARY FOR KNOWN GENES

Following testing of the vibrissa follicle end bulb cDNA library, it was screened using a variety of methods. The library test results are shown in table 2.5. The genes which have been isolated by screening the end bulb library (in this and other studies) are shown in table 2.6. Several of the clones isolated were used in this study.

Table 2.5: Test results for skin, whole follicle and follicle end bulb cDNA libraries.

LIBRARY	method of construction Vector	starting material	unamplified titer (pfu/ml)	amplified titer (pfu/ml)	proportion non-recombinant plaques	mean insert size and range	% GAPDH
END BULB	conventional UNI-ZAP XR	6μg mRNA	$8.1x10^6$	$4.2x10^9$	3%	2kb 0.5-5.0kb	>0.1
FOLLICLE	conventional UNI-ZAP XR	4.5μg mRNA	$6.0x10^6$	$1.2x10^9$	7%	1.5kb 0.5-5.0kb	0.09
SKIN	conventional UNI-ZAP XR	6μg mRNA	$3.0x10^5$	$8x10^8$	8%	1.0kb 0.2-3.0kb	0.07

Figure 2.3: Screening the vibrissa follicle end bulb cDNA library for known genes - methods used for characterisation of clones obtained.

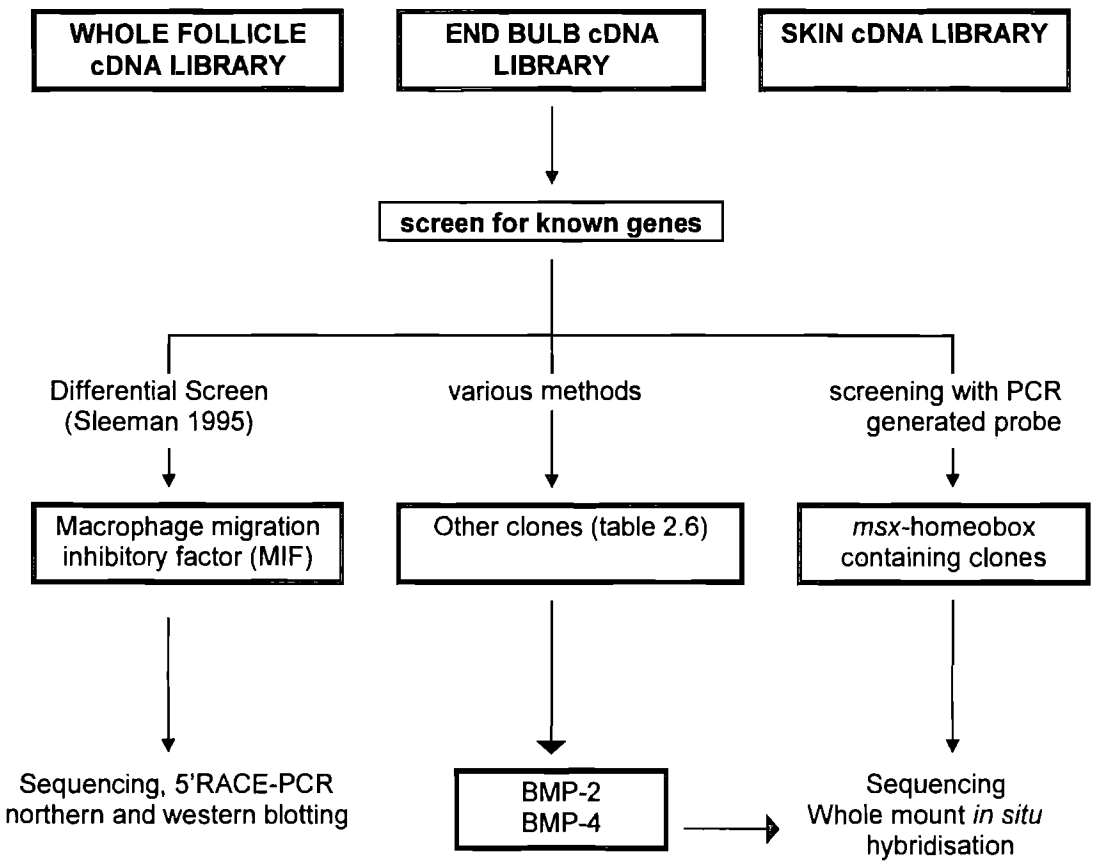


Table 2.6: Examples of Clones Isolated From The Vibrissa Follicle End Bulb cDNA Library.

Clones in bold type were isolated as a result of this study.

CLONE	METHOD OF ISOLATION	REFERENCE/ ACKNOWLEDGEMENT
GAPDH	**screening with human GAPDH clone (1.1kb)**	**human clone provided by Dr. I. H. Still, University of Durham**
RL3A	**screening with human ribosomal L3A protein clone**	"
BMP2	screening with mouse BMP2 clone (1.1kb) (screening performed by M. A. Sleeman)	mouse clone provided by Dr. B. Hogan, Vanderbilt University.
BMP4	screening with mouse BMP4 clone (1.4kb) (screening performed by M. A. Sleeman)	"
msx2	**screening with rat PCR product (~250bp) (screening performed in association with Dr. J. W. Huckle)**	
hoxd1	screening with human Hoxd1 (hox 4G) clone (screening performed by Dr. J. W. Huckle)	human clone provided by Dr. I. H. Still, University of Durham
MIF	differential screen using probes derived from different phenotypic stages of cultured DP cells (performed by M. A. Sleeman)	M. A. Sleeman, Ph. D. thesis, University of Durham, 1995.
Tcl-30	"	"
glycine/tyrosine rich hair keratin	"	"
16s rRNA	**Differential screening using probes generated from regions of the follicle end bulb**	**See chapter 3**

3:1.1 Screening the End Bulb Library for *msx-2*

Since *msx* gene expression has previously been shown in adult follicles (Stelnicki *et al* 1997), the end bulb cDNA library was screened with a ^{32}P-labelled *msx*-homeobox specific probe (section 2:4.1). The probe was generated by PCR using degenerate primers designed from homologous regions of the murine *msx-1* and *msx-2* homeobox sequences (Monaghan *et al* 1991, see figure 2.2), to give a PCR product of 200bp. The library screen resulted in the isolation of six putative positive clones with inserts ranging from 0.9-1.2kb (figure 2.4) These were recovered to the plasmid stage as described in section 2:5, and their inserts sequenced from the 5' end. Analysis of the sequence data generated (using the GenBank database,) revealed that all six clones were rat homologues of *msx-2* (sequence alignment shown in figure 2.5).

Table 2.7: Rat *msx*-homeobox containing clones isolated by screening of the vibrissa follicle end bulb cDNA library.

CLONE	Length of cDNA insert determined by gel electrophoresis	Predicted length of cDNA insert when sequence aligned with full length cDNA (1550bp)	% identity with murine *msx-2*
pmsx2-1	~1kb	1kb	95.9
pmsx2-2	~1.2kb	1.13kb	84.2
pmsx2-3	~1.2kb	1.13kb	83.2
pmsx2-4	~0.9kb	0.92kb	97.9
pmsx2-5	~0.95kb	0.93kb	92.1
pmsx2-6	~1.05kb	1.01kb	92.4

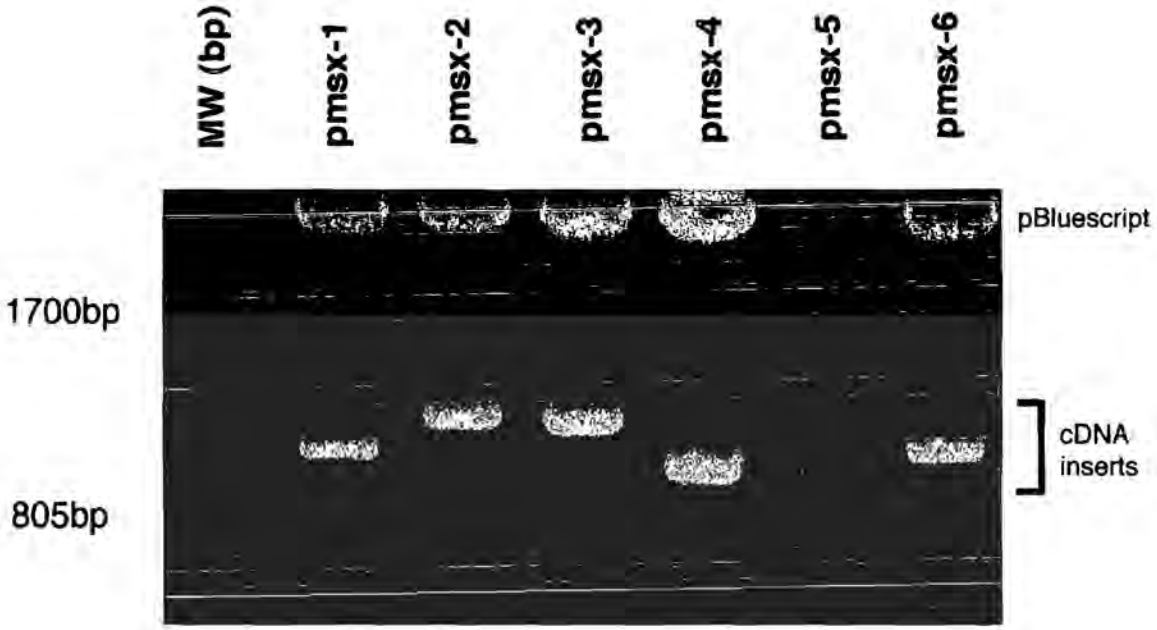
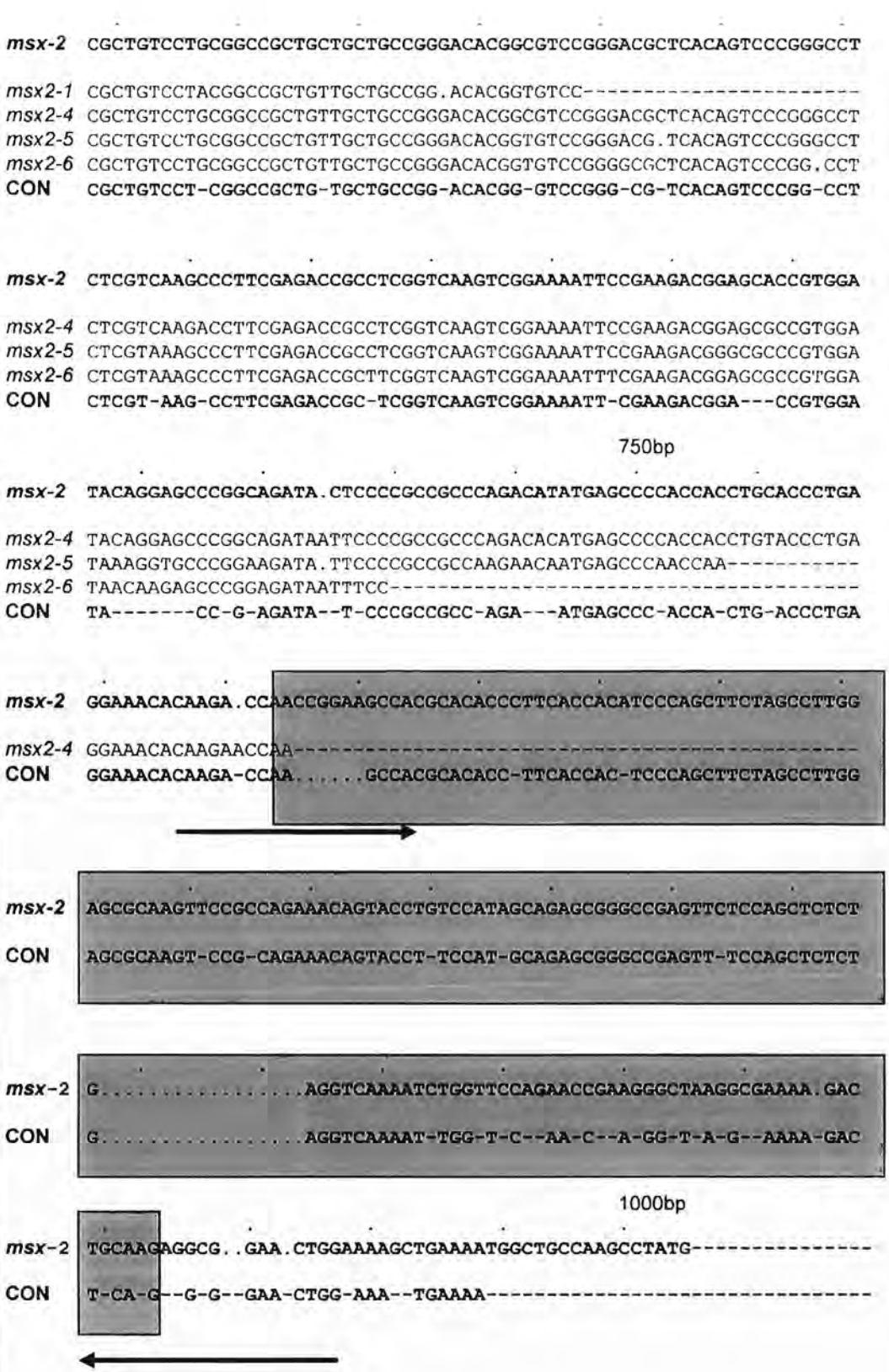


Figure 2.4: Insert sizes of the six msx-homeobox containing clones isolated from the vibrissa follicle end bulb cDNA library. Plasmids containing the cDNA inserts of interest were digested with *Eco* RI and *Xho* I to release the insert, and electrophoresed on a 1.5% agarose/TAE gel.
The six clones had inserts of around 0.9-1.2kb, with pmsx2-4 being the shortest at ~920bp, and pmsx2-2 and -3 being the longest at ~1130bp. The ends of the inserts were sequenced and aligned with the murine msx-2 cDNA sequence to confirm their identity (figure 2.5).

Figure 2.5: 5' sequence alignment of the six *msx*-homeobox containing clones isolated from the vibrissa follicle end bulb cDNA library (*pmsx2-1 - pmsx2-6*), compared with murine *msx-2* (Monaghan et al 1991). Clones pmsx2-1 - pmsx2-6 were sequenced from the 5' end as described in section 2.7, generating 110-260bp of sequence data. This was then aligned with murine msx-2. Conserved nucleotides are indicated beneath the alignment (CON). The homeobox region is indicated by the shaded box, and the primers used to generate the probe are indicated by the arrows. All six clones show significant homology to *msx-2* in the regions sequenced.

```
        330bp
         .         .         .         .         .         .
msx-2   CGCGCGCCGACAGCTACGCGGCGCAGAAAGTCATGGCTTCTCCGACTAAAGGCGGTGACTTGTT

msx2-2                    GCGGCGCAGAAAGTCATGGCTTCTCCGTCCAAAGGCGGTGACTTGTT
msx2-3                    GCGGCGCAGAAAGTCATGGCTTCTCCGTCCAAAGGCGGTGAATTGTT
CON                       GCGGCGCAGAAAGTCATGGCTTCTCCG-C-AAAGGCGGTGA-TTGTT


         .         .         .         .         .         .         .
msx-2   TTTTTCGTCGGATGAGGAGGGCCCCGCGGTACTGGCCGGCCCGGGTCCTGGGCCTG-GAGGAGC

msx2-2  TT...CGTCTGATGAGGAGGACCCCGCGGTGCTGGCCGGCCCGGGCCCCGAGTCTGTGAGGAGC
msx2-3  TT...CGTCTGATGAGGAGGGCCCCGCGGTGCTT.CCGGCCCGGGCCCCGGGCTGGAGA.AAGC
CON     TT---CGTC-GATGAGGAGG-CCCCGCGGT-CT--CCGGCCCGGG-CC-G-G---G-GA--AGC

                                                          500bp
                  .         .         .         .         .         .
msx-2   CGAGGGCAGCGC.AGAGGAGCGCAGGGTCAAGGTCTCCAGCCTGCCCTTCAGCGTGGAGG.CGC

msx2-1                         CGCAGGGTCAAGGTCTCCAGCCTGCCCTTCAGCGTGGAGGGCGC
msx2-2  CGAAGTCGGCGCGGGAGGAGTGAAGTT-------------------------------------
msx2-3  CCAGGGTGGCGCG-----------------------------------------------------
msx2-6                    GGAGCGCAGGGTCAAGGTCTCCAGCCTGCCCTTCAGCGTGGAGG.CGC
CON     C-A-G---GCGC--GAGGAG---AG--TCAAGGTCTCCAGCCTGCCCTTCAGCGTGGAGG-CGC


                  .         .         .         .         .         .
msx-2   TCATGTCCGACAAGAAGCCGCCCAAGGAATCGCCCGCGGTGCCACCCGACTGCGCCTCGGCTGG

msx2-1  TCATGTCCGACAAGAAGCCGCCCAAGGAATCGCCCGTGGTGCCACCCGACTGCGCCTCGGCTGG
msx2-4                                        GCGGTGCCACCCGACTGCGCCTCGGCTGG
msx2-5                                  ATCGCCCGCGGTGCCACCCGACTGCGCCTCGGCTGG
msx2-6  TCATGTCCGACAAGAAGCCGCCCAAGGAATCGCCCGCGGTGCCACCCGACTGCGCCTCGGCTGG
CON     TCATGTCCGACAAGAAGCCGCCCAAGGAATCGCCCG-GGTGCCACCCGACTGCGCCTCGGCTGG
```


msx-2 CGCTGTCCTGCGGCCGCTGCTGCTGCCGGGACACGGCGTCCGGGACGCTCACAGTCCCGGGCCT
msx2-1 CGCTGTCCTACGGCCGCTGTTGCTGCCGG.ACACGGTGTCC----------
msx2-4 CGCTGTCCTGCGGCCGCTGTTGCTGCCGGGACACGGCGTCCGGGACGCTCACAGTCCCGGGCCT
msx2-5 CGCTGTCCTGCGGCCGCTGTTGCTGCCGGGACACGGTGTCCGGGACG.TCACAGTCCCGGGCCT
msx2-6 CGCTGTCCTGCGGCCGCTGTTGCTGCCGGGACACGGTGTCCGGGGCGCTCACAGTCCCGG.CCT
CON CGCTGTCCT-CGGCCGCTG-TGCTGCCGG-ACACGG-GTCCGGG-CG-TCACAGTCCCGG-CCT
msx-2 CTCGTCAAGCCCTTCGAGACCGCCTCGGTCAAGTCGGAAAATTCCGAAGACGGAGCACCGTGGA
msx2-4 CTCGTCAAGACCTTCGAGACCGCCTCGGTCAAGTCGGAAAATTCCGAAGACGGAGCGCCGTGGA
msx2-5 CTCGTAAAGCCCTTCGAGACCGCCTCGGTCAAGTCGGAAAATTCCGAAGACGGGCGCCCGTGGA
msx2-6 CTCGTAAAGCCCTTCGAGACCGCTTCGGTCAAGTCGGAAAATTTCGAAGACGGAGCGCCGTGGA
CON CTCGT-AAG-CCTTCGAGACCGC-TCGGTCAAGTCGGAAAATT-CGAAGACGGA---CCGTGGA
750bp
msx-2 TACAGGAGCCCGGCAGATA.CTCCCCGCCGCCCAGACATATGAGCCCCACCACCTGCACCCTGA
msx2-4 TACAGGAGCCCGGCAGATAATTCCCCGCCGCCCAGACACATGAGCCCCACCACCTGTACCCTGA
msx2-5 TAAAGGTGCCCGGAAGATA.TTCCCCGCCGCCAAGAACAATGAGCCCAACCAA----------
msx2-6 TAACAAGAGCCCGGAGATAATTTCC----------
CON TA-------CC-G-AGATA--T-CCCGCCGCC-AGA---ATGAGCCC-ACCA-CTG-ACCCTGA
msx-2 GGAAACACAAGA.CCAACCGGAAGCCACGCACACCCTTCACCACATCCCAGCTTCTAGCCTTGG
msx2-4 GGAAACACAAGAACCAA----------
CON GGAAACACAAGA-CCAA.....GCCACGCACACC-TTCACCAC-TCCCAGCTTCTAGCCTTGG
msx-2 AGCGCAAGTTCCGCCAGAAACAGTACCTGTCCATAGCAGAGCGGGCCGAGTTCTCCAGCTCTCT
CON AGCGCAAGT-CCG-CAGAAACAGTACCT-TCCAT-GCAGAGCGGGCCGAGTT-TCCAGCTCTCT
msx-2 G.....AGGTCAAAATCTGGTTCCAGAACCGAAGGGCTAAGGCGAAAA.GAC
CON G.....AGGTCAAAAT-TGG-T-C--AA-C--A-GG-T-A-G--AAAA-GAC
1000bp
msx-2 TGCAAGAGGCG..GAA.CTGGAAAAGCTGAAAATGGCTGCCAAGCCTATG----------
CON T-CA-G--G-G--GAA-CTGG-AAA--TGAAAA----------

The lengths of the cDNA inserts as determined by agarose gel electrophoresis are very similar to the predicted lengths when the sequence data obtained for the clones is aligned with the full length cDNA (about 1550bp), indicating that all the clones obtained are not truncated at the 3' end (table 2.7). A single clone (pmsx2-1) was then selected for non radioactive whole mount *in situ* hybridisation on rat pelage follicles.

3:1.2 Whole Mount *In Situ* Hybridisation of Rat Pelage Follicles.

Of the clones isolated from the end bulb library during the course of this and other studies, *msx*-2, *BMP*-2 and *BMP*-4 have been shown to be particularly important in follicle initiation and development, having temporally and spatially overlapping expression patterns, though their role in the adult follicle is less clear. Non radioactive *in situ* hybridisation was performed on rat pelage follicles to determine the expression patterns of these genes in adult follicles. The 1kb *msx*-2 clone used for ISH was isolated as described in section 3:1.1, and the 1.7kb *BMP*-2 and *BMP*-4 clones were isolated from the end bulb library as described by Sleeman (1995). Almost all of the pelage follicles used for ISH were in anagen. To demonstrate that the ISH method used was effective on rat pelage follicles, the constitutively expressed 16s rRNA was used as a control probe to show that penetration of probe through all the layers of the follicle was occurring. Follicles hybridised with a 16s rRNA probe are shown in figure 2.6a (whole follicle) and 2.6b (transverse section). A strong hybridisation signal is obtained throughout the hair follicle with this probe, with the exception of the keratinised hair fibre itself, in which the cells are no longer living. Negative control follicles hybridised to 16s RNA probe digested with RNase A, and sense *msx*-2, *BMP*-2 and *BMP*-4 probes gave no hybridisation signal in the base of the follicles studied (not shown). However, most of the follicles studied showed an artefactual hybridisation signal in the upper portion of the hair fibre itself, whether they were probed with antisense, sense or no probe at all. This is quite common with both whole mount and sections of hair follicles, and is seen with both radioactive and non-radioactive probes. This effect may be due to probe becoming "trapped"

in the meshwork of keratin filaments in the hair fibre during the long hybridisation step, which the relatively short post-hybridisation washes cannot remove.

3:1.2-1 msx-2

Msx-2 expression in adult rat anagen pelage follicles was studied by ISH using an RNA probe transcribed from a 1kb rat msx-2 cDNA clone. In contrast to the result obtained with the 16s probe, very little expression was observed in the lower portions of the follicle. The hybridisation signal appeared to be confined largely to the epidermis around the base of the end bulb (figure 2.6c), with little or no staining observed in other regions of the follicle. Of the three genes studied, msx-2 had the most restricted expression pattern in anagen pelage follicles.

3:1.2-2 BMP-2

BMP-2 expression in adult rat pelage follicles was studied by ISH using an RNA probe transcribed from a 1.7kb rat BMP-2 cDNA clone. In anagen, a strong hybridisation signal was obtained in a band around the epidermis of the end bulb above the level of the germinative cells (figure 2.6d). However, in telogen, this pattern of hybridisation was not apparent (figure 2.6e), and expression appeared to be limited to the lower outer root sheath, around the club fibre.

3:1.2-3 BMP-4

BMP-4 expression in adult rat anagen pelage follicles was studied by ISH using an RNA probe transcribed from a 1.7kb rat BMP-4 cDNA clone. This probe gave the strongest hybridisation signal in anagen pelage follicles, with heavy staining apparent throughout the end bulb of all follicles studied (figure 2.6f).

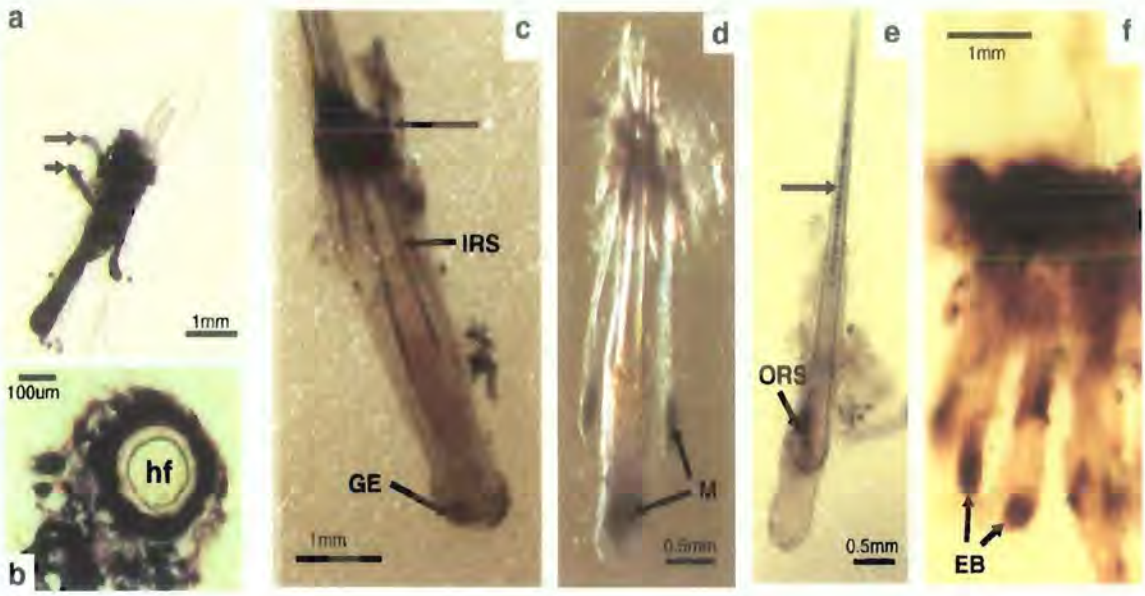


Figure 2.6: Whole mount non radioactive *in situ* hybridisation of rat pelage follicles with msx-2, BMP-2 and BMP-4 probes.

a: Whole follicles hybridised to a 16s rRNA probe, showing strong hybridisation throughout. The labelling is particularly strong in the end bulbs (arrowed).

b: A transverse section through a follicle hybridised to a 16s rRNA probe. The labelling is strong through all the layers of the follicle except the keratinised epidermis making up the hair fibre itself (hf), in which the cells are no longer living. This indicates that the probe is penetrating through the follicle at least to the level of the hair fibre.

c: An anagen follicle hybridised to a rat msx-2 probe. Little staining is apparent in most of the dermal and epidermal tissues; however, labelling is present in the epidermis around the base of the end bulb (GE) and in the inner root sheath above the middle of the follicle (IRS). The staining in the tissue around the top of the follicle (arrowed) is non specific, common to several of the probes used in this study.

d: Anagen follicles hybridised to a rat BMP-2 probe. Strong staining is evident in the epidermis of the end bulb (M) above the GE cells. It appears that there is little overlap between regions hybridising to the msx-2 and BMP-2 probes.

e: A telogen follicle hybridised to a rat BMP-2 probe. There is now no evidence of BMP-2 staining in the end bulb, with all the labelling appearing in the ORS at the base of the club fibre (ORS). This follicle shows the typical pattern of non-specific staining seen in the hair fibre itself (arrowed).

f: A clump of pelage follicles hybridised to a rat BMP-4 probe. Staining is particularly apparent throughout the end bulbs (EB) of these follicles, in a similar pattern to BMP-2.

3:1.3 Macrophage Migration Inhibitory Factor (MIF)

The rat homologue of macrophage migration inhibitory factor was isolated from the vibrissa follicle end bulb cDNA library by Dr. M. A. Sleeman using a differential screening technique (Sleeman, 1995). Since the full length rat MIF cDNA sequence had not been reported and expression of this gene had not been shown in adult hair follicles or skin at the time of this study, it was included in further work.

3:1.3-1 Sequence Of MIF cDNA Isolated From Adult Rat Vibrissa Follicles.

Full length rat MIF cDNA was obtained from rat vibrissa follicle end bulb total RNA by 5'RACE-PCR, as described in section 2:11. Three overlapping clones designated masCL-32, MIF-B2 and MIF5'-7 had inserts of 413, 237 and 100bp respectively, giving a full length cDNA of 551bp, with a 5' untranslated region (UTR) of 83bp. The sequencing strategy performed is shown in figure 2.7. This represented the full length rat MIF cDNA. A comparison between full length rat, mouse and human MIF sequences is given in figure 2.8.

Table 2.8: cDNA sequence identity between regions of the rat, mouse (Mitchell *et al* 1995) and human (Paralkar and Wistow 1994) transcripts

cDNA Segment	**% identity rat: mouse**	**% identity rat: human**	**length (rat: mouse: human)**
5'UTR	85.9%	42.9%	83bp: 85bp: 101bp
ORF	97.1%	89.7%	348bp
3'UTR	80.9%	43.7%	119bp: 120bp: 126bp
Full length	92.2%	70.7%	551bp: 553bp: 575bp

Figure 2.7a: Sequencing strategy used to obtain full length rat MIF cDNA sequence. The boxes labelled MIF5'-7, MIF-B2 and masCL-32 represent the three clones obtained in this study, with the arrows above and below indicating the direction of sequencing. Boxes 1 and 2 with associated bold arrows indicate the position of the two 5' RACE primers used to obtain the full length cDNA. The position of the open reading frame (ORF) and untranslated regions (UTR's) are indicated at the top of the figure.

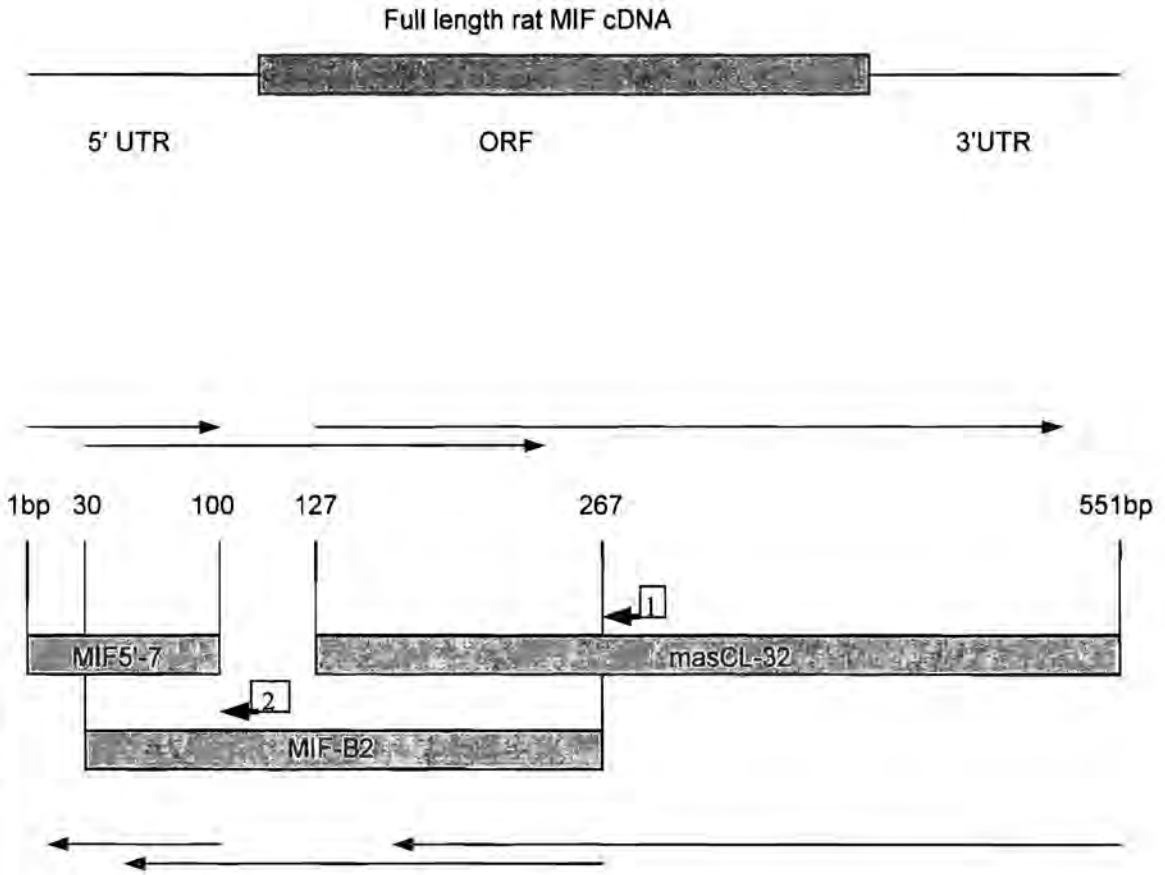
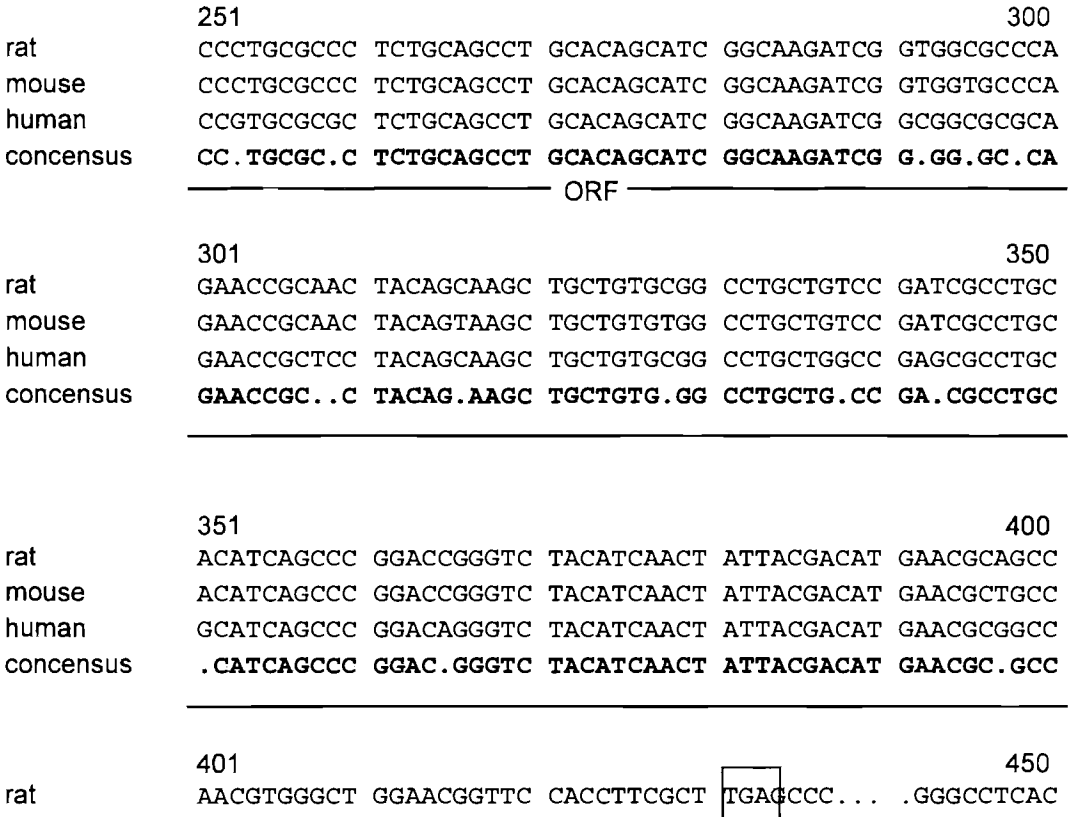


Figure 2.7b: Sequence of full length rat MIF cDNA. The open reading frame is indicated in upper case, with the predicted protein sequence shown below the DNA sequence in single letter code. The potential stable stem-loop structure is underlined, and the polyadenylation signal is indicated in bold type.

```
1
gggtcacgtagtcaggtcccagacttgggtcacaccgcacttaacaccgtcctccggccg

61
tcgttcgcagtctctccgccaccATGCCTATGTTCATCGTGAACACCAATGTTCCCCGCG
                        M  P  M  F  I  V  N  T  N  V  P  R  A

121
CCTCCGTGCCAGAGGGGTTTCTCTCCGAGCTCACCCAGCAGCTGGCGCAGGCCACCGGCA
  S  V  P  E  G  F  L  S  E  L  T  Q  Q  L  A  Q  A  T  G  K

181
AGCCGGCACAGTACATCGCAGTGCACGTGGTCCCGGACCAGCTCATGACTTTTAGTGGCA
  P  A  Q  Y  I  A  V  H  V  V  P  D  Q  L  M  T  F  S  G  T

241
CGAGCGACCCCTGCGCCCTCTGCAGCCTGCACAGCATCGGCAAGATCGGTGGCGCCCAGA
  S  D  P  C  A  L  C  S  L  H  S  I  G  K  I  G  G  A  Q  N

301
ACCGCAACTACAGCAAGCTGCTGTGCGGCCTGCTGTCCGATCGCCTGCACATCAGCCCGG
  R  N  Y  S  K  L  L  C  G  L  L  S  D  R  L  H  I  S  P  D

361
ACCGGGTCTACATCAACTATTACGACATGAACGCAGCCAACGTGGGCTGGAACGGTTCCA
  R  V  Y  I  N  Y  Y  D  M  N  A  A  N  V  G  W  N  G  S  T

421
CCTTCGCTTGAgcccgggcctcacttacctgcaccgctgttcttcgagtcttgctgcacg
  F  A  *

481
ccccgttctgtgtttatccacccgtaatgatggccaccttccggtcgggagaataaaat

541
ggtttgagacc
```

Figure 2.8: A comparison between full length rat, mouse and human MIF cDNA sequences. DNA sequence alignment between full length rat, mouse (Mitchell *et al* 1995) and human (Paralkar and Wistow 1994) MIF cDNA sequences. The start codons are in bold type, the stop codons are boxed, the polyadenylation signals are underlined and the open reading frame (ORF) is arrowed. A putative stable stem-loop structure at the 5' end of the rat and mouse sequences is indicated by the shaded box. Sequence identity (concensus) is shown below the alignment.

```
                1                                                    50
rat             CGCGTCACGT AG.TCAGGTC CCAGACTTGG GTCAGACCGC ACTTAACACC
mouse           TGCGTCACGT AGCTCAGGTC CCTGGCTTGG GTCAGACCGC GCTTTGTACC
human           .......... .......... .......... .....CTCGA GCTGCAGAGC
concensus       .......... .......... .......... .......CG. .CT....A.C

                51                                                   100
rat             GTCCTCCGGC CGTCGTTCGC AGTCTCTCCG CCACCATGCC TATGTTCATC
mouse           GTCCTCCGGT CCACGCTCGC AGTCTCTCCG CCACCATGCC TATGTTCATC
human           TGCCTCTGCG CGGGTCTCCT GGTCCTTCTG CCATCATGCC GATGTTCATC
concensus       ..CCTC.G.. C.....TC.. .GTC..TC.G CCA.CATGCC .ATGTTCATC
                                                              <-------

                101                                                  150
rat             GTGAACACCA ATGTTCCCCG CGCCTCCGTG CCAGAGGGGT TTCTCTCCGA
mouse           GTGAACACCA ATGTTCCCCG CGCCTCCGTG CCAGAGGGGT TTCTGTCGGA
human           GTAAACACCA ACGTGCCCCG CGCCTCCGTG CCGGACGGGT TCCTCTCCGA
concensus       GT.AACACCA A.GT.CCCCG CGCCTCCGTG CC.GA.GGGT T.CT.TC.GA
                ---------------------------------------------------------

                151                                                  200
rat             GCTCACCCAG CAGCTGGCGC AGGCCACCGG CAAGCCGGCA CAGTACATCG
mouse           GCTCACCCAG CAGCTGGCGC AGGCCACCGG CAAGCCCGCA CAGTACATCG
human           GCTCACCCAG CAGCTGGCGC AGGCCACCGG CAAGCCCCCC CAGTACATCG
concensus       GCTCACCCAG CAGCTGGCGC AGGCCACCGG CAAGAA..C. CAGTACATCG
                ---------------------------------------------------------

                201                                                  250
rat             CAGTGCACGT GGTCCCGGAC CAGCTCATGA CTTTTAGTGG CACGAGCGAC
mouse           CAGTGCACGT GGTCCCGGAC CAGCTCATGA CTTTTAGCGG CACGAACGAT
human           CGGTGCACGT GGTCCCGGAC CAGCTCATGG CCTTCGGCGG CTCCAGCGAG
concensus       C.GTGCACGT GGTCCCGGAC CAGCTCATG. C.TT..G.GG C.C.A.CGA.
                ---------------------------------------------------------
```


251
300
rat CCCTGCGCCC TCTGCAGCCT GCACAGCATC GGCAAGATCG GTGGCGCCCA
mouse CCCTGCGCCC TCTGCAGCCT GCACAGCATC GGCAAGATCG GTGGTGCCCA
human CCGTGCGCGC TCTGCAGCCT GCACAGCATC GGCAAGATCG GCGGCGCGCA
concensus CC.TGCGC.C TCTGCAGCCT GCACAGCATC GGCAAGATCG G.GG.GC.CA
ORF
301
350
rat GAACCGCAAC TACAGCAAGC TGCTGTGCGG CCTGCTGTCC GATCGCCTGC
mouse GAACCGCAAC TACAGTAAGC TGCTGTGTGG CCTGCTGTCC GATCGCCTGC
human GAACCGCTCC TACAGCAAGC TGCTGTGCGG CCTGCTGGCC GAGCGCCTGC
concensus GAACCGC..C TACAG.AAGC TGCTGTG.GG CCTGCTG.CC GA.CGCCTGC
351
400
rat ACATCAGCCC GGACCGGGTC TACATCAACT ATTACGACAT GAACGCAGCC
mouse ACATCAGCCC GGACCGGGTC TACATCAACT ATTACGACAT GAACGCTGCC
human GCATCAGCCC GGACAGGGTC TACATCAACT ATTACGACAT GAACGCGGCC
concensus .CATCAGCCC GGAC.GGGTC TACATCAACT ATTACGACAT GAACGC.GCC
401
450
rat AACGTGGGCT GGAACGGTTC CACCTTCGCT TGAGCCC... .GGGCCTCAC
mouse AACGTGGGCT GGAACGGTTC CACCTTCGCT TGAGTCC... .TGGCCCCAC
human AGTGTGGGCT GGAACAACTC CACCTTCGCC TAAGAGCCGC AGGGACCCAC
concensus A..GTGGGCT GGAAC...TC CACCTTCGC. T.AG..C... ..GG.C.CAC
451
500
rat TTACCTGCAC CGCTGTTCTT CGAGTCTTGC TGCACGCCCC GTTCTGTGTT
mouse TTACCTGCAC CGCTGTTCTT TGAGCCTCGC TCCACGTAGT GTTCTGTGTT
human GCTGTCTGCG CTGGCTCCAC CCGGGAACCC GCCGCACGCT GTGTTCTAGG
concensus .... C....T.C.. ...G....GC ..C.C..... GT..T.T...
501
550
rat TATCCACCCG TAATGATGGC CACCTTCCGG TCGGGAGAAA TAAATGGTTT
mouse TATCCACCGG TAGCGATGCC CACCTTCCAG CCGGGAGAAA TAAATGGTTT
human CCCGCCCACC C.....C AACCTTCTGG TGGGGAGAAA TAAACGGTTT
concensus ....C.C... .....C .ACCTTC..G ..GGGAGAAA TAAA..GTTT
551
rat GAGACC.... ....
mouse ATAAGAG... ....
human AGAGACAGCT CTGCAG.... ....
concensus ....

3:1.3-2 Northern Blotting

A northern blot of adult rat liver, skin and vibrissa follicle end bulb total RNA probed with a ^{32}P labelled rat MIF cDNA clone confirmed that MIF mRNA is expressed in these tissues, present as a single transcript of approximately 600 nucleotides (nt). (figure 2.9). Liver has been shown to express high levels of MIF (Sakai *et al* 1994), and MIF expression in skin has recently been shown by Shimizu *et al* (1996).

3:1.3-3 Western Blotting

An antibody raised against human MIF (Zeng *et al* 1993) was used to detect rat and human MIF in total protein extracts of rat and human tissue. A western blot of adult human follicle total protein, adult rat vibrissa follicle total protein, adult human skin total protein and adult rat skin total protein extracts revealed a single protein of approximately 13kDa (figure 2.10a). A comparison of equal amounts of total protein from balding and non bald areas of human scalp skin showed that MIF was more highly expressed in bald areas, though significant amounts were present in both samples. (figure 2.10b). A western blot of equal amounts of total protein extracted from 14.5dpc, 16dpc, and 18dpc embryonic rat skin revealed that MIF was expressed in embryonic skin at 18dpc (figure 2.10c).

3:1.3-4 Localisation of MIF in anagen follicle end bulbs by immunofluorescence.

Preliminary studies to localise MIF in human anagen follicles by indirect immunofluorescence using an antibody to human MIF (Zeng *et al*, 1993) revealed that during anagen, MIF was localised in the epidermis immediately above the dermal papilla in a very restricted pattern (figure 2.11).

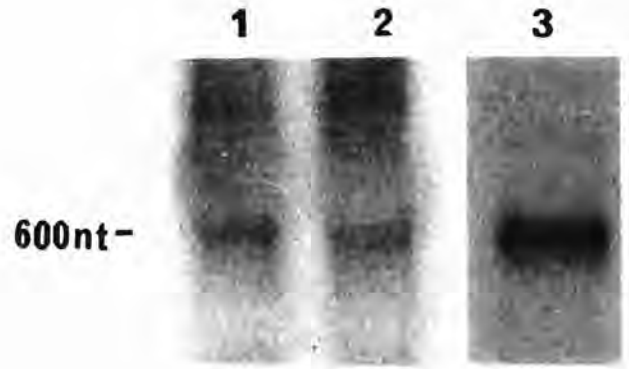


Figure 2.9: Northern blots of total RNA from rat liver (lane1), skin (lane 2) and vibrissa follicle end bulb (lane 3), probed with a 32-P labelled MIF cDNA probe. A single transcript of ~600 nucleotides (nt) is present in each case.

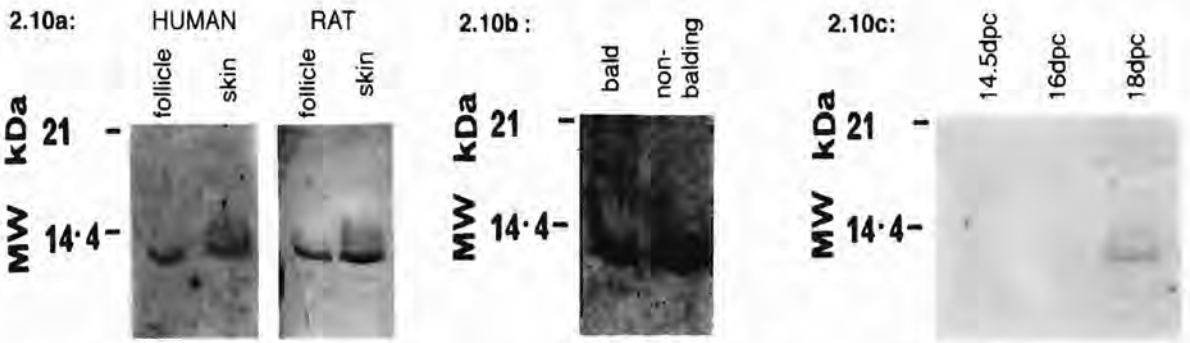


Figure 2.10: Western blots of total protein extracts probed with an antibody to human MIF.
a: Western blot of adult rat and human skin and follicle total protein (100ug/lane) with anti-human MIF antibody (Zeng *et al* 1993) diluted 1/500 in PBS. The antibody cross-reacts with a single protein of 13kDa.
b: A comparison of 100ug total protein from balding and non-balding human scalp skin suggests that MIF may be more highly expressed in balding skin.
c: A comparison of 20ug total protein extracted from 14.5dpc, 16dpc and 18dpc embryonic rat skin shows that MIF is most highly expressed at 18dpc.

Figure 2.11: A 5um section through a human scalp follicle (anagen) stained with an antibody to human MIF (Zeng *et al* 1993) diluted 1/40 in PBS. The section was counterstained with Evans Blue.
MIF staining (arrowed) appears only in the epidermis at the apex of the papilla (DP), in a very restricted pattern.

Bar = 100um

4: DISCUSSION

4:1 Production of a cDNA Library Containing Clones Specific To The Vibrissa Follicle End Bulb.

We have produced cDNA libraries derived from rat skin in which all the follicles are in anagen, whole vibrissa follicles from all stages of the hair growth cycle, and vibrissa follicle end bulbs from all stages of the hair growth cycle. All the work described in this study is based on screening of the end bulb cDNA library, which has the largest inserts, the lowest proportion of non-recombinant clones and the highest representation of housekeeping type genes (see library test results, table 2.5). Since we used end bulbs from all stages of the hair growth cycle, all the genes which are expressed in the tissues of the end bulb throughout all stages of anagen, catagen and telogen should be represented to a greater or lesser degree in this library.

All of the clones that have been isolated from the libraries so far have been truncated at the 5' end. This may be due to partial degradation of the mRNA during preparation and cDNA synthesis, or the presence of particularly stable RNA secondary structure. The use of an oligo-dT primer in first strand cDNA synthesis inevitably results in over-representation of the 3' ends of cDNA's in the resulting library, and it is difficult to obtain full length cDNA, especially if the starting mRNA is greater than 3kb long. This problem can be overcome by using random hexamer primers in first strand cDNA synthesis, so that priming events are evenly distributed over the whole RNA molecule and not confined to the 3' end. However, when using random primers, the ratio of primer:RNA is important, as the higher the ratio, the shorter the resulting cDNA molecules will be, and thus more overlapping clones have to be sequenced in order to obtain full length cDNA for any given gene. A problem which may be encountered when screening a random primed library, especially if shorter probes are used, is that the probe will "miss" cDNA clones produced by priming upstream of the probe sequence. Further rounds of sequencing with "downstream" clones will then be required in order to obtain

sequence data towards the 5' end of the cDNA. The presence of RNA secondary structure will result in loss from the library of the region containing and immediately upstream of the secondary structure, whichever priming method is used. This may be overcome by using thermostable reverse transcriptases such as *Tth* DNA polymerase, which has reverse transcriptase activity in the presence of Mn^{2+}, and is stable at temperatures in excess of 90°C, since RNA secondary structure is denatured at temperatures above 70°C.

A limitation of using cDNA library screening to isolate and characterise genes expressed in a particular tissue is that the cDNA clones isolated are copies of the mRNA and not the gene itself. Therefore, no information about intron position and sequence or upstream promoters can be obtained directly from cDNA library screening. However, cDNA clones can be used as highly specific probes for the isolation of the desired genomic sequences, and clones obtained from cDNA libraries can be used for expression of putative open reading frames in both bacterial and mammalian systems.

Several methods have recently been described for the construction of PCR generated cDNA libraries from small amounts of tissue, enabling the cloning of genes specifically expressed at the level of very small cell populations. Belyavsky *et al* (1989) described a method based on amplification of total cDNA following first strand synthesis from total RNA using a poly-T primer. Library production based on the use of total RNA as the template for a PCR must be primed from the polyadenylated region of the mRNA, since the use of random primers will result in extensive amplification of ribosomal RNA sequences. The amplification of all first strand cDNA's regardless of their 5' sequence was achieved by the addition of common priming sites to the 3' end of the first strand cDNA. This may be accomplished by either homopolymeric tailing with a single nucleotide using terminal deoxynucleotidyl transferase (TdT) (Belyavsky *et al* 1989), or by ligation of a single stranded anchor primer of known sequence to the 3' end of the first strand cDNA's using T4 RNA ligase (Edwards *et al* 1991). Homopolymeric tailing has limitations in that the TdT reaction is difficult to control, and the use of homopolymeric primers in the PCR amplifications may result in non-specific amplification and interference with the relative abundance of primary cDNA clones. Edwards *et al* (1991) suggest that methods involving single strand ligation of oligonucleotide anchors to first strand cDNA (SLIC) results in more efficient and reliable production of full length cDNA's for cloning. A number of commercially available kits have been described for production of

cDNA libraries by PCR. In particular, a novel PCR-based method for making cDNA libraries from as little as 50ng total RNA has been utilised by Clontechs Capfinder™ PCR cDNA library construction kit (Clontechniques No. 1, Jan 1996). This utilises a CapSwitch™oligonucleotide attached to the 5' cap at the end of the mRNA molecule. Briefly, the mRNA is reverse transcribed from an oligo-dT primer at the 3' end, and when the RT reaches the 5' end of the mRNA, the enzyme switches templates and continues replication to the end of the CapSwitch oligonucleotide. The resulting full length single stranded cDNA contains the complete 5' end of the mRNA, as well as the sequence complimentary to the CapSwitch oligonucleotide. This then serves as a universal anchor in subsequent PCR reactions. The major advantage of this procedure is that truncated first strand cDNA's and cDNA produced from rRNA or contaminating genomic sequences will lack the CapSwitch anchor sequence and so cannot be amplified, virtually eliminating library contamination by polyA- and genomic sequences. This method could therefore be used in future to generate libraries from limited tissue, for example DP or GE cells alone, or telogen vibrissa follicle end bulbs.

A method has also been described for global amplification of mRNA from single cells using poly-A primed RT-PCR method (Brady and Iscove 1993). This method is designed to generate a pool of 3'EST's which can be subsequently screened and/or sequenced, and compared with cDNA pools from cells from related lineages. This method may therefore become a useful tool for the analysis of lineage- and stage- specific gene expression, and may identify genes which distinguish between progenitor cells at different stages of commitment.

4.2 Non Radioactive In Situ Hybridisation Using DIG Labelled Antisense RNA Probes on Whole Pelage Follicles From Adult Wistar Rats.

The detection of specific nucleic acid sequences by *in situ* hybridisation has important applications in many areas of biology, ranging from studies of the spatial and temporal regulation of gene expression in multiple tissues to genome mapping and analysis of chromosomal rearrangements. We have used *in situ* hybridisation to study the spatial

localisation of three genes thought to be important in hair follicle development in adult pelage follicles.

Many different methods detailing probe and tissue preparation for *in situ* hybridisation have been reported. Probes may be RNA, DNA or short oligonucleotide, with radioactive or non radioactive labels, and tissue under study can be fixed, permeabilised and probed using several different techniques. However, it is of primary importance that the target nucleic acid is retained *in situ*, is not degraded, and is accessible for hybridisation to the probe. Understanding of hybridisation parameters under the conditions encountered for *in situ* hybridisation is still poor, and many methodological variations are empirical.

Recently, Steel *et al* (1998) described a comparison of radioactively and non-radioactively labelled cRNA probes used for *in situ* hybridisation of various mRNA targets on paraffin embedded tissue sections. They found that although ^{35}S labelled probes gave excellent contrast, sensitive detection and low background under dark field illumination, the resolution obtained was poorer than that obtained with digoxygenin labelled probes, which could localise the target sequences to regions of individual cells. However, although the sensitivity obtained with digoxygenin labelled probes was equivalent to that obtained with ^{35}S, especially when the target sequence was abundant, the contrast between positive signal and background was less obvious. Therefore, they concluded that the choice of probe labelling method was dependent on the resolution required.

A more sensitive method for *in situ* hybridisation based on deposition of biotinylated tyramine at the location of the ISH probe hybridisation site has been described by several groups (Poddighe *et al* 1996, Hacker 1998). This catalysed reporter deposition (CARD) method is a useful tool for routine detection of low copy targets such as viral sequences, which are difficult to detect by conventional *in situ* hybridisation.

A comparison of RT-PCR, conventional ISH and *in situ* RT-PCR was performed by Bates *et al* (1997). This demonstrated that *in situ* RT-PCR was the most sensitive of these techniques for the detection of low titer rhinovirus infection in paraffin embedded human tissue sections, although the thermal cycling technique involved resulted in a slight loss of morphology and fine localisation when using this method.

All of the methods described here involve paraffin sections and low copy target sequences. Such techniques are constantly being refined to produce even greater sensitivity

and resolution. However, it can be difficult to reconstruct a complex three dimensional expression pattern from the signals seen in sectioned tissue, and interpretation of sections through more complex organs is dependant on the quality and availability of morphological markers to determine the position of any particular section relative to the whole organ. It is for these reasons that we decided to use a whole mount procedure to examine the expression of known genes in rat pelage follicles, even though the sensitivity of detection is lower than for hybridisation to sectioned tissues, since analysis of the spatial expression of genes is particularly important in studies following development and follicle cycling (Wilkinson 1992, Tautz *et al* 1992). Many genes are known to be switched on and off in a temporally asymmetric pattern, and growth of the fibre itself is "switched off" at one side of the matrix first. It is therefore important that *in situ* hybridisation methods should be able to show this asymmetry in the whole follicle, as this eliminates the need for standardised orientation of follicle sectioning, the quality of which varies widely between laboratories. The whole mount technique described here is therefore a useful tool for studying the spatial expression of genes in the follicle as the hair growth cycle proceeds.

A particular problem associated with whole mount procedures is the difficulty of ensuring complete probe diffusion throughout the whole mount. Digestion and permeabilisation steps must be included in the protocol, along with some degree of probe hydrolysis. These introduce their own problems, especially in particularly tough tissues like skin and hair, in that a balance must be struck between permeability of the tissue and preservation of morphology and target sequences.

The complete penetration of the follicles used in this study was demonstrated using a constitutively expressed 16s rRNA probe, which stained all tissues of the follicle except the keratinised hair fibre, as expected. The retention of target sequences in particular cell layers was demonstrated using various mouse and rat hair keratin probes (data not shown). The major problem found in interpreting the results of this technique was in compartmentalising the expression patterns produced - when looking through the thickness of a follicle, it is difficult to determine in exactly which tissue layer the expression is occurring. This could be overcome by examining transverse sections taken through the follicles after staining.

4:3 *msx-2*

The vibrissa follicle end bulb cDNA library was tested by screening with a *msx*-homeobox specific probe to demonstrate that the library contains clones derived from *msx* genes, as these are known to be expressed in the hair follicle. All six positive clones isolated from the library screen were shown to be derived from rat *msx-2*. Non-radioactive whole mount *in situ* hybridisation analysis using a rat msx-2 probe on rat pelage follicles showed that msx-2 expression in adult anagen follicles appears to be localised specifically in the epidermis of the lower end bulb.

Studies of *Drosophila* homeobox genes have shown that homeobox containing genes are involved in controlling pattern formation and segment identity. Class I homeobox containing genes (*hox*) play a key role in determination of the anterior-posterior axis, segment formation and polarity, and the identities of the segments themselves during insect development (reviewed in Ingham 1988). Homeobox genes are also involved in normal growth control and tumourigenesis (Song *et al* 1992). A common feature of all homeobox genes is a conserved 180bp nucleotide sequence encoding a helix-turn-helix protein domain which has been shown to bind DNA (Scott *et al* 1989). Since their discovery in *Drosophila*, homeobox genes have also been found in vertebrates. Extensive analysis in the mouse has yielded the sequences and chromosomal locations of many murine homeobox containing genes. A second group of homeobox genes have also been isolated in both *Drosophila* and vertebrates which are not involved in segmentation, but appear to play a more important role in organogenesis and cell differentiation. This family includes the *Drosophila* muscle segment homeobox (*msh*) gene and the related vertebrate *msx* genes. The *msx-1* and *-2* homeobox containing genes (formerly *hox-7* and *-8*) belong to the *Drosophila msh*-like family. In chicken and mouse embryonic development, where their expression pattern has been studied in detail, they are expressed in overlapping but distinct sites in a variety of organs including the developing skin appendages (MacKenzie *et al* 1991a, 1991b, 1992, Jowett *et al* 1993, Noveen *et al* 1995, Stelnicki *et al* 1997). During the development of skin appendages, *msx* gene expression is associated with epithelial-mesenchymal interactions in a highly dynamic fashion, suggesting that these genes may play a role in inductive events in embryonic development. A

recent study by Stelnicki *et al* (1997) demonstrated that msx-1 and msx-2 are differentially expressed in the dermis and epidermis of fetal and adult skin. Msx-1 and -2 expression was localised to the epidermis, hair follicles and dermal fibroblasts of fetal skin by *in situ* hybridisation, with expression in the adult confined to hair follicle epidermis. Stelnicki *et al* (1998) also demonstrated that several HOX homeobox genes are expressed during human fetal skin development using RT-PCR of RNA from defined cell populations in developing human skin. Strong *hoxa4* expression was detected in the basal layer of ten week fetal epidermis, and throughout the skin of 17 week embryos. Hox a5 and hoxa7 were detected in a similar pattern, but with weaker expression, and hoxb4, hoxb7 and hoxc4 were also detected. Differential hox gene expression was observed in developing hair follicles, sebaceous glands and sweat glands. However, none of the hox genes examined were detected in the adult dermis, with very weak expression of hoxa5 and hoxa7 apparent in adult epidermis. These data imply that individual homeobox genes each play a specific role in skin and follicle development. A similar study was performed on embryonic chick skin by Kanzler *et al* (1997). This demonstrated that hoxc-8 was expressed in dorsal dermal and epidermal cells between E6.5 and E8.5 during the first stages of feather morphogenesis, and hoxd-13 was expressed in the dermis between E10.5 and E12.5. However, expression of both of these genes disappears shortly after anlage. It was also shown that the ability of the epidermis to form feathers is already established at the time of skin formation, using a series of heterotypic dermal/epidermal recombinations.

The isolation of six msx-2 clones from an adult rat vibrissa follicle end bulb library indicates that msx-2 is reasonably highly expressed in this tissue. From the sequence data generated from these six clones, it appears that two (pmsx2-2 and pmsx2-3) are derived from the same clone in the initial unamplified library. In order to localise the expression of msx-2 to a particular region of the end bulb, we performed whole mount *in situ* hybridisation on rat pelage follicles. This demonstrated that msx-2 expression is confined to the lower epidermis of the end bulb. This has also been shown by Stelnicki *et al* (1997).

4:4 Bone Morphogenetic Proteins

We used whole mount non radioactive *in situ* hybridisation to localise the expression of BMP-2 and BMP-4 in adult rat pelage follicles. Bone morphogenetic proteins (BMP's) were originally identified from extracts of bone by their capacity to induce ectopic bone formation. There are several bone morphogenetic proteins - members of the TGF-β superfamily, of which BMP-2 and BMP-4 are the most conserved, having 92% identity at the carboxy terminus. They are also strongly similar to the *Drosophila* gene *decapentaplegic dpp* (Padgett *et al* 1987), and the *Xenopus Vg-1* gene (Weeks and Melton 1987). BMPs, in common with other members of the TGFβ superfamily, are synthesised as large precursor molecules which are cleaved to release carboxy-terminal domains containing a characteristic motif of seven conserved cysteines. Biologically active BMPs are homo- or hetero-dimers of these C-terminal domains (reviewed in Kingsley 1994). It is clear that BMPs are highly conserved throughout evolution, and that related genes can perform similar functions in very different organisms. For example, *Drosophila* dpp can induce endochondral bone formation in mammals, and mammalian BMPs can replace dpp in *Drosophila* (Padgett *et al* 1993, Sampath *et al* 1993). These proteins have also been shown to be crucial for tissue interactions and differentiation (Winnier *et al* 1995, reviewed in Tickle 1994).

Both BMP2 and BMP4 are widely expressed throughout embryogenesis (Lyons *et al* 1990, Jones *et al* 1991), though BMP4 has greater association with the mesoderm at sites of dermal-epidermal interactions (Jones *et al* 1991, Vainio *et al* 1993), whereas BMP2 adopts a more epithelial expression (Lyons *et al* 1990). Receptors for BMP-2 and -4 have also been localised to many tissues in the developing embryo (Dewulf *et al* 1995). BMP-6 has also been identified in developing murine skin, from 15dpc, in post mitotic keratinocytes undergoing terminal differentiation in the interfollicular epidermis (Lyons *et al* 1989, Wall *et al* 1993).

BMPs have been shown to have a variety of functions in various cell types, for example, they have been shown to be chemotactic for monocytes (Cunningham *et al* 1992), to have a regulatory role in keratinocyte differentiation (Drozdoff *et al* 1994), and they have been shown to stimulate collagen synthesis in osteoblasts and proteoglycan and collagen synthesis in chondroblasts (Vukicevic *et al* 1989). Recently, BMP2 has been shown to be involved in

regulation of cell adhesion in two osteogenic sarcoma cell lines, as well as chondrocytes and fibroblasts, by downregulating the expression of integrin $\alpha 3\beta 1$. BMP-2 can also decrease $\alpha 3\beta 1$ integrin mediated cell adhesion to laminin 5, and it has been shown that BMP-2 reduces the expression of $\alpha 2$ integrin in the human keratinocyte cell line HaCaT (Nissinen *et al* 1997). BMPs have therefore been shown to be involved in extracellular matrix production and cell attachment to laminin 5. BMP's may also have widespread signalling functions in organ development that have been conserved during evolution and that may be associated with the regulation of transcription factor expression.

4:5 Macrophage Migration Inhibitory Factor (MIF)

A cDNA clone isolated from the rat vibrissa follicle end bulb cDNA library (Sleeman 1995) designated masCL-32, was shown to have 93.5% homology over 398bp with the 3' end of mouse macrophage migration inhibitory factor (MIF). This study confirmed that MIF was expressed in rat vibrissa follicle end bulbs and skin by northern blotting and 5'RACE-PCR from vibrissa follicle end bulb total RNA, and reports the full length sequence of rat MIF cDNA. Using indirect immunofluorescence, MIF expression was localised in adult human anagen end bulbs. It was also demonstrated that MIF is expressed in both rat and human follicles and skin by western blotting. Furthermore, western blots of equal amounts of total protein extracted from adult and embryonic rat skin show that there is a peak of MIF expression in developing rat skin at 18dpc. Results from a similar study involving protein extracted from human male bald and non-bald scalp suggests that the level of MIF expression is raised in bald areas. These findings suggest multiple functions of MIF in hair growth and differentiation.

Recently, macrophage migration inhibitory factor (MIF) was shown to be expressed in the basal layer of normal human epidermis (Shimizu *et al* 1996). Macrophage migration inhibitory factor was one of the first lymphokines to be isolated, being found in crude culture fluids of activated T-lymphocytes (Bloom and Bennet 1966, David 1966). Until relatively recently, MIF was thought to be purely a product of circulating T-cells, with expression occurring in response to antigens. However, it is now apparent that a variety of cells express MIF (Malorny *et al* 1988, Bernhagen *et al* 1993, Wistow *et al* 1993, Calandra *et al* 1994, Sakai

et al 1994, Shimizu *et al* 1996, Wen *et al* 1996, Hirokawa *et al* 1998) and its effects extend beyond the immune system. For example, MIF has been shown to be essential for growth factor induced mitogenesis (Paralkar and Wistow 1995) and it has been proposed that MIF may be a delayed early response gene (Lanahan *et al* 1992), and may be involved in cellular proliferation control (Wen *et al* 1996). Furthermore, MIF has been shown to exhibit tautomerase activity during melanin biosynthesis (Rosengren *et al* 1996, 1997), and may in part mediate its biological effects by similar enzymatic reactions.

The human MIF gene was cloned in 1994 (Paralkar and Wistow 1994) and localised to chromosome 22q11.2 in 1997 (Budarf *et al* 1997), and the mouse MIF gene was cloned in 1995 (Kozak *et al* 1995, Mitchell *et al* 1995). Primer extension analysis by Mitchell *et al* (1995) showed that the mouse MIF mRNA has a stable 30bp stem-loop structure at its 5' end. A similar potentially stable 31bp stem-loop structure at the extreme 5' end of the full length rat MIF transcript is reported in this study.

MIF has previously been shown to be expressed in a variety of normal and abnormal tissues at various stages of development by northern or western blotting and immunohistochemical studies (Wistow *et al* 1993, Sakai *et al* 1994, Imamura *et al* 1996, Tampanaru-Sarmesiu *et al* 1997, Wada *et al* 1997).

MIF clearly belongs to a class of active polypeptides with assorted growth and mitogenic effects, and it may be involved in processes of cell growth and differentiation. This is supported by the work of Shimizu *et al* (1996) suggesting that MIF expression in human epidermis is confined to the highly proliferative basal layer.

Specific pro-inflammatory stimuli have been shown to increase the levels of MIF expression in various cell types above the level present constitutively (Neumann *et al* 1987, Malorny *et al* 1988, Czarnetzki *et al* 1989, Malorny *et al* 1990, Bernhagen *et al* 1993, Calandra *et al* 1994, reviewed in Bucala 1996). In cultured mouse 3T3 fibroblasts, MIF is induced in a delayed early response to various growth factors (Lanahan *et al* 1992), and it has been shown that MIF is highly expressed in subconfluent cultures of rat follicular dermal papilla and skin fibroblast cells (Sleeman 1995). This appears to be a culture specific phenomenon, as we have been unable to show that MIF is present in the dermal papilla during anagen. Studies using antisense MIF oligonucleotides on cultured end bulbs appear to

confirm that MIF is present in the epidermal layer adjacent to the apex of the dermal papilla during the later stages of anagen (data not shown), as indicated by the immunofluorescence data. It therefore appears that MIF expression in hair follicles is both spatially and temporally very restricted, suggesting that it may have a hair cycle dependant role in this tissue.

Paralkar and Wistow (1995) suggest that MIF has an essential role in the mitogenic response and in control of cell cycle progression, and studies of the mouse MIF gene have shown that several consensus transcription factor binding sites are present upstream of the transcription start site including the *CRE* site which has been associated with the expression of certain peptide hormones (Mitchell *et al* 1995). In spite of the apparently constitutive expression of MIF in many tissues, its upstream sequences bear few features of housekeeping-type promoters, instead appearing consistent with induction and regulation by external signals (Bozza *et al* 1995). The presence of MIF protein in all tissues studied so far may in part be explained by the presence of quantities of the protein in circulating macrophages both prior to and following their activation (Calandra *et al* 1994). However, expression of MIF at 18dpc in embryonic rat skin cannot simply be explained by an increase in macrophage numbers, as macrophages are present throughout the tissues of the embryo from 14dpc, and can be readily recruited to wound sites from 14.5dpc (Hopkinson-Woolley *et al* 1994). Several groups have suggested that MIF is involved in differentiation control and proliferation in various tissues (Lanahan *et al* 1992, Wistow *et al* 1993, Paralkar and Wistow 1995, Wen *et al* 1996) and it may fulfil a similar function in the differentiating embryonic hair follicle epidermis, as epidermal proliferation and differentiation into distinct layers is occurring in the developing rat hair follicle from around 17dpc. In the adult hair follicle, a similar process of epidermal proliferation and differentiation occurs at the start of each anagen phase. However, it has been suggested that macrophages may play an important role in the hair cycle, appearing at catagen as fibre growth is switched off (Westgate *et al* 1991).

4.6: SUMMARY

To comprehensively describe all of the molecules whose expression has been localised to the hair follicle or its component tissues throughout its development would require

more space than is available in this chapter. Instead, I have tried to briefly describe some of the more significant and well documented examples in order to demonstrate what an incredibly complex system our experimental models are based upon. More importantly, I have described and discussed several of the methods used to isolate and characterise genes expressed in particular tissues or at specific developmental stages. In any such study, the merits and disadvantages of the available methods must be carefully scrutinised in order to determine which combination of these will give the most useful and convincing information.

We have used the construction of conventional cDNA libraries from follicle tissues as the basis of this study, as a single round of reverse transcription without subsequent amplification is likely to give a more balanced representation of the genes expressed than the other methods described here, which all include some form of cDNA amplification. We decided to use a poly T primed reverse transcription reaction rather than a random priming method in order to bias the libraries produced in favour of cDNA 3' ends. This was because the methods that we subsequently intended to use to characterise particular cell types in the follicle were likely to result in the generation of large numbers of 3' EST's which we could then use for library screening. If we had adopted a random primed approach, it is possible that the screening efficiency of subsequent experiments would be reduced. The major disadvantage of this is that most of the clones obtained from this library are likely to be 5' truncations, as for all of the msx clones and the MIF clone described in this chapter. However, it was felt that missing 5' sequence could be relatively easily obtained by 5'RACE-PCR, and the disadvantages of missing 5' ends were considered less than missing 3' ends, which would directly affect results obtained in the subsequent experiments.

CHAPTER 3

DIFFERENTIAL SCREENING OF THE END BULB cDNA LIBRARY TO OBTAIN CLONES HIGHLY EXPRESSED IN THE GERMINATIVE EPIDERMIS

1: INTRODUCTION

Current approaches to understanding the molecular basis of hair growth often involves isolation and manipulation of specific follicular cell populations. Many groups have principally concentrated on the cells of the dermal papilla (DP) since this structure has been shown to be persistent throughout the life of the follicle (Wessels and Roesner 1965), and DP cells can be readily maintained in culture (Jahoda and Oliver 1981, Messenger 1984, AlmondRoesler *et al* 1997). Furthermore, isolated dermal papillae and cultured papilla cells retain the ability to induce hair growth, and even follicle formation (Oliver 1967a, Oliver 1970, Jahoda *et al* 1984, Horne *et al* 1986, Reynolds and Jahoda 1991a, Jahoda 1992, Reynolds and Jahoda 1992, Jahoda *et al* 1993). The culture of follicular dermal papilla cells is discussed in more detail in chapter 1.

Attempts to culture the epidermal components of the follicle have principally concentrated on plucked outer root sheath (ORS) material (Wells 1982, Weterings *et al* 1982, Vermorken and Bloemendal 1986, Limat *et al* 1996a, 1996b). Attempts have also been made to culture cells from the matrix regions of plucked hairs on dermal feeder layers (Jones *et al* 1988), but these populations have not been shown to be morphologically or biochemically different from cultured ORS cells. However, Reynolds and Jahoda (1991b) have been able to isolate and culture a population of germinative epidermal (GE) cells from the base of the end bulb in close association with the dermal papilla. These cells, a subpopulation of hair follicle matrix cells, are the group of acknowledged progenitor cells found at the extreme base of the mature follicle bulb. Germinative epidermal cells differ from other adult epidermal cell types, and have certain stem cell-like characteristics. They (a) are very small with a high nucleus to cytoplasm ratio; (b) do not express the hair specific keratins found in other hair follicle epidermis; and (c) cannot be cultured except in the presence of their functionally and anatomically intimate partners *in situ* - DP cells. In co-culture with DP cells, GE cells undergo division and form organotypic structures with the production of follicle specific basement membrane components (Reynolds and Jahoda 1991b, Reynolds *et al* 1993). GE cells have also been shown to exert an effect on dermal cell differentiation (Reynolds 1989). When GE cells are transplanted to ectopic skin sites with follicle derived dermal sheath (DS) cells (which

do not normally induce hair growth) they induce the DS cells to undergo a phenotypic change from DS-like to DP-like cells, and stimulate new follicle morphogenesis (Reynolds and Jahoda 1996).

The other epidermal components of the end bulb appear secondary in importance to the germinative epidermal cell population, although the site of the epidermal stem cells in the follicle remains a subject of much debate (discussed in chapters 1 and 4). It has been suggested that all of the epidermal differentiation products present in the hair follicle may be derived from the GE cell population, acting as either a stem- or progenitor-cell compartment (Reynolds and Jahoda 1993). It therefore appears that the germinative cell population, in close communication with the dermal papilla, is at the core of activities involving the fundamental processes of cell signalling and differentiation, where the signal to "make hair" is being received and transcribed. The gene products controlling this process will include growth factors and growth factor receptors, transcription factors and other molecules involved in dermal-epidermal signalling, molecules involved in the control of hair cycling, the earliest stages of hair keratin differentiation and other molecules involved in fundamental cellular control processes. A study of genes expressed in the GE cells could therefore be important for our understanding of the molecular mechanisms operating at the site of epidermal proliferation and differentiation. However, it is quite possible that the genes responsible for hair growth control in the GE population have not yet been characterised, as our understanding of these control mechanisms remains relatively limited. It therefore becomes important to determine which genes expressed in the GE cells are downregulated or not expressed in other epidermal cell types in the hair follicle. Such differentially expressed genes may provide important insights into the molecular control of hair production.

1:1 IDENTIFICATION OF NOVEL GENES ON THE BASIS OF THEIR DIFFERENTIAL EXPRESSION

Several methods have been employed for the isolation of genes whose expression is differential in different tissues. These include dual-labelling differential screening (Olszewski *et al* 1989); screening of PCR generated cDNA libraries (Belyavsky *et al* 1989, Smith and Gridley

1992); differential display reverse transcriptase PCR (DDRT-PCR) (Liang and Pardee 1992, Bauer *et al* 1993, Wan *et al* 1996); global cDNA amplification from single cells (Brady and Iscove 1993, Trumper *et al* 1993, Brady *et al* 1995) and cDNA library subtraction (Deleersnijder *et al* 1996). To date, few of these methods have been used to isolate genes from the hair follicle. However, DDRT-PCR has been used to isolate a gene expressed in cultured dermal papilla cells and not other cultured dermal fibroblasts by Yu *et al* (1995) (section 1:2.1) and a similar method has identified differential expression of *msx-1*, *msx-2* and *mox-1* in fetal and adult human skin (Stelnicki *et al* 1997, section 1:2.1), a differential hybridisation method has been used to identify androgen regulated genes in hamster flank organs (Seki *et al* 1991, section 1:2.2), a subtractive method has accidentally identified a gene which is highly expressed in the hair follicle matrix (Deleersnijder *et al* 1996, section 1:2.3), and a combination of subtractive and differential hybridisation methods has localised high levels of CD24 (nectadrin) expression to the rat vibrissa follicle bulge (Magnaldo and Barrandon 1996, section 1:2.3). This information is summarised in table 3.1. In other systems, these methods have been shown to be very useful for the isolation of novel genes, not on the basis of their similarities to known genes, but by virtue of the fact that they are expressed more highly in the tissue or developmental stage of interest, and less or not at all elsewhere or at other times. Therefore, it is possible to isolate genes which are more highly expressed in the tissue of interest, suggesting that they may fulfil some important function in that tissue, without knowing anything at all about their structure or DNA sequence.

1:2 IDENTIFICATION OF NOVEL GENES EXPRESSED IN THE HAIR FOLLICLE

Several methods to identify genes on the basis of their differential expression have been applied to skin and hair follicles, serving to illustrate that novel genes can readily be isolated from particular tissues within the hair follicle. Some of these are summarised below.

Table 3.1: Methods used for the isolation of genes which are differentially expressed in tissues of the hair follicle.

Method used	Gene isolated	Expression Pattern	Reference
DDRT-PCR	nexin-1	Expressed in cultured DP cells but not other cultured fibroblasts. Also expressed in the DP *in vivo*.	Yu *et al* 1995
Differential hybridisation	FAR-17a	Expressed in the flank organs of normal male Chinese hamsters (in ORS and sebaceous gland), not expressed post-castration.	Seki *et al* 1991
Subtractive hybridisation	E25	Most highly expressed in hair follicle matrix cells.	Deleersnijder *et al* 1996
Subtractive and differential hybridisation	nectadrin (CD-24)	Highly expressed in the follicle bulge.	Magnaldo and Barrandon 1996

1:2.1 Differential Display RT-PCR (DDRT-PCR)

A technique based on DDRT-PCR (Liang and Pardee 1992, Bauer *et al* 1993) was used by Yu *et al* (1995) to identify mRNA species which are expressed by cultured dermal papilla cells but not other cultured dermal fibroblasts, since a better understanding of the molecules which are synthesised and secreted by papilla cells would greatly enhance understanding of the mechanisms involved in papilla-epidermal interactions throughout the hair cycle. An arbitrarily primed RT-PCR technique using poly-T 3' primers and random 10-mer oligonucleotide 5' primers was performed, using total RNA from cultured DP cells and fibroblasts as the PCR template. It was found that a great majority of the PCR products obtained from both templates were identical, but there were several species which were specific to either the DP cells or the fibroblasts. In particular, one of the DP specific PCR products was shown to be nexin-1, a potent protease inhibitor known to inactivate a number of serine proteases.

Northern blot analysis of mRNA isolated from cultured cells confirmed that the relative abundance of *nexin*-1 mRNA is much higher in cultured DP cells than rat skin fibroblasts and human embryonic lung fibroblasts. *In situ* hybridisation analysis of rat lip skin demonstrated that this gene is also expressed in the dermal papilla *in vivo*, and is temporally restricted to the anagen phase of the hair growth cycle. The fact that *nexin*-1 mRNA is synthesised in large quantities during anagen suggests that nexin-1 may protect follicular keratinocytes from apoptosis, since apoptosis of the matrix cells occurs extensively during catagen when nexin-1 is no longer being synthesised. Alternatively, nexin-1 may be involved in promotion of differentiation of matrix cells, since this protein has been shown to affect cellular growth and differentiation in other organs.

A similar method was used by Stelnicki *et al* (1997) to study the differential expression of related homeobox genes in human skin. Amplified human fetal and adult skin cDNA populations were screened by PCR using degenerate oligonucleotide primers designed against highly conserved regions within the homeobox. Three non-*hox* homeobox genes (*msx*-1, *msx*-2 and *mox*-1) were shown to be differentially expressed in the populations studied, and their expression patterns were confirmed by *in situ* hybridisation (see chapter 2).

1:2.2 Differential Hybridisation Of A cDNA Library

A technique based on differential hybridisation of an adult male hamster flank organ cDNA library with two ^{32}P-labelled cDNA probes derived from normal male flank organ mRNA (+) and 3 week post-castrated male flank organ mRNA (-) was utilised by Seki *et al* (1991) in order to isolate androgen dependant cDNA clones. Duplicate filters of hamster flank organ cDNA library were hybridised individually to the two probes, and plaques which hybridised to the (+) probe but not the (-) probe were isolated for secondary screening. This resulted in the isolation of 18 potentially androgen dependant cDNA clones.

One of these, designated FAR-17a, was shown to be strongly differentially expressed in normal and castrated male flank organ mRNA by northern blotting, suggesting that the expression of this mRNA is regulated by androgens. The expression of FAR-17a was shown to decrease dramatically following castration, but its expression could be stimulated in both

castrated males and females by administration of testosterone. *In situ* hybridisation studies performed by Puy *et al* (1996) showed that FAR-17a mRNA was localised to the sebaceous glands and outer root sheath keratinocytes.

1:2.3 cDNA Library Subtraction

A method based on cDNA library subtraction of a cultured mouse mandibular condyle cDNA library by Deleersnijder *et al* (1996) isolated a cDNA clone which was shown to be highly expressed in mouse hair follicle epidermal matrix cells. cDNA libraries were constructed from cultured condyles and the Balb/3T3 cell line. The Balb/3T3 library was used to synthesise biotinylated driver cRNA, and the condyle library was used to synthesise target cRNA by *in vitro* transcription. The target cRNA was then used to synthesise first strand cDNA for use as the target in the subtraction protocol. Target cDNA was hybridised to a 50-fold excess of biotinylated driver cRNA, and hybrids containing biotinylated cRNA were removed by incubation with streptavidin. The remaining target cDNA hybrids were then used as the positive probe in a differential screen of the original condyle library with a negative probe derived from the unsubtracted original library. Plaques which hybridised more strongly to the subtracted probe than the unsubtracted probe could then be isolated for further characterisation. The ten clones isolated by this method were all shown to be markers of chondro-osteogenic differentiation, including H19, collagen II and bone sialoprotein. However, one of the clones, designated E25, was shown to be strongly expressed in hair follicle matrix cells and tooth odontoblasts by *in situ* hybridisation. The deduced amino acid sequence of the E25 protein suggests that it is a novel integral membrane protein containing a single putative transmembrane domain.

A method using both subtractive and differential hybridisation of a rat keratinocyte colony-forming cell (K-CFC) cDNA library was used by Magnaldo and Barrandon (1996) to isolate genes expressed in keratinocyte clonogenesis. They found that CD24 (nectadrin, a heat stable antigen) was highly expressed in keratinocytes located in the bulge of the rat vibrissa follicle. CD24 is a glycoprotein thought to be involved in cell-cell adhesion and

signalling. Further studies demonstrated that CD24 expression was not restricted solely to K-CFC's, and is therefore not a specific marker of clonogenic keratinocytes.

1:3 A STUDY OF GENES PREFERENTIALLY EXPRESSED IN HAIR FOLLICLE GERMINATIVE EPIDERMAL CELLS

1:3.1 The Importance Of The Germinative Epidermal Cells.

A study of genes differentially expressed in the GE cells when compared to the epidermal matrix is important for our understanding of the molecular mechanisms operating at the site of epidermal proliferation and differentiation. Furthermore, it may be possible to determine whether the GE cells are a transit amplifying cell population derived from stem cells elsewhere in the epidermis, or are stem cells in their own right. Currently, the site and origin of epidermal stem cells in the follicle remains a point of interest and debate. Evidence has been put forward that cells of the bulge region of the outer root sheath (ORS), present as a distinct anatomical feature in the upper half of some follicles, are the follicular epidermal stem cells (Cotsarelis *et al* 1990). The unknown inter-relationship between follicular GE and ORS populations during the hair growth cycle is probably the main factor preventing any absolute classification in this area. However, the plurality of function and complex differentiation of hair follicle epidermis suggests that this tissue may have more than one stem cell location, or alternatively, it may involve a system of stem and progenitor cell populations from which are derived at least six different differentiated cell types. This is supported by the work of Kamimura *et al* (1997), whose work on hair follicle reconstitution suggested that primary mouse keratinocyte cultures contain two or three progenitor cell types with distinct localisations and differentiation pathways. The "stem cell question" is discussed in chapters 1 and 4. Regardless of the location of the follicular epidermal stem cells, it is clear that the germinative cell population is at the core of activities involving fundamental processes of cell signalling and differentiation.

Several methods have been employed to identify differentially expressed genes in various cell types when the amount of tissue available for the study is limiting. These are

briefly discussed in sections 1:1 and 1:2. A similar type of study based on the GE cells may result in the isolation of putative epidermal stem cell markers and other molecules involved in the fundamental processes underlying the growth of hair.

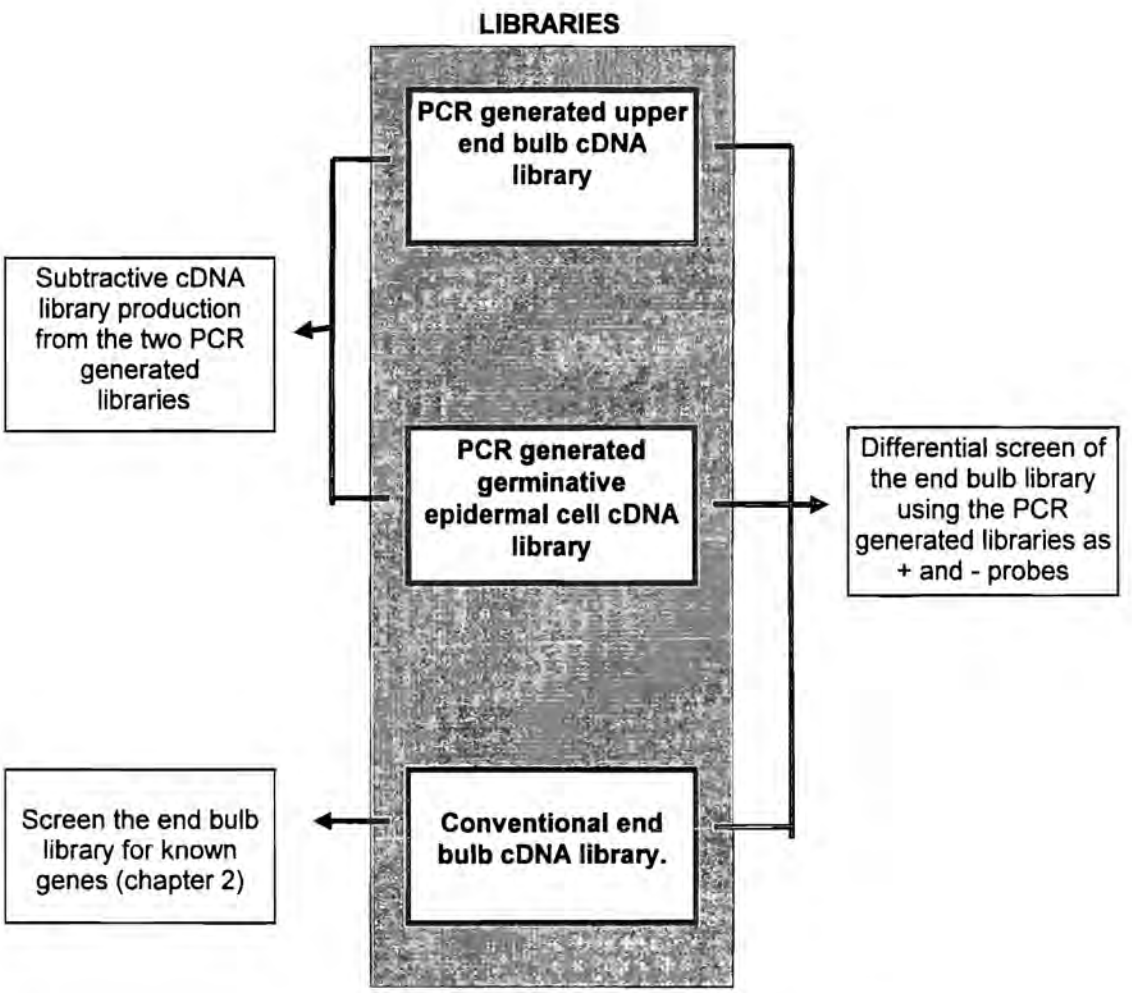
1:3.2 Characterisation Of The Germinative Epidermal Cells Based On Differential Screening Of A cDNA Library.

In order to identify genes preferentially expressed in the germinative epidermis, a dual labelling differential screen (Olszewski *et al* 1989) of a rat vibrissa follicle end bulb cDNA library was performed. The creation of a conventional cDNA library from germinative cells was not feasible due to the very limited amounts of material available. Therefore, a PCR approach (Belyavsky *et al* 1989, Gurr and McPherson 1991) was used to produce cDNA libraries for both the germinative cells, and the non-germinative components of the end bulb. The cDNA inserts of the two PCR generated libraries were then used as probes in the differential screen protocol.

This method was chosen in preference to DDRT-PCR due to the difficulty of obtaining quantities of RNA from the GE and non germinative tissues. The production of PCR generated libraries from both of these tissues relied on a single set of PCR reactions from a single round of dissection and RNA isolation, rather than the multiple PCR reactions and dissections required for DDRT-PCR. Also, at the time, it was felt that DDRT-PCR may be less reliable and reproducible than a differential screening method, since in 1992, DDRT-PCR was a relatively new technique, whereas results had been obtained using differential screening protocols for a number of years.

In parallel with the differential hybridisation approach, a subtractive cDNA library was also produced from the same starting material. However, the differential hybridisation approach is reported here in preference to the subtractive approach because clones isolated in the differential screen arise from a conventional cDNA library, whereas the clones isolated in the subtractive approach originate from a PCR generated library having shorter inserts and more chance of disproportionate representation of individual clones. The different strategies used for the molecular characterisation of the GE cells are summarised in figure 3.1.

Figure 3.1: Methods used for the characterisation of the germinative epidermal cells based on the production of three cDNA libraries from different regions of the follicle.



2: MATERIALS AND METHODS

2:1 MATERIALS

Essentially, the materials, bacterial strains and vectors used are as described in chapter 2 (section 2:1), with the addition of the following vectors - pUC19 (NBL); pGEM4Z, (Promega).

2:2 STRATEGY FOR THE CONSTRUCTION OF PCR GENERATED cDNA LIBRARIES SPECIFIC TO THE GERMINATIVE EPIDERMIS AND THE UPPER END BULB.

Several methods have been employed to construct cDNA libraries from very limited amounts of tissue. Essentially, the libraries were constructed as described by Gurr and MacPherson (1991).

2:2.1 Preparation of Tissue for the Construction of Germinative Epidermal and Upper End Bulb Libraries.

Germinative epidermal (GE) cells were dissected from the vibrissa follicles of adult PVG rats (Durham University) using the method described by Reynolds (Reynolds and Jahoda 1991b). Batches of about 50 follicles were dissected in MEM on ice, and the GE cells accumulated. The pellets of GE cells were then transferred to RNase free microcentrifuge tubes, and snap frozen in liquid nitrogen. The cells were then stored at -80°C until a sufficient number had been obtained. The cell pellets were then combined in a single 1.5ml microcentrifuge tube and stored at -80°C.

Material for the upper end bulb library was also obtained from the vibrissa follicles of PVG rats. About 50 follicles were plucked, removing the fibre and inner and outer root sheaths. The fibres were transferred to a dish of MEM on ice. The lowermost portion of the matrix was then removed and discarded, and the fibre cut to a length of about 1cm (Figure 3.2). This epidermal material was then washed in clean MEM and accumulated in an RNase free microcentrifuge tube on ice. The tissue was then snap frozen in liquid nitrogen and stored at -80^{o}C. Dermal sheath tissue was also obtained for construction of the upper end bulb library. Briefly, dermal sheath tissue was dissected from the vibrissa follicles of PVG rats and cultured in MEM supplemented with 10% FBS and antibiotics for a single passage in order to eliminate GE cell contamination. The cultured cells were then scraped into 1ml sterile PBS, transferred to an RNase free microcentrifuge tube, and pelleted by centrifugation at 1800g. The pellet was then snap frozen in liquid nitrogen, and the tissue combined with the epidermal material.

2:2.2 Extraction of RNA

The frozen tissue was placed on ice, and 50μl Guanidine HCl buffer was added (8M guanidine HCl, 20mM MES, 20mM Na_2EDTA, 50mM 2-mercaptoethanol, pH 7.0), followed by the addition of 50μl phenol/chloroform/ isoamyl alcohol (25:24:1) pH 7.0. The tissue was then homogenised for 10 minutes while allowing it to thaw on ice. The homogenate was transferred to an RNase free microcentrifuge tube, and the phases were separated by centrifugation at 13000g for 10 minutes at 4^{o}C. The aqueous phase was removed and transferred to a fresh tube. This was then extracted twice with an equal volume of phenol/chloroform/isoamyl alcohol (pH 7.0), with the aqueous phase being transferred to a fresh tube after each extraction. 1M acetic acid (0.2 volumes) and cold absolute ethanol (0.7 volumes) was then added, and the RNA allowed to precipitate at -20^{o}C overnight. The RNA was then pelleted by centrifugation at 13000g for 10 minutes at 4^{o}C, resuspended in 200μl 3M sodium acetate (pH 5.5) and incubated for 10 minutes at 4^{o}C to remove low molecular weight RNA's and polysaccharides. The RNA was then collected by centrifugation, washed in 500μl ice cold

70% ethanol and dried under vacuum. The pellet was then resuspended in 10μl DEPC treated H_2O, and stored at -80°C.

Since the amount of tissue available for each extraction was limited, it was not possible to directly assess the quality of the RNA after the extraction. However, a total RNA sample was prepared from an equivalent amount of cultured cells using this method, and this was examined by denaturing agarose gel electrophoresis as described in appendix A, to approximately validate the RNA extraction protocol.

2:2.3 Production of PCR Generated cDNA Libraries

The methods involved are summarised in figure 3.3, and are briefly described below.

2:2.3-1 First Strand cDNA Synthesis From Total RNA

The total RNA sample was denatured by heating to 70°C for 5 minutes in a water bath, and cooling rapidly on ice. A reaction mixture was then prepared, containing 200ng of poly-T primer,(5' ATC CTC GAG CCT TTT TTT TTT TTT TTT 3'), dNTP's at 1mM final concentration, 0.5mM spermidine HCl, 4mM sodium pyrophosphate and 1x RT buffer (50mM Tris-HCl, pH 8.3, 75mM KCl, 3mM $MgCl_2$). To this was added 25U human placental ribonuclease inhibitor, and 10μl of denatured total RNA. M-MuLV reverse transcriptase (10U) was then added to give a final reaction volume of 20μl, and the reaction incubated at 37°C for 2 hours. The reaction was then stopped by the addition of 20μl 0.1M NaCl, 40mM EDTA.

2:2.3-2 Removal of Oligo-dT Primers

At this point, it was important to remove the oligo-dT primers from the reaction mixture to ensure that spurious products did not arise following tailing and PCR amplification. This was achieved by cetyltrimethylammonium bromide (CTAB) precipitation, which allowed collection of cDNA/RNA hybrids as a precipitate, while leaving the single stranded primers in solution. (Belyavsky *et al* 1989).

0.5μg polyI.polyC was added to the reaction mixture, followed by 3μl 10% CTAB. The cDNA/RNA hybrids were allowed to precipitate at room temperature for 5 minutes, then pelleted by centrifugation at room temperature for 20 minutes at 13000g. The supernatant was then carefully removed, and the pellet resuspended in 14μl 1M NaCl. 25μl H_2O and 1μl 10% CTAB was then added, and the nucleic acid pelleted once more by centrifugation at room temperature for 20 minutes at 13000g. The pellet was then resuspended in 10μl 1M NaCl and precipitated with 27μl ethanol overnight at -20°C. The nucleic acid was then collected by centrifugation at 13000g for 20 minutes at 4°C, washed in 500μl 70% ethanol and dried.

2:2.3-3 Oligo-dG Tailing

A tailing reaction was performed in order to provide the annealing site for the 5' amplimer during the PCR reaction. This involved the addition of several G residues to the 3' end of the first strand cDNA using terminal transferase. The concentration of dGTP and enzyme and reaction temperature were controlled to give tail lengths of 17-20 bases.

A reaction mixture was prepared containing 1x tailing buffer (200mM potassium cacodylate, 25mM Tris-HCl pH 6.6, 5mM $CoCl_2$, 0.25mgml-1 BSA) and 5μM dGTP. The pellet of nucleic acid was resuspended in 7μl ddH_2O, and added to the reaction mixture. Terminal transferase (25U, Promega) was then added to give a final reaction volume of 20μl. This was then incubated at 37°C for 20 minutes. The reaction was stopped by the addition of 4μl 100mM EDTA and 2μl 1M NaCl. Unincorporated dGTP was removed by the addition of 1μl 10% CTAB, followed by centrifugation at 13000g for 20 minutes at 4°C. The supernatant was carefully removed and the pellet of nucleic acid resuspended in 10μl 1M NaCl. Glycogen (0.5μl, 0.5mgml^{-1}) was then added as a carrier, and the nucleic acid precipitated overnight at -20°C by the addition of 30μl cold absolute ethanol. The precipitate was collected by centrifugation at 13000g for 20 minutes at 4°C and the pellet washed in 70% ethanol and dried under vacuum.

2:2.3-4 RNA Hydrolysis

The RNA template was removed from the tailed first strand cDNA by alkaline hydrolysis. Briefly, the pellet of nucleic acid was resuspended in 20μl 50mM NaOH, 2mM EDTA, and incubated at 65°C for 1 hour. 3μl 3M sodium acetate, pH 5.2, and 70μl ethanol was then added, and the cDNA allowed to precipitate overnight at -20°C. The precipitate was collected by centrifugation at 13000g for 20 minutes at 4°C, and the pellet washed in 70% ethanol and vacuum dried. The cDNA was then resuspended in 20μl ddH_2O.

2:2.3-5 PCR Amplification

The cDNA was then used as the template in a PCR reaction. Primers (0.5μg of each 5' ATC CTC GAG CCT TTT TTT TTT TTT TTT 3' and 5' ATC GAA TTC CCC CCC CCC CCC CCC 3') were added to 20μl cDNA in a 100μl reaction volume containing 1.5mM dNTP's, 1.5mM $MgCl_2$, 10mM Tris-HCl pH 8.8, 50mM KCl, 0.08% Nonidet-P40, and 1U Taq DNA polymerase (ImmunoGen International). Fifteen cycles of denaturation at 94°C for one minute, primer annealing at 50°C for one minute, and primer extension at 72°C for five minutes were then performed. The PCR products were then size fractionated by agarose gel electrophoresis on a 1.0% agarose/TAE gel, and portions of the gel excised corresponding to size ranges of 100-500bp (fraction 1), 500-1000bp (fraction 2), 1000-1500bp (fraction 3) and >1500bp (fraction 4). The DNA was recovered from the gel using silica fines, as described in section 2:8.1(chapter 2). Each fraction was then used as the template in a second PCR reaction, using the conditions described above, with varying numbers of cycles performed depending on the size of the template cDNA, in order to minimise disproportionately high amplification of short cDNA's (fraction 1 = 3 cycles, fraction 2 = 6 cycles, fraction 3 = 9 cycles, fraction 4 = 12 cycles, see figure 3.7). Each PCR reaction was then precipitated by the addition of 10μl 3M sodium acetate and 300μl ice cold absolute ethanol, followed by incubation at -20°C overnight. The amplified cDNA was then pelleted by centrifugation at 12,000g for 15 minutes at 4°C, and

the supernatant carefully removed. The pellets were then washed with 1ml 70% ethanol, dried, resuspended in 5µl ddH_2O and pooled.

2:2.3-6 Ligation of Amplified cDNA into Lambda ZAP XR Vector, Packaging, and Amplification of cDNA Libraries.

This protocol was modified from that supplied with the Stratagene ZAP-cDNA library synthesis kit. Briefly, the pooled cDNA isolated following PCR amplification was restricted with *Eco*RI and *Xho*I (sites incorporated at the 5' ends of the PCR primers, as well as internal sites) to produce fragments suitable for ligation into precut vector arms (Stratagene). The digested cDNA was precipitated and resuspended in 2µl ddH_2O. To this was added 0.5µl 10x ligase buffer, 1.0µl of a 10^{-1} dilution of precut Uni-ZAP XR vector (100ng), and 0.5µl T4 DNA ligase (4U/µl). The reaction was incubated at $4^{o}C$ for 48 hours, and the complete ligation was packaged into a single Gigapack II Gold packaging extract (Stratagene), according to the manufacturers instructions. The titer of the packaged GE and Upper End Bulb libraries was then determined, (as described in section 2:2.4-3, chapter 2) and the libraries amplified to produce stable stocks as described in section 2:2.4-4 (chapter 2). The amplified libraries were then tested as described in section 2:2.5 (chapter 2).

2:3 DIFFERENTIAL SCREENING OF THE END BULB LIBRARY

A differential screen approach based on a method described by Olszewski *et al* (1989) was used to screen the vibrissa follicle end bulb cDNA library (produced as described in chapter 2) with probes derived from the PCR generated GE and upper end bulb libraries, in order to isolate cDNA clones which were more highly expressed in the germinative epidermal cells than in the other cell types present in the end bulb.

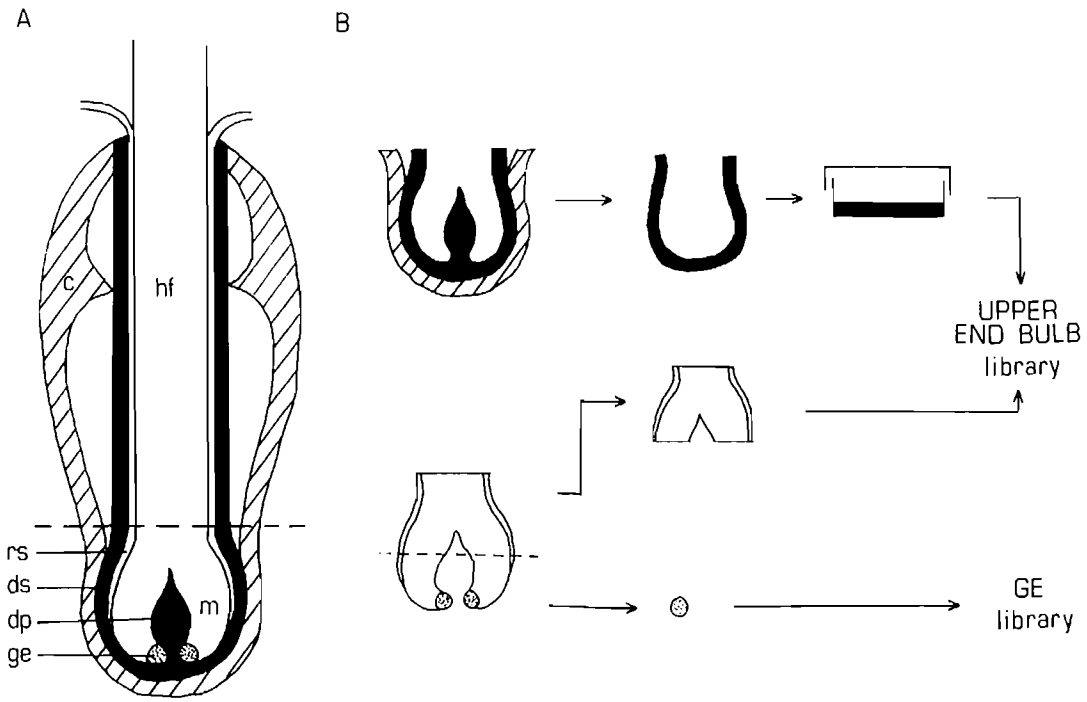
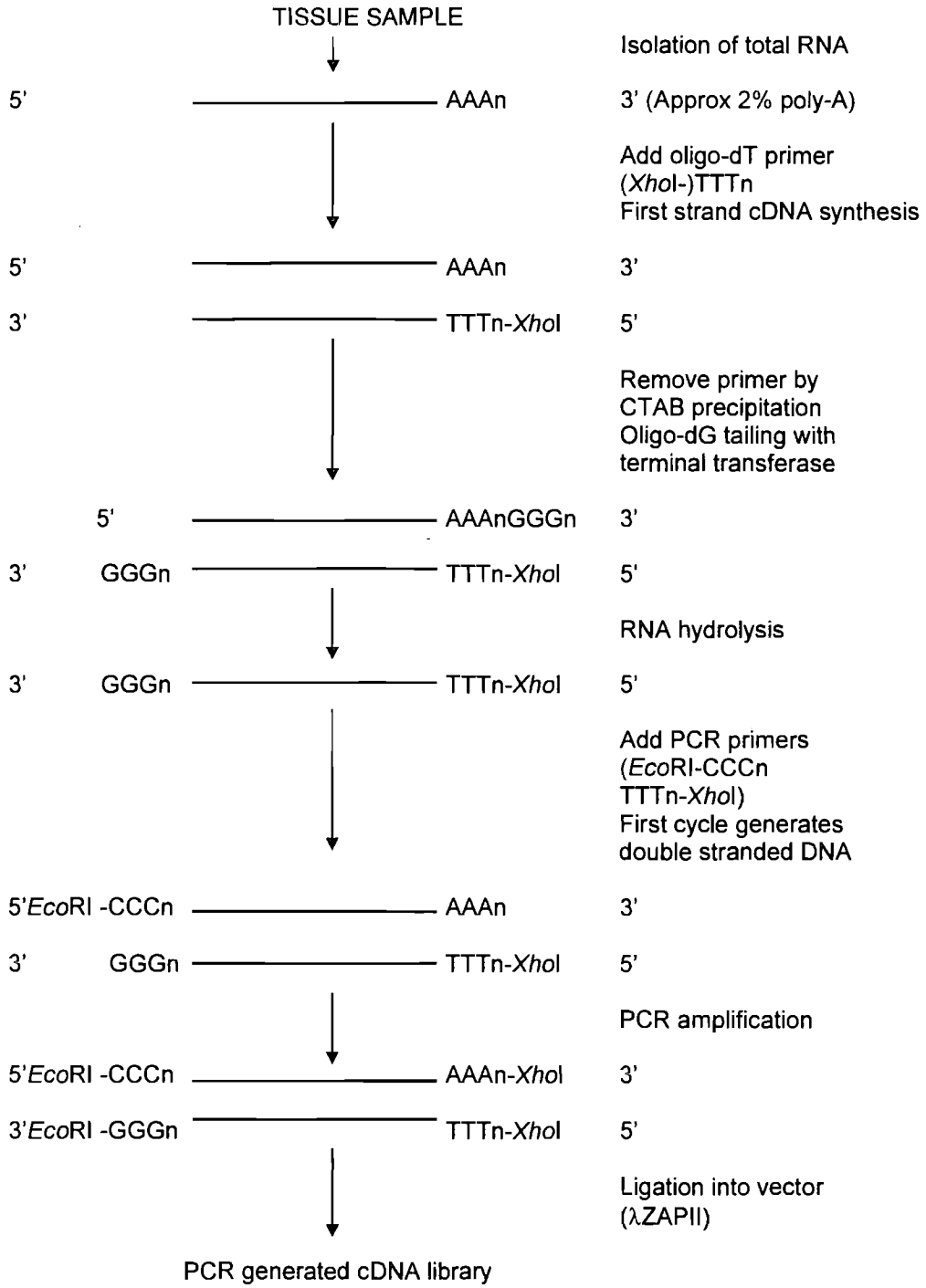


Figure 3.2: Isolation of tissue for the production of PCR generated cDNA libraries from the germinative epidermis and upper end bulb regions.

A: Schematic diagram of a vibrissa follicle showing the hair fibre (hf), germinative epidermis (ge), dermal papilla (dp), dermal sheath (ds), inner and outer epidermal root sheath (rs), epidermal matrix (m) and collagen capsule (c). The end bulbs were removed from follicles at the level indicated by the dotted line, and accumulated in a dish of MEM on ice.

B: The portions of hair fibre were then plucked from the accumulated end bulbs and transferred to a fresh dish of medium. The dermal tissue was removed from the collagen capsule and the dermal papilla discarded. The dermal sheath tissue was transferred to a dish of MEM and cultured for a single passage to eliminate epidermal contamination. The ring of germinative epidermal cells was removed from the base of the plucked hair fibres and stored at -80^{o} C until required. The lower portion of the matrix was then removed from the plucked hair fibres and discarded. The remaining portions of upper matrix and lower hair fibre were combined with the cultured dermal tissue and stored at -80^{o} C until required.

Figure 3.3: Strategy for the construction of PCR generated cDNA libraries from the GE cells and the non germinative components of the end bulb (adapted from Gurr and MacPherson 1991).



2:3.1 Generation of Radiolabelled Probes

Probes for the differential screen were generated by PCR from a sample of GE and upper end bulb library equivalent to 800,000 pfu. PCR reactions were performed as described in section 2:2.3-5 using vector specific forward and reverse primers, and the PCR products recovered using silica fines (section 2:8.1, chapter 2) following agarose gel electrophoresis. GE and upper end bulb cDNA was then labelled by random priming essentially as described by Feinberg and Vogelstein (1983) (section 2:3, chapter 2), except that the GE (+) probe was labelled with 50μCi α-^{35}SdATP (low energy β-emitter, >400Cimmol^{-1}), and the upper end bulb (-) probe was labelled with 50μCi α-^{32}PdATP (high energy β-emitter, >400Cimmol^{-1}). Unincorporated nucleotides were then removed using chromatography biospin columns (Biorad), and a 1μl aliquot of each probe reaction counted using a scintillation counter (^{35}S labelled (+) probe = 3.3 x 10^5 counts/μl, ^{32}P labelled (-) probe = 1 x 10^6 counts/μl).

2:3.2 Control Probing of EB library

Aliquots of end bulb library to yield approximately 200 plaques were plated on 90mm NZY agar plates as described in section 2:2.4-3 (chapter 2). Duplicate lifts onto 90mm nitrocellulose filters were then performed as described in section 2:2.5-3 (chapter 2). The filters were prehybridised at 65°C for two hours, and one of each pair of filters was probed with 10^5 counts of either (+) or (-) probe. Following autoradiography, the relative number and intensity of (+) and (-) specific spots was determined. There were approximately ten times more plaques which hybridised to the (-) probe, and these were about 50 times more intense than the plaques hybridising to the (+) probe since ^{32}P has a higher energy and therefore a greater relative signal intensity than ^{35}S. Therefore, in order to obtain a similar signal intensity from (-) and (+) specific plaques, taking into account their relative abundance, the ratio of counts of (+) probe:counts of (-) probe used for the differential screen was estimated at 5:1.

2:3.3 Differential Screen

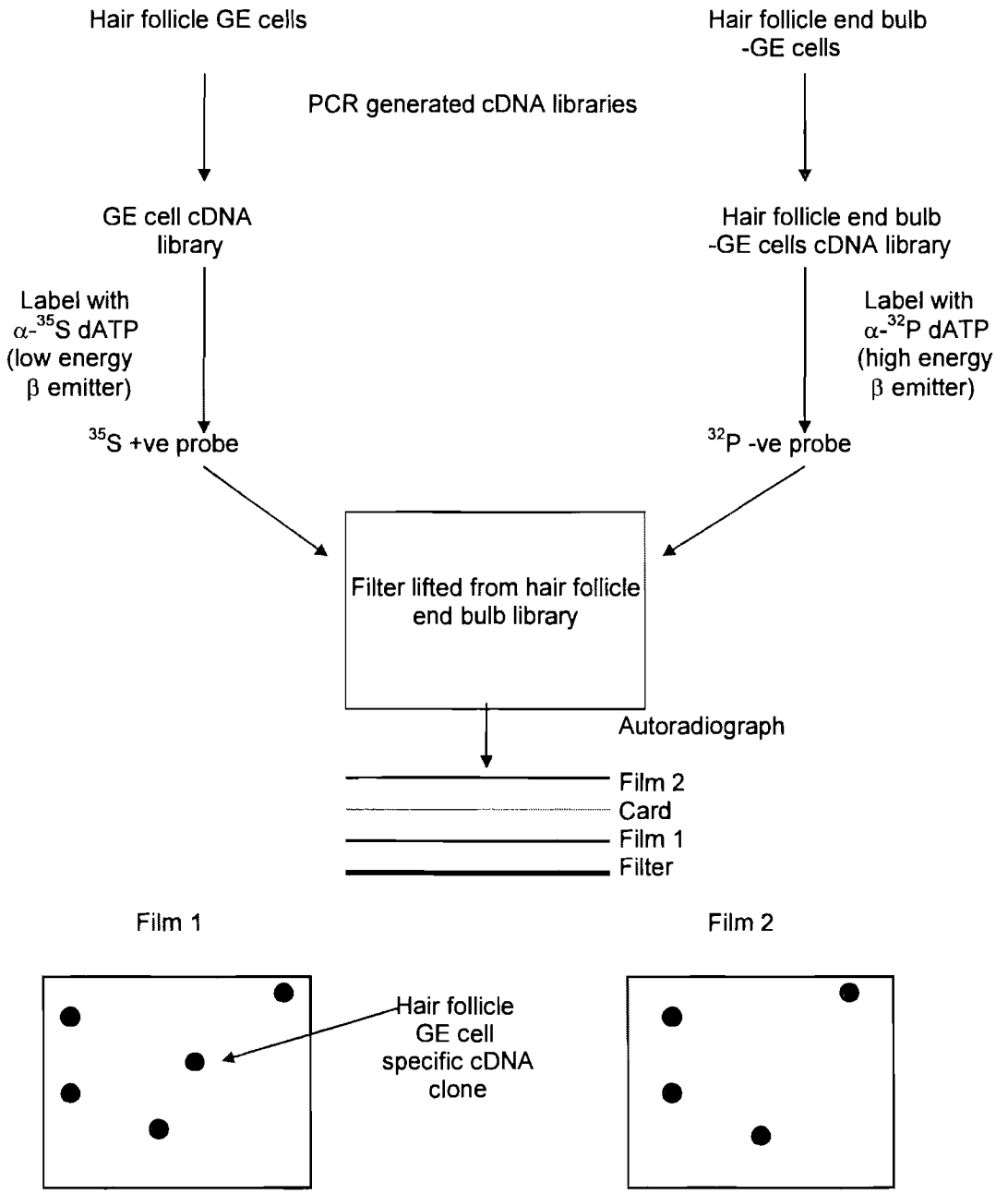
The end bulb cDNA library was plated at a low density (25000pfu/500cm^2 x 6 plates). Duplicate lifts of nitrocellulose were taken from the plate and the filters prehybridised in 6xSSC, 5xDenhardts reagent, 0.5% SDS, 100μgml^{-1} denatured herring sperm DNA, 0.1% sodium pyrophosphate at 65°C for 2 hours, as described by Sambrook *et al.* (1989). All filters were then hybridised with equivalent amounts (as determined in section 2:3.2) of (+) and (-) probes simultaneously in 6xSSC, 5xDenhardts reagent, 0.5% SDS, 1mM EDTA, 0.1% sodium pyrophosphate at 62°C for 16 hours. Filters were then washed, mounted on Whatman 3MM paper, and exposed to film as described. This strategy is summarised in figure 3.4.

Fifty-three putative differentially expressed clones were identified, cored from the primary screens and incubated in 1ml SM buffer with 20μl chloroform at 4°C overnight to allow diffusion of the phage particles into the buffer. Serial dilutions of the supernatant containing the phage from the primary core were then plated onto 90mm NZY agar plates. Secondary screens were then performed using equivalent amounts of (+) and (-) probe to isolate single plaques containing putative differentially expressed cDNA inserts. Plasmid recovery was then performed as described in section 2:5 (chapter 2). Plasmid DNA was then prepared and sequenced as described in sections 2:6 and 2:7 (chapter 2).

2:4 INITIAL CHARACTERISATION OF PUTATIVE DIFFERENTIALLY EXPRESSED cDNA CLONES

Single clones isolated from the fifty-three primary cores containing putative differentially expressed cDNA inserts were recovered to the plasmid stage. Plasmids were initially numbered and digested with *Eco*RI and *Xho*I to release the cDNA insert. Insert sizes were determined by electrophoresis on a 1.5% agarose/TAE gel containing 0.5μgml^{-1} ethidium bromide. Gel slices containing cDNA inserts to be isolated were cut from the gel using a clean scalpel blade. The DNA was purified from the gel block using one of the methods described in section 2:8 (chapter 2).

Figure 3.4: Strategy for a differential screen of the vibrissa follicle end bulb cDNA library to isolate clones which are preferentially expressed in the germinative epidermal cells (adapted from Olszewski *et al* 1989).



2:4.1 Cross-Hybridisation of Putative Differentially Expressed Clones

Cross hybridisation analysis of the 53 clones isolated from the differential screen was performed to determine how many discrete cross hybridisation groups were present (consisting of identical or closely related sequences). The cDNA inserts of the clones which were apparently "most differential" according to the primary differential screens were recovered from gel slices using one of the methods described in section 2:8 (chapter 2). The inserts were then labelled with ^{32}P as described in section 2:3 (chapter 2).

Plasmid DNA (500ng) prepared from each of the 53 putative differentially expressed clones was dot-blotted onto a nylon membrane (Hybond-N, Amersham), using a Biorad Dot-blotter according to the manufacturers protocol. The filter was denatured and neutralised as described in section 2:2.5-3 (chapter 2), and allowed to air dry for 30 minutes at room temperature. The DNA was then fixed onto the membrane by U.V. crosslinking for 5 minutes. The membrane was then prehybridised as described in section 2:2.5-3 (chapter 2), and probed with ^{32}P-labelled inserts from the selected clones. Following each round of probing and autoradiography, the filter was stripped by three ten minute washes in boiling 0.1% SDS prior to the application of the next probe (for examples see figure 3.8).

Putative differential clones from the cross hybridisation groups identified by this screening were then compared with a GAPDH control. Duplicate dot blots of equal amounts of these clones with a GAPDH control plasmid were then probed with ^{32}P-labelled (+) and (-) probes. The exposure times were adjusted so that the GAPDH control produced signals of equal intensity with both probes. The (+) and (-) signal intensity of the putative differential clones could then be compared, to verify that they were differentially expressed (figure 3.8).

2:4.2 Restriction Mapping and Subcloning

The length of the longest cDNA insert in each of the differential hybridisation groups was determined by agarose gel electrophoresis of plasmids digested with *Eco*RI and *Xho*I or *Kpn*I and *Pst*I to release the cDNA insert. The size of the longest clone from each group

ranged from ~100bp to ~4kb. Of the clones of interest, only four were short enough to obtain full length sequence data by sequencing from both ends of the insert. The remaining clones were restriction mapped using *Eco*RI, *Xho*I, *Sst*I, *Pst*I, *Sma*I, *Kpn*I, *Bam*HI, *Hin*cII, *Hin*dIII, and *Xba*I (sequencing strategies shown in figure 3.9). Restriction digests of 500ng plasmid were performed as described by Sambrook *et al.*(1989), using one or two enzymes in order to produce overlapping restriction fragments for sequencing. Restriction fragments were recovered following agarose gel electrophoresis as described in section 2:8 (chapter 2). These fragments were then subcloned into pBluescript cut to give compatible cohesive ends, and transformed into competent *E. coli* cells, as described in section 2:12 (chapter 2). The subcloned fragments were then sequenced as described in section 2:7 (chapter 2), and the sequences combined to give sequence data for the full length insert. In cases where the cross hybridisation group contained less than four clones, all of these were sequenced to confirm that they were independent clones arising from transcription of the same gene, rather than related gene products or identical clones arising from the library amplification step. These sequences were then analysed using the GenBank and EMBL databases (BLASTN 2.0.6, Altschul *et al* 1997).

Expression of the differential clones representing the discrete cross-hybridisation groups was then studied in adult rat follicles using non-radioactive *in situ* hybridisation and RNase protection analysis.

2:5 NON-RADIOACTIVE *IN SITU* HYBRIDISATION

In situ hybridisation was performed using digoxigenin (DIG) labelled antisense riboprobes on paraformaldehyde fixed wax sections, using methods based on those described by Wilkinson (1992).

2:5.1 Riboprobe Construction

Anti-sense riboprobes were transcribed *in vitro* from plasmid DNA containing the cDNA insert of interest, prepared as described in section 2:6 (chapter 2). Plasmid DNA was linearised by restriction with *Eco*RI, and purified using silica fines as described in section 2:8.1 (chapter 2). Linearised plasmid DNA (1μg) was then used in a transcription reaction with 15 units T7 RNA polymerase (Promega) in 1x transcription buffer (40mM Tris-HCl pH 7.5, 6mM $MgCl_2$, 2mM spermidine-HCl, 10mM NaCl, 10mM DTT) with 1mM ATP, 1mM CTP, 1mM GTP, 0.65mM UTP and 0.35mM DIG-UTP (Boehringer). The reactions were incubated at 37°C for 2 hours, and the efficiency of the reaction was determined by electrophoresis of a 1μl aliquot of each reaction on a 1.0% agarose/TAE gel containing 0.5μgml^{-1} ethidium bromide. The template DNA was then removed from the reaction by incubation with 2U RNase free DNase (Promega) at 37°C for 15 minutes. The RNA probe was then precipitated with 0.1 volume 4M LiCl and 2.5 volumes 100% ethanol at -20°C for 2 hours, and collected by centrifugation at 12000g for ten minutes at 4°C. The pellet was washed with 1ml 70% ethanol, air dried and resuspended in 100μl H_2O to give an approximate concentration of 0.1μgml^{-1}.

2:5.2 Preparation of Follicle Sections for ISH

Anagen vibrissa follicles were dissected from the mystacial pads of PVG/C rats and immediately fixed in 4% paraformaldehyde in PBS at 4°C overnight. The follicles were then briefly washed in PBS and dehydrated in ethanol (50% ethanol, 20 minutes; 70% ethanol, 2x 30 minutes; 90% ethanol, 2x 30 minutes; 100% ethanol, 2x 30 minutes). The tissue was then cleared in Histoclear (2x 1 hour), transferred to paraffin wax at 60°C for 2x 2 hours and embedded in fresh wax. Ten micron sections were then cut using a Leitz microtome and dried onto 3-aminopropyltriethoxysilane (TESPA) coated slides overnight.

2:5.3 Treatment of Slides with TESPA

Slides were treated with TESPA to maximise adhesion of sections through the harsh pre-treatments and washes required for ISH. All of the treatment steps were performed in baked glass staining dishes, with batches of ten slides at a time. Clean slides were baked at 180°C overnight and allowed to cool. They were then transferred to acid alcohol (70% ethanol, 1.7N HCl) for 5 minutes, followed by three five minute washes in DEPC treated H_2O. The water was then removed with two five minute washes in 100% ethanol, and the slides immersed in 4% TESPA in acetone for one minute, and allowed to air dry for two minutes. The slides were then washed twice for 5 minutes in 100% acetone and once for 5 minutes in H_2O, and dried at 37°C overnight.

2:5.4 Pre-treatment of Follicle Sections

The sections were dewaxed in Histoclear for 2 x 5 minutes, and rehydrated through an ethanol series to PBS (100%, 2x 5 minutes; 90%, 5 minutes; 70%, 5 minutes; 50%, 5 minutes; PBS, 2x 5 minutes). The slides were then immersed in 0.1M Tris-HCl pH 8.0, 50mM EDTA at 37°C, and proteinase K (Sigma) was added to 5$\mu g ml^{-1}$ to permeabilise the tissue. The incubation was continued for 15 minutes at 37°C, and the slides were then washed 2x 5 minutes in PBS and submerged in 0.2% glycine in PBS for ten minutes at room temperature. The slides were then placed in 0.1M Triethanolamine in PBS, acetic anhydride was added to a final concentration of 0.25%, and the slides incubated at room temperature for ten minutes. This acetylation step reduces non-specific electrostatic binding of the probe to the sections by acetylating positively charged amino groups. The slides were then rinsed twice in PBS.

2:5.5 *In Situ* Hybridisation

The slides were then placed in a humidified chamber, 50μl prehybridisation buffer (50% deionised formamide, 4x SSC, 1x Denhardts solution, 125$\mu g ml^{-1}$ tRNA, 100$\mu g ml^{-1}$

freshly denatured salmon sperm DNA) was added to each section and the slides were prehybridised at 42°C for one hour. The prehybridisation buffer was then removed, and 30µl fresh prehybridisation buffer containing 1µgml^{-1} labelled RNA probe (denatured by heating to 70°C for 5 minutes) was added to each section. The sections were then covered with parafilm coverslips and incubated in a humidified chamber at 50°C overnight. Following hybridisation, the coverslips were carefully removed, and the slides rinsed briefly in 2x SSC. Unhybridised probe was then removed by incubation with 20µgml^{-1} RNase A in 2x SSC for 20 minutes at room temperature.

2:5.6 Post Hybridisation Washes

Following RNase treatment, the slides were rinsed twice in 2x SSC. Three washes of increasing stringency (2x SSC, 50% formamide; 1x SSC, 50% formamide; 0.5x SSC, 50% formamide) were then performed at 50°C for 30 minutes each to reduce background signal. The slides were then rinsed in 2x SSC for ten minutes at room temperature.

2:5.7 Immunological Detection

The slides were submerged in buffer 1 (150mM NaCl, 100mM Tris-HCl pH 7.5) for 5 minutes at room temperature, and placed in a humidified chamber. Non-specific binding was then blocked by the addition of 100µl 2% normal sheep serum in buffer 1 to each section, and incubation of the slides at room temperature for 30 minutes. The blocking buffer was then replaced with 100µl sheep anti-DIG alkaline phosphatase conjugated antibody (Boehringer) diluted 1:500 in 1% normal sheep serum in buffer 1, and the slides incubated at room temperature for 3 hours. Unbound antibody was removed by washing 3x 5 minutes in buffer 1. The slides were then incubated for 5 minutes at room temperature in buffer 2 (100mM Tris-HCl pH 9.5, 100mM NaCl, 50mM $MgCl_2$), and bound antibody was revealed by incubation in light excluded conditions for 2-16 hours with 75µgml^{-1} NBT and 50µgml^{-1} BCIP in buffer 2. The slides were then rinsed in TE buffer to inactivate the alkaline phosphatase, and mounted in glycerol gelatin (Sigma).

2:6 RNase PROTECTION ANALYSIS

RNase protection analysis was performed using the commercially available RNase I RNase protection assay system (Promega). RNA was obtained from adult rat vibrissa follicles using the method described by Chomczynski and Sacchi (1987), and mRNA was isolated from total RNA using the Pharmacia Quick-Prep micro™ mRNA purification kit, according to the manufacturers protocol, as described in chapter 2. The concentration of each mRNA sample was then determined by ultra violet spectrophotometry at 260nm.

2:6.1 Synthesis of ^{32}P Labelled RNA Probes for RNase Protection Analysis

Antisense RNA probes were synthesised by *in vitro* transcription using T7 RNA polymerase from the T7 promoter present in the vector at the 3' end of the cDNA insert. Plasmids containing the clones of interest were linearised with restriction enzymes giving a 5' overhang and a probe length of 200-800 bases. However, in cases where the cDNA insert contained a common repeat element at the 3' end, it was important not to include this in the antisense RNA probe. This sequence is common to a great many mRNA's and would produce a major protected fragment of 90-100bp (the length of the repeat element) at the expense of any clone specific protected fragment produced in the RNase protection assay. Therefore, clones containing the common repeat element were prepared as follows. Plasmid DNA (50ng) containing the clone of interest was used as the template in a PCR reaction using 20pM of a 5' primer hybridising to the vector sequence at the 5' end of the insert (5' CAGGAAACAGCTATGAC 3'), and 20pM of a 3' primer hybridising to the 5' end of the common repeat element (5' CTCGAGCTCTACCACTGAGCTAAATCC 3'), in a 50µl reaction volume containing 1.5mM dNTP's, 1.5mM $MgCl_2$, 10mM Tris-HCl pH 8.8, 50mM KCl, 0.08% Nonidet-P40 and 1U Taq DNA polymerase (ImmunoGen International). Thirty five cycles of denaturation at 94°C for 1 minute, annealing at 55°C for 1 minute and primer extension at 72°C for 2 minutes were then performed, and an aliquot of the reaction (5µl) was analysed by

agarose gel electrophoresis to check that a single product of the expected size was produced. The remainder of the PCR reaction was then precipitated with 4.5µl 3M sodium acetate and 125µl absolute ethanol at -20°C for 2 hours. The precipitated DNA was collected by centrifugation, washed with 1ml 70% ethanol, dried and resuspended in 15µl ddH_2O. The purified PCR product was then blunt ended with Klenow DNA polymerase and digested with *Xho*I to give a product with a 5' blunt end and a 3' *Xho*I sticky end. This was then ligated into *Sma*I/*Xho*I cut pBluescript and transformed into competent *E. coli* as described in section 2:12 (chapter 2). Recombinant plasmids were isolated as described in section 2:6 (chapter 2), and sequenced as described in section 2:7 (chapter 2) to confirm their identity. The plasmids were then linearised with appropriate restriction enzymes chosen to cut the cDNA insert 200-800bp upstream of its 3' end. Linearised plasmid DNA (0.5µg) was used as the template in a transcription reaction containing 40mM Tris-HCl pH 7.5, 6mM $MgCl_2$, 2mM spermidine, 10mM NaCl, 20mM DTT, 20U RNase inhibitor (RNAGuard, Promega), 15U T7 RNA polymerase (Boehringer), 50µCi α-^{32}P UTP, 10µM UTP and 200µM ATP, CTP, GTP in a 10µl reaction volume. The reaction was incubated at 37°C for 40 minutes, and terminated by the addition of 1µl 20mM EDTA. The plasmid template DNA was then removed from the transcription reaction by incubation with 2U RQ1 RNase free DNase (Promega) at 37°C for 10 minutes.

The labelled RNA probe was then made up to 50µl with DEPC H_2O, and extracted once with 50µl phenol:chloroform:isoamyl alcohol (25:24:1). The aqueous phase was transferred to a fresh tube and extracted once with an equal volume of chloroform. Probes greater than 300 bases long were precipitated with 0.5 vol 7.5M ammonium acetate and 2.5 vol absolute ethanol at -20°C for 10 minutes. The RNA was then collected by centrifugation at 12000g for 5 minutes, washed with 1ml 70% ethanol, dried under vacuum and resuspended in 20µl H_2O. Probes less than 300 bases long were not ethanol precipitated as the percentage recovery of short probes by this method varies directly with the length of the probe.

2:6.2 Gel Purification Of RNA Probes

Probes of less than 300 bases were purified directly from a denaturing polyacrylamide minigel (Biorad mini-ProteanII mini gel system) while probes of greater than 300 bases were

gel purified following ethanol precipitation. Each RNA probe sample was mixed with 20μl gel loading buffer (80% deionised formamide, 1mM EDTA, 0.1% bromophenol blue, 0.1% xylene cyanol, 0.1% SDS), heated to 95°C for 5 minutes, and electrophoresed on a 1mm thick 8M urea/5% polyacrylamide TBE minigel using 1xTBE as the gel running buffer. Following electrophoresis, the gel was wrapped in cling film and exposed to X-ray film (Fuji) for 5 minutes. The autoradiogram was developed to locate the full length probe band, and this was excised from the gel, chopped into small pieces and transferred to a clean microcentrifuge tube. 200μl elution buffer (0.5M ammonium acetate, 1mM EDTA, 0.2% SDS) was then added, and the RNA probe eluted at 60°C for one hour. Gel fragments were then removed by centrifugation at 12000g for 5 minutes, and the supernatant transferred to a fresh tube and stored at -80°C until required.

2:6.3 RNase Protection

RNase protection assays were then performed using 1 μg mRNA as the target for each radiolabeled antisense RNA probe. The mRNA (1μg) was combined with 10^5cpm RNA probe in a 1.5ml microcentrifuge tube. The combined RNA samples (target + probe) were mixed with 0.1 volume 3M sodium acetate and 2.5 volumes 100% ethanol and precipitated at -20°C overnight. The RNA was then collected by centrifugation at 12000g for 15 minutes at 4°C. The RNA pellet was carefully washed with 1ml 70% ethanol, air dried for 10 minutes and resuspended in 20μl hybridisation buffer (80% deionised formamide, 1mM EDTA, 40mM PIPES pH 6.4, 0.2M sodium acetate). The samples were then incubated at 45°C overnight to allow the probe to hybridise to the target mRNA.

Following hybridisation, 180μl RNase ONE digestion buffer and 0.1U RNase ONE were added to each sample, and the reactions incubated at 37°C for 30 minutes to remove unhybridised probe and target RNA. The reaction was then terminated with 20μl stop solution (10% SDS, 1mgml^{-1} tRNA), and the target/probe hybrids were precipitated with 550μl cold 100% ethanol at -80°C for 30 minutes. The RNA was then collected by centrifugation at 12000g for twenty minutes at 4°C, washed with 300μl cold 70% ethanol, resuspended in 10μl gel loading buffer (80% deionised formamide, 1mM EDTA, 0.1% bromophenol blue, 0.1%

xylene cyanol, 0.1% SDS) and heated to 85°C for 5 minutes to denature the sample. Protected fragments were then resolved by electrophoresis through a 7% polyacrylamide/8M urea sequencing gel (Biorad) using 1xTBE buffer according to the manufacturers protocol. Following electrophoresis, the gel was dried under vacuum onto Whatman 3MM paper, and exposed to X-ray film overnight.

2:7 FURTHER CHARACTERISATION OF CLONES

Following DNA sequencing, *in situ* hybridisation and RNase protection analysis, putative differentially expressed clones which did not correspond to known genes in the GenBank and EMBL databases were selected for further studies.

2:7.1 Northern Blotting

A rat multiple tissue northern blot (Clontech) consisting of 2μg each testis, kidney, skeletal muscle, liver, lung, spleen, brain and heart mRNA was used to study the expression of the differentially expressed clones in other tissues. This blot was sequentially probed with ^{32}P-labelled inserts of the selected cDNA clones and autoradiographed, as described by the manufacturer.

Total RNA and mRNA was extracted from whole vibrissa follicles, liver and skin as described in sections 2:2.2 and 2:10 (chapter 2). Alternatively, total RNA was extracted from vibrissa follicle end bulbs using TRIzol™ (Gibco) as described in section 2:10 (chapter 2). Total RNA samples were also extracted from whole 10, 13, 14 and 15dpc rat embryos, and skin dissected from 14dpc and 20dpc embryos. This soft tissue was finely chopped with scissors and transferred to an RNase free microcentrifuge tube. TRIzol™ (1ml) was then added, and the tube vortexed to allow the tissue to break up. The RNA extraction was then completed as described in section 2.10 (chapter 2).

The RNA (10μg/lane total, 5μg/lane mRNA for adult tissues, 3.5μg/lane total for embryonic tissues, determined by UV spectrophotometry) was then subjected to denaturing agarose/formaldehyde gel electrophoresis on a 1.4% agarose gel, and blotted onto nylon

membrane as described by Sambrook *et al.* (1989). Ribosomal RNA bands were used as size markers for these gels. The blots were then sequentially probed with ^{32}P-labelled inserts of the selected cDNA clones and autoradiographed.

2:7.2 cDNA LIBRARY SCREENING BY PCR.

In order to confirm that the putative differentially expressed clones were expressed in both the end bulb and follicle cDNA libraries, and to determine whether any longer clones could be isolated, the libraries were screened by PCR as described below. DNA was prepared from a volume of each of the libraries corresponding to 10^8pfu as follows.

10^8pfu of cDNA library was made up to 10ml with SM buffer. The phage particles were then precipitated by the addition of NaCl to a final concentration of 1M, followed by incubation on ice for 1 hour. Bacterial debris was removed by centrifugation at 11000g for 10 minutes at 4°C. The supernatant was then carefully transferred to a clean tube, and PEG 8000 was added to a final concentration of 10%. The phage particles were then allowed to precipitate on ice for several hours. The precipitate was collected by centrifugation at 11000g for 10 minutes at 4°C, and the supernatant discarded. The pellet of phage particles was then resuspended in 500µl SM buffer, transferred to a 1.5ml microcentrifuge tube and extracted with an equal volume of chloroform to remove excess PEG 8000. Following extraction, the aqueous layer was transferred to a fresh tube, and EDTA, proteinase K and SDS were added to final concentrations of 20mM, 50µgml^{-1} and 0.5% respectively, followed by incubation at 56°C for 1 hour. The reaction was then extracted twice with an equal volume of phenol:chloroform:isoamyl alcohol (25:24:1) and once with chloroform to remove digested protein. The phage DNA was then precipitated from the aqueous layer by the addition of 0.1 volume 3M sodium acetate and 2.5 volumes 100% ethanol, followed by incubation overnight at -20°C. The precipitate was then collected by centrifugation at 13000g for 10 minutes at 4°C, washed in 1ml 70% ethanol and dried. The DNA was then resuspended in 200µl TE buffer and used as the template in a series of PCR reactions.

Clone specific antisense oligonucleotide primers were designed from 5' sequence data for each of the clones under investigation from sequence data obtained as described in section 2:4.2. Segments of the sequence (30bp) were analysed by comparison with sequences in the GenBank database to determine which regions had least homology to other known sequences in the database. Primers were then designed so that an overlap of 30-300bp of known sequence would be produced (primer sequences given in table 3.2).

PCR reactions were then performed using 20pM clone specific primer, 20pM of a primer specific to the vector sequence at the 5' end of the cDNA insert (primer 332, 5' CAG GAA ACA GCT ATG AC 3'), 1.5mM dNTP's, 1.5mM $MgCl_2$, 10mM Tris-HCl pH 8.8, 50mM KCl, 0.08% Nonidet-P40, 1U Taq DNA polymerase (ImmunoGen International) and phage DNA prepared as described above corresponding to 10^5pfu in a 50μl reaction volume. Thirty five cycles of denaturation for 30s at 94°C, annealing for 30s at 57°C, and primer extension for 2 minutes at 72°C were then performed. PCR products were then analysed by electrophoresis on a 2.0% agarose/TAE gel containing 0.5μgml^{-1} ethidium bromide.

Table 3.2: Primer sequences used for cDNA library screening. All reactions used a vector specific forward primer hybridising to the vector 120bp upstream of the *Eco*RI site at the 5' end of the cDNA insert. The PCR product size indicated is the size obtained when the template for the PCR reaction was the putative differential clone itself; both longer and shorter PCR products are expected when using total cDNA library as the PCR template.

Clone	Vector Specific Forward Primer	Clone Specific Reverse primer	PCR Product Size
DIF-R6b	332: 5' CAGGAAACAGCTATGAC 3'	1214: 5' CCCCTCGAGGTGGACCCACAGACAAATCTA 3'	~320bp
DIF-14b	"	1215: 5' CCCCTCGAGCACAAATCCAGTACCGTCCAC 3'	~390bp
DIF-15	"	15b: 5' TTTGCTGAGAGAAGTGAAAGCC 3'	~250bp
DIF-19b	"	19b: 5' ACTGCTGAGCCATTTCACTGGC 3'	~175bp

The PCR products obtained in each reaction were excised from the gel and recovered using one of the methods described in section 2:8 (chapter 2). The DNA obtained was then

blunt ended by incubation with 1U Pfu DNA polymerase at 60°C for 20 minutes, and subcloned into pCRscript (Stratagene) as described below.

Approximately 300ng blunt ended PCR product was ligated into 10ng precut pCRscript SK+ in a 10μl reaction containing 1x pCRscript buffer, 0.5mM rATP, 2.5U T4 DNA ligase and 2.5U *Srf*I. The reaction was incubated at room temperature for 1 hour, and the enzymes inactivated by heating to 65°C for 10 minutes. 3μl of the ligation reaction was then transformed into competent XLI-Blue cells, prepared as described in section 2:12 (chapter 2).

The cDNA inserts were then sequenced as described in section 2:7 (chapter 2). The sequence data obtained was then compared with the known sequence data for each of the clones under study to confirm that each of the clones was expressed in both the end bulb and follicle cDNA libraries.

2:7.3 SOUTHERN BLOTTING

Genomic DNA for Southern blotting was prepared from rat or mouse liver. The liver was removed from a freshly killed adult animal. The tissue was then finely chopped with a scalpel blade, and transferred to a 10ml glass Corex tube. DNA extraction buffer (100mM Tris-HCl pH 8.0, 0.5% SDS, 100μgml^{-1} proteinase K, 20μgml^{-1} RNase A) was then added (4ml per ml of tissue), and the tube incubated at 37°C for 16 hours. An equal volume of phenol:chloroform (1:1), pH 8.0 was then added, and the contents of the tube vortexed thoroughly. The phases were then separated by centrifugation at 10.000g for 10 minutes at 4°C, and the aqueous phase transferred to a fresh tube. The phenol extraction was then repeated, followed by a single extraction with chloroform. An equal volume of isopropanol was then added to the aqueous phase, and the tube incubated at room temperature for ten minutes to allow the DNA to precipitate. The precipitated DNA was collected by centrifugation at 5,000g for 15 minutes at 4°C, and the supernatant aspirated off. The pellet was washed three times with 1ml 70% ethanol, air dried and resuspended in 500μl water.

Southern blots were prepared using 10μg genomic DNA digested with 60U *Hin*dIII, and electrophoresed on a 0.8% agarose/TAE gel. Blotting onto nylon membrane was then carried out essentially as described by Sambrook *et al.* (1989). DNA was denatured prior to

transfer by soaking agarose gels in an excess of denaturing solution (0.5M NaOH, 1.5M NaCl) for one hour, followed by neutralisation of the gel by soaking in an excess of neutralisation solution (0.5M Tris-HCl, pH 7.5, 1.5M NaCl) for one hour. Gels were blotted for 16 hours using 10x SSC as the transfer buffer, after which time complete transfer of DNA had occurred. The DNA was fixed to the membrane by U.V. cross-linking. The Southern blots were then sequentially probed with partial or full length ^{32}P-labelled cDNA inserts of the clones of interest.

2:8 5' RACE-PCR

Primers were designed for the differentially expressed clones as described in section 2:7.2. The primer sequences used for each clone are given in table 3.2. Individual 5' RACE-PCR reactions were then performed using clone specific primers, as described in section 2:7.2. PCR products obtained were then blunt ended with Klenow DNA polymerase, cloned into *Sma*I cut pGEM4Z (Promega), and transformed into *E. coli* as described in section 2:12 (chapter 2). Plasmids were then sequenced as described in section 2:7(chapter 2), and the sequence data analysed.

2:9 LIGATION MEDIATED, OR ANCHORED PCR (APCR)

Anchored PCR (Robinson *et al* 1990) with single sided specificity was used to obtain 5' sequence data when secondary structure of mRNA made 5' RACE-PCR unreliable. Rat genomic DNA (10μg) was digested for sixteen hours with 15U either *Eco*RI, *Xho*I or *Hin*dIII using incubation conditions recommended by the manufacturer. An aliquot (3μg) was then ligated to 1μg pBluescript plasmid DNA previously restricted with *Sma*I and either *Eco*RI, *Xho*I or *Hin*dIII using 5U T4 DNA ligase in a 20μl reaction volume. Aliquots (2μl) of each ligation reaction were then used as template DNA for three separate APCR's using combinations of gene specific primers (sequences given in table 3.2) as shown in figure 3.5 with universal M13 forward or reverse sequencing primers (which hybridise to sites on pBluescript) as the anchor primer. Thirty five cycles of denaturation at 94°C for 1 minute, annealing at 62°C for 1 minute

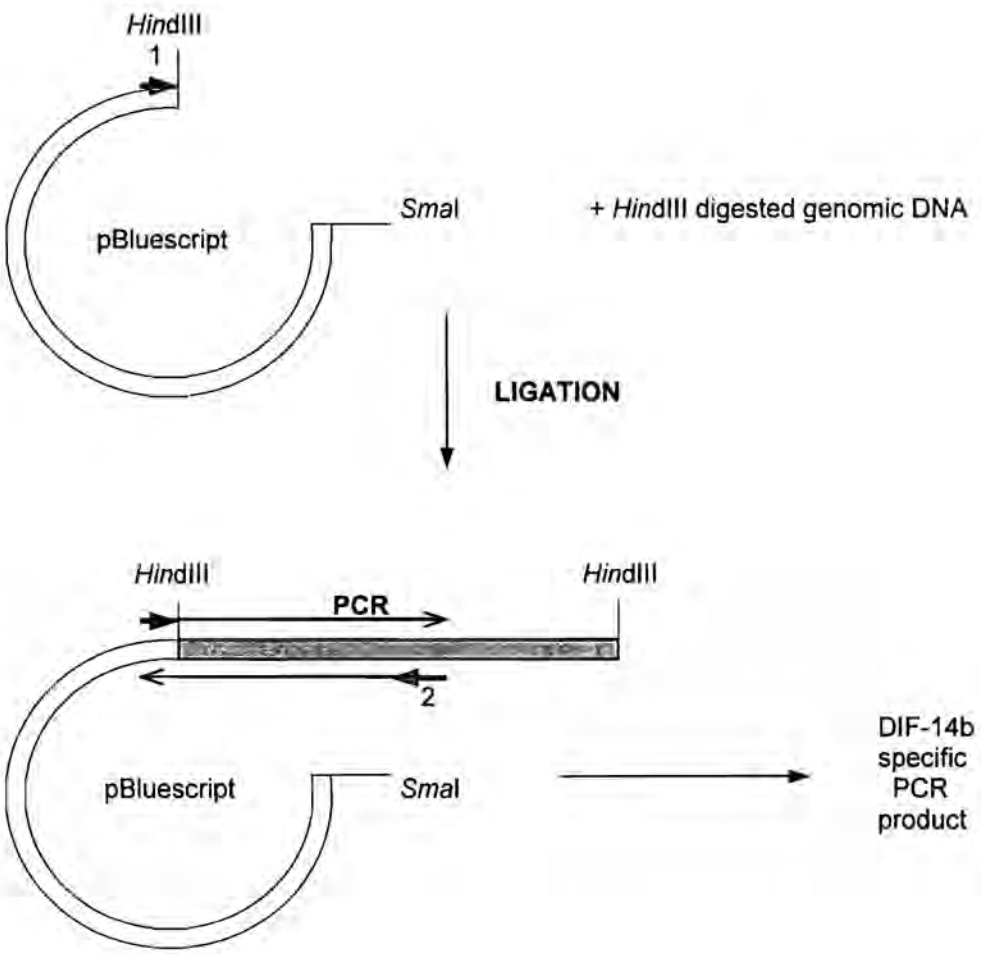
and primer extension at 72°C for 3 minutes were performed, in 100µl reaction volumes containing 1.5mM dNTP's, 1.5mM $MgCl_2$, 10mM Tris-HCl pH 8.8, 50mM KCl, 0.08% Nonidet-P40, and 2U *Taq* DNA polymerase per reaction. Aliquots (10µl) of each PCR were analysed by electrophoresis on a 1.0% agarose/TAE gel, and blotted onto nylon membrane as described by Sambrook *et al* (1989). The blot was then probed with a ^{32}P-labelled gene specific probe as described in section 2:2.5-3 (chapter 2) to identify the PCR product corresponding to the desired clone. PCR products were then purified using one of the methods described in section 2:8 (chapter 2), blunt ended with Klenow DNA polymerase, and cloned into *Sma*I cut pBluescript. New sequence data generated using this method will contain introns since the sequence is derived from genomic DNA and not mRNA.

2:10 Preparation of a Genomic Zooblot

Genomic DNA was prepared from rat, mouse, chicken, goldfish (*Carassius auratus*) and frog (*Xenopus borealis*). The liver was removed from freshly killed adult animals, except for the chicken, for which a whole 15 day embryo was used. The tissue was finely chopped with a scalpel blade, and transferred to a 10ml glass Corex tube. DNA was then extracted from the chopped tissue using a proteinase K digestion method as described in section 2:7.2, precipitated with isopropanol and collected by centrifugation. The pellet was then washed three times with 1ml 70% ethanol, air dried and resuspended in 500µl water.

Southern blots were prepared using 10µg of each genomic DNA sample and 10µg human genomic DNA (a gift of Dr. D. Pearson, University of Durham) digested with 60U *Hin*dIII, and electrophoresed on a 0.8% agarose/TAE gel. Blotting onto nylon membrane was then carried out essentially as described by Sambrook *et al.* (1989). Gels were blotted for 16 hours using 10x SSC as the transfer buffer, after which time complete transfer of DNA had occurred. The DNA was fixed to the membrane by U.V. cross-linking. The Southern blots were then sequentially probed with partial or full length ^{32}P-labelled cDNA inserts of the clones of interest.

Figure 3.5: Strategy for ligation mediated anchored PCR to obtain sequence data for the putative differentially expressed genes, using genomic DNA as the template for the reactions. *Hin*dIII digested rat genomic DNA (shaded box) was ligated to pBluescript cut with *Hin*dIII and *Sma*I. The vector specific primer 332 (1, black arrow) will prime unidirectional single strand synthesis of all ligated products, but only ligated *Hin*dIII genomic fragments containing the DIF-14b reverse primer site (2, grey arrow) will be exponentially amplified.



3: RESULTS

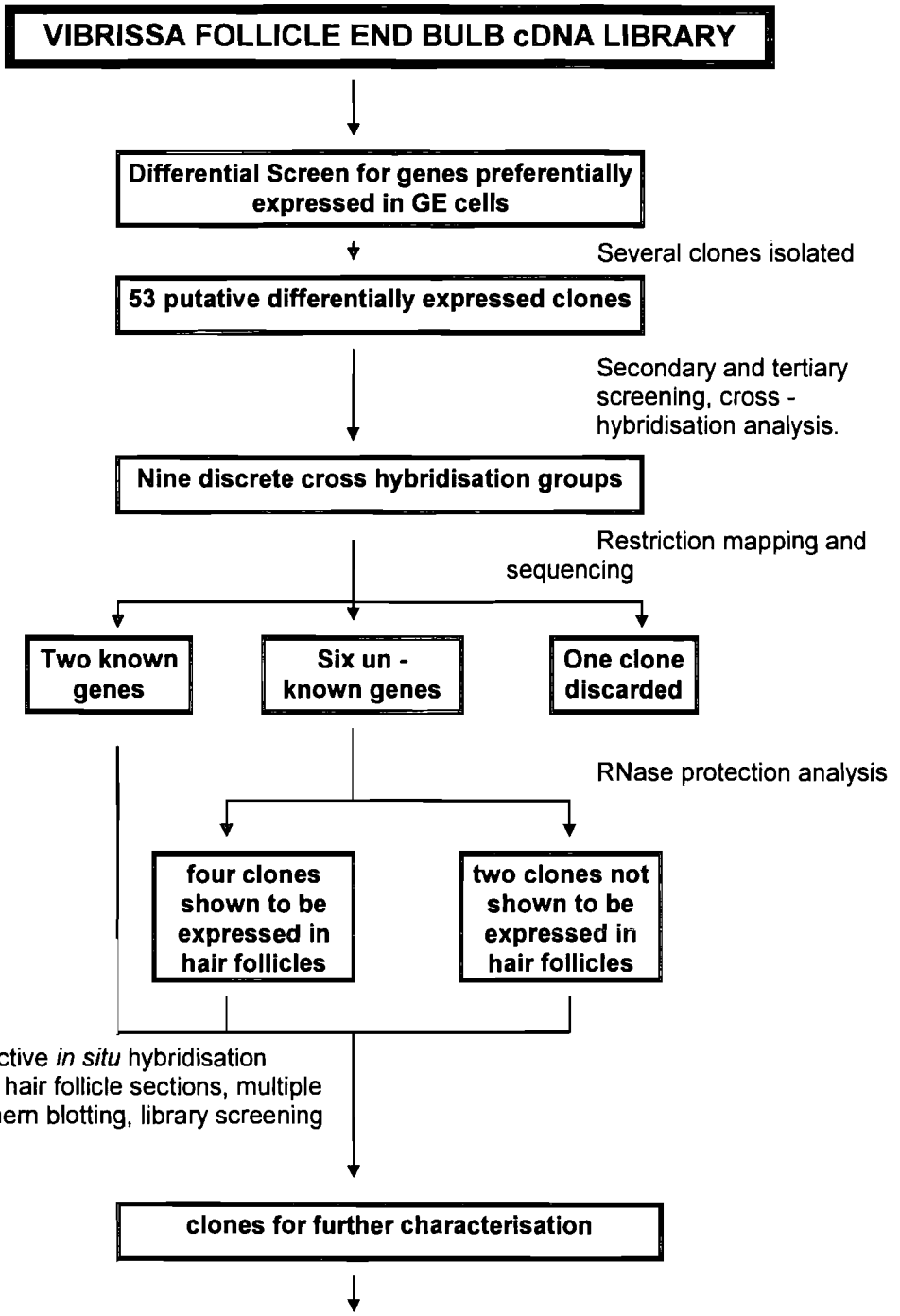
The strategies employed for the characterisation of the genes isolated by differential screening from the end bulb cDNA library are summarised in figure 3.6. The PCR generated cDNA libraries used in this protocol were tested as described in section 2:2.5 (chapter 2). The test results are summarised in table 3.3.

Table 3.3: Library test results

LIBRARY	method of construction vector	starting material	unamplified titer (pfu/ml)	amplified titer (pfu/ml)	proportion non recombinant plaques	mean insert size and range	% GAP DH
Germin-ative epidermal (+)	PCR generated UNI-ZAP XR	~1μg total RNA	6.3×10^5	1.5×10^8	12%	0.5kb 0.1-1.8kb	0.01
Upper end bulb (-)	PCR generated UNI-ZAP XR	~1μg total RNA	3.2×10^5	1.0×10^8	14%	0.3kb 0.1-1.0kb	0.01

The mean size and range of inserts in these two libraries were smaller than those in the libraries generated by conventional means (chapter 2). The attempts to size fractionate the inserts prior to the PCR amplification step in order to minimise disproportionate amplification of small inserts was partially successful in that some large inserts were present in a sample of 15 plaques selected at random from the GE library (figure 3.7). Even so, these libraries predominantly consist of regions of 3' UTR.

Figure 3.6: Strategies employed for the isolation and characterisation of genes which are preferentially expressed in the germinative epidermal cells.



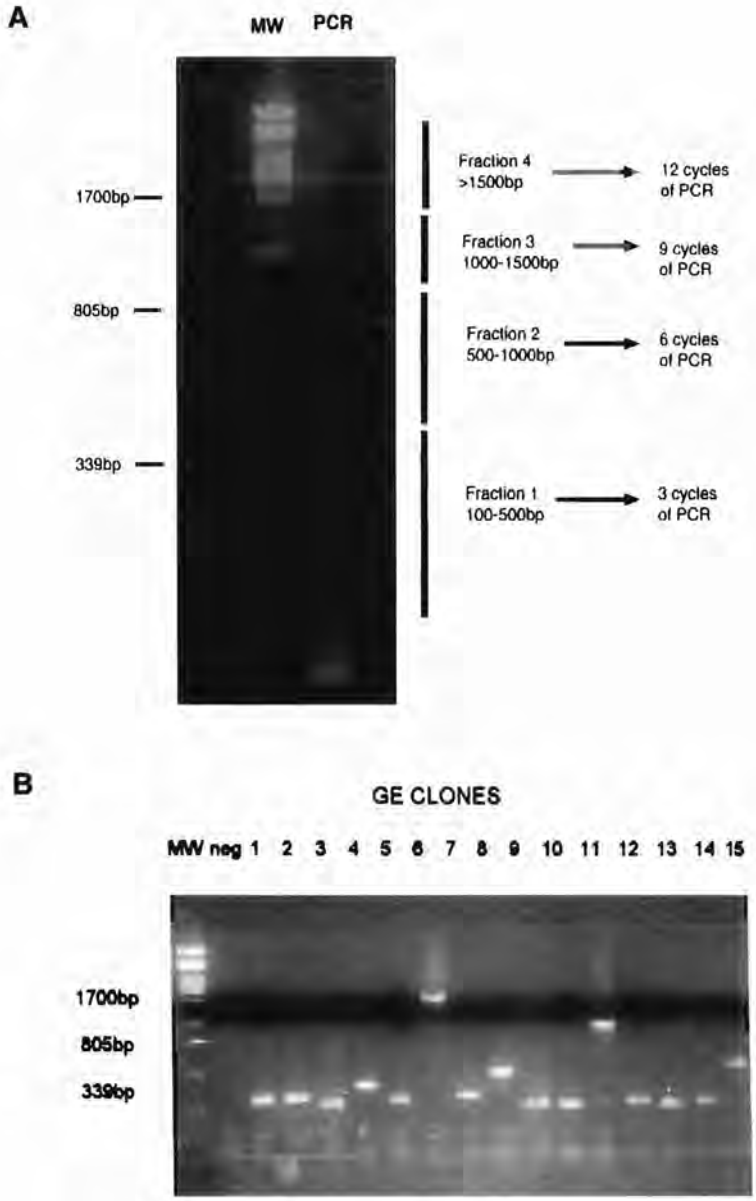


Figure 3.7: Construction and testing of a PCR generated cDNA library: amplification strategy and insert sizes produced from GE cells.

A: Size fractionation and reamplification of PCR products following initial round of amplification from dissected tissue. After electrophoresis, portions of the gel containing PCR product size ranges corresponding to fractions 1-4 as shown were excised, and the DNA recovered and reamplified as described prior to ligation into the vector.

B: Plaques were selected at random from the GE library and the inserts amplified using vector specific forward and reverse primers to determine the range of insert sizes present in the library. Though most of the inserts are between 300 and 500bp long, the range from this small sample extends up to around 1700bp.

3:1 CHARACTERISATION OF CLONES ISOLATED BY DIFFERENTIAL SCREEN

3:1.1 Secondary and Tertiary Screening

The primary differential screen resulted in the isolation of fifty three putative differentially expressed clones. Secondary and tertiary screening revealed that forty two of these appeared to be preferentially expressed in the germinative epidermis. The remaining eleven clones were discarded, as they were not shown to be differentially expressed following secondary and tertiary screening. Of the forty-two remaining putative differentially expressed clones, cross hybridisation analysis revealed that these were represented by nine independent cross hybridisation groups, containing 1-29 clones. Duplicate dot blots of equal amounts of these nine with a GAPDH control were then probed with ^{32}P-labelled (+) and (-) probes, adjusted so that the GAPDH control produced signals of equal intensity with both probes. The (+) and (-) signal intensity of the nine putative differential clones could then be compared, to verify that they were differentially expressed. Examples of the cross hybridisation analysis and dot blotting are shown in figure 3.8a.

3:1.2 Restriction Mapping, Subcloning And Sequencing

Insert sizes of the largest clones in each of the nine putative differentially expressed hybridisation groups were determined by agarose gel electrophoresis on a 1.5% agarose/TAE gel with a λ DNA molecular weight marker, following restriction of the plasmids containing the cDNA inserts with *Pst*I and *Kpn*I (fig 3.8b). Eight of the nine clones had inserts of greater than 200bp. These were initially sequenced using vector specific primers to obtain sequence data for the 5' and 3' ends of the cDNA clones. This sequence data was used to search the GenBank and EMBL databases for homology to known genes. Clone DIF-20 was shown to be 98.1% identical to a rat mitochondrial rRNA transcript, and so was not sequenced further. The remaining seven clones were restriction mapped, subcloned and sequenced as described in

section 2:4.2. The sequencing strategy, restriction maps and ORF maps are shown in figures 3.9a-3.9g. (The arrows indicate the direction of sequencing of subcloned fragments of each clone, and the restriction map derived from the sequence data shows unique sites present in each clone sequence. The map of the ORF, also generated from the sequence data, shows the position of stop codons (long bars) and start codons (short bars) in the predicted protein sequence translated from each of the three reading frames of the cDNA. The full sequence of each of the clones is given in appendix C. Sequence data for DIF-15 is derived from both the original clone isolated from the EB library and a clone obtained by PCR from the follicle library (section 3:2.1)).

Table 3.4: The nine independent hybridisation groups are represented by the nine clones shown below, giving the number of clones in each hybridisation group, the cDNA insert length of the longest clone in each group, the sequence of any concensus polyadenylation signal at the 3' end of the clone and the presence or absence of any putative ORF in each clone.

CLONE	No. of clones in hybridisation group	Length (bp)	Polyadenylation signal?	Putative ORF?
DIF-1	1	717	NO	NO
DIF-R6b	1	508	ATTAAA	NO
DIF-7	1	4200	AATAAA	NO
DIF-14b	4	1568	NO	YES, partial, 283 amino acids
DIF-15	1	947(EB clone) 202(Foll clone)	AATAAA	YES, C-terminal end, 36 amino acids, from combined EB + Foll sequences
DIF-16	2	935	NO	NO
DIF-17a	1	<100	NO	NO
DIF-19b	1	314	AATAAA	NO
DIF-20	29	900-1200	not done	not done

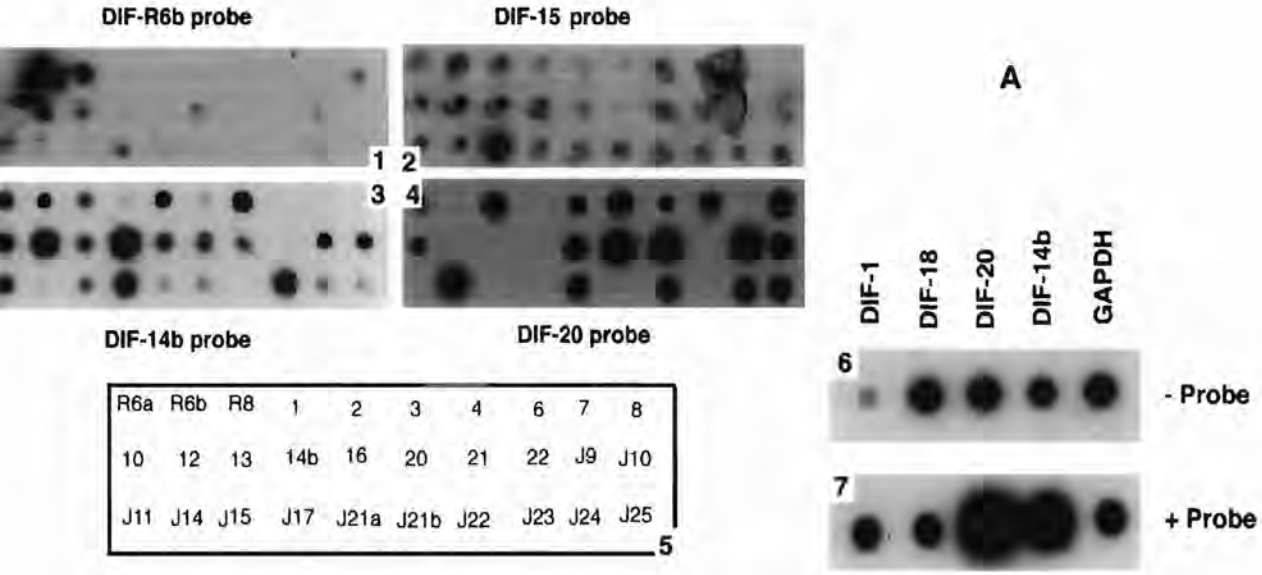
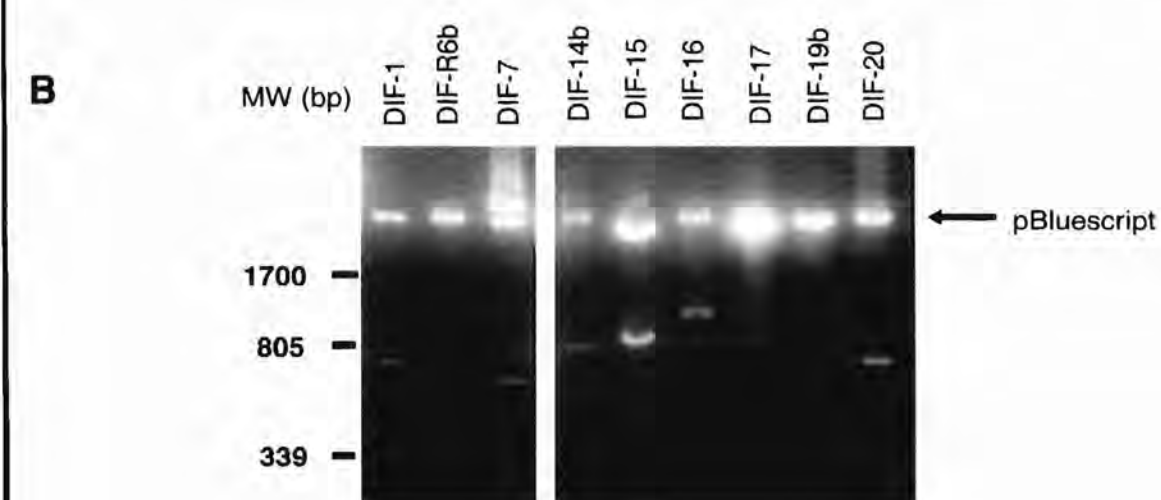
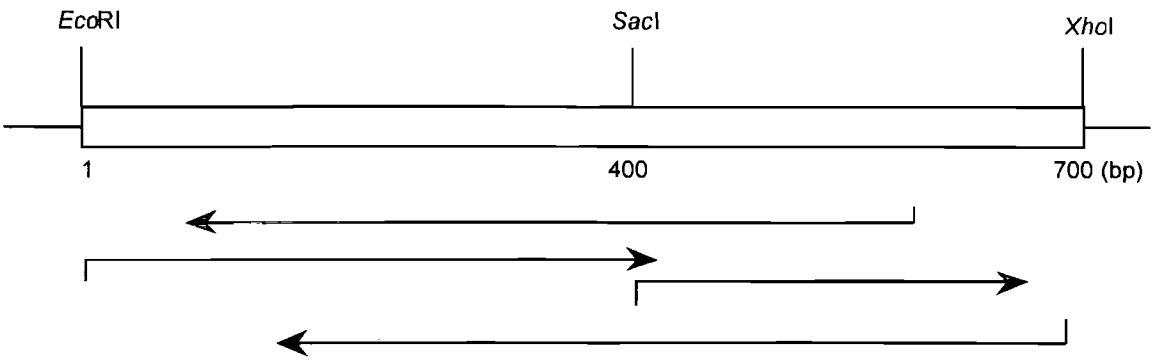


Figure 3.8A: Examples of the cross hybridisation screening performed on the fifty-three putative differentially expressed cDNA clones isolated from the EB cDNA library. A series of duplicate dot blots were prepared, and hybridised with probes generated from those clones which appeared to be "most differential" from the rounds of primary and secondary screening. Panels 1-5 show an example of a dot blot of the clones detailed in panel 5 probed with DIF-R6b (1), DIF-15 (2), DIF-14b (3) and DIF-20 (4). This shows that DIF-R6b and DIF-15 are represented by a single clone, DIF-14b is represented by four clones, and DIF-20 hybridises to multiple clones. Panels 6 and 7 show hybridisation of duplicate dot blots of equal amounts of four of the putative differentially expressed clones and a GAPDH control with probes generated from the GE (+) and upper end bulb (-) cDNA libraries. Three of the clones in this example are clearly differentially expressed when compared to GAPDH, and the fourth clone, DIF-18, is apparently not. Such non-differential clones were not used in further studies.

Figure 3.8B: Insert sizes of the longest putative differentially expressed cDNA clones in the nine cross hybridisation groups. Excised plasmids containing the clones of interest were digested with *Pst*I and *Kpn*I to release the cDNA insert. These were then analysed by agarose gel electrophoresis.

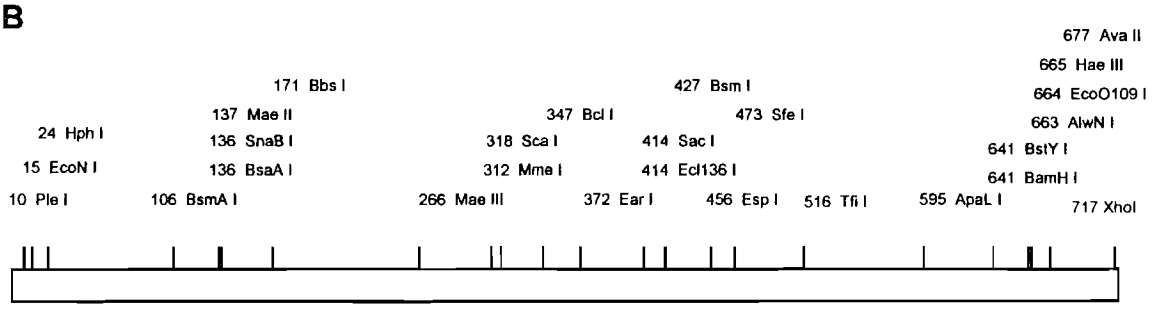


A



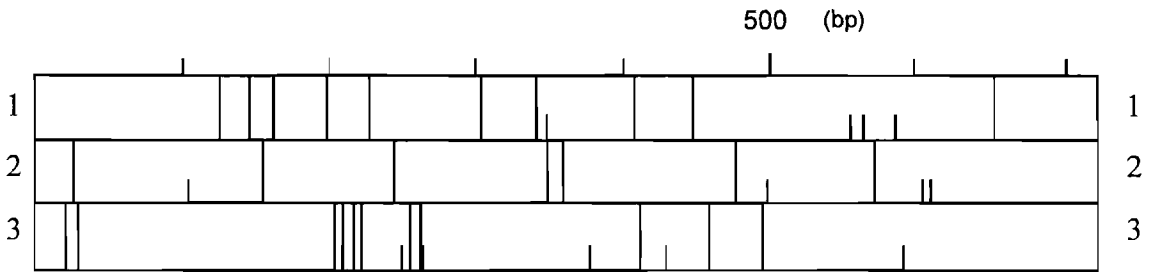
DIF-1 Sequencing strategy

B



DIF-1 Restriction map Unique Sites

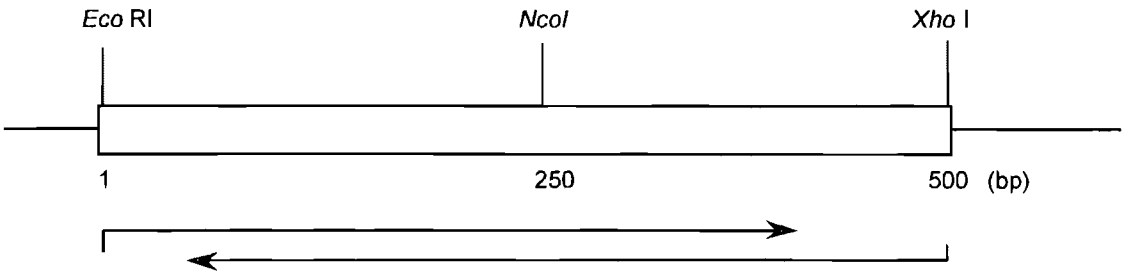
C



DIF-1 ORF map

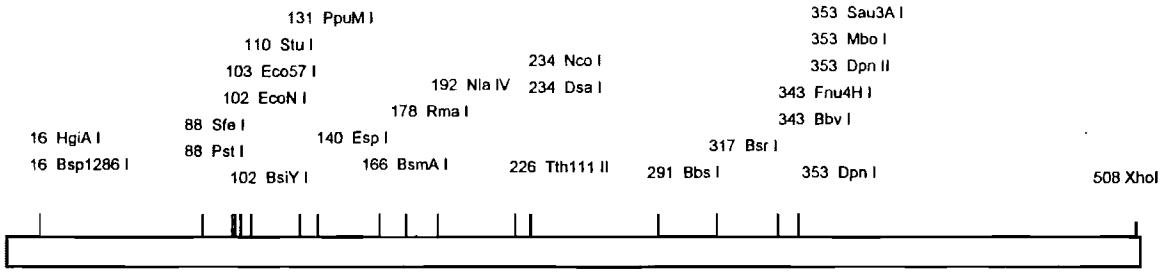
Figure 3.9a: Sequencing strategy (A), restriction map (B) and ORF map (C) for DIF-1.

A



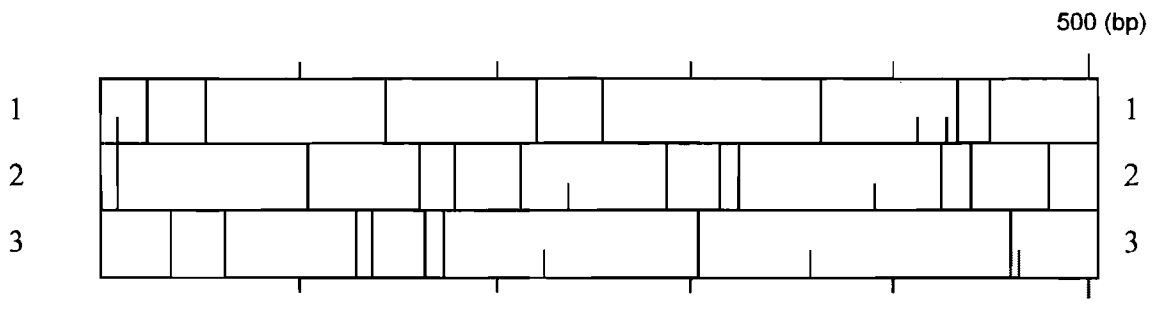
DIF-R6b Sequencing strategy

B



DIF-R6b Restriction map Unique sites

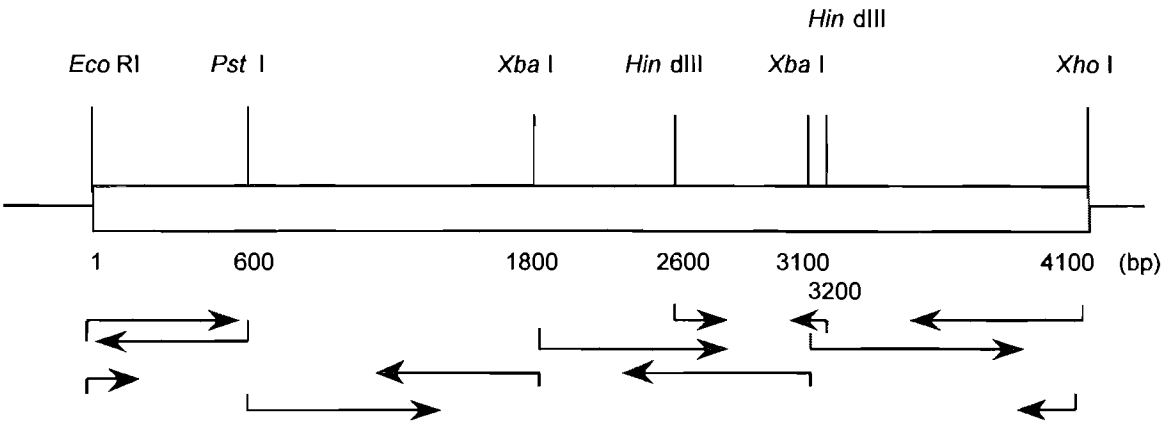
C



DIF-R6b ORF map

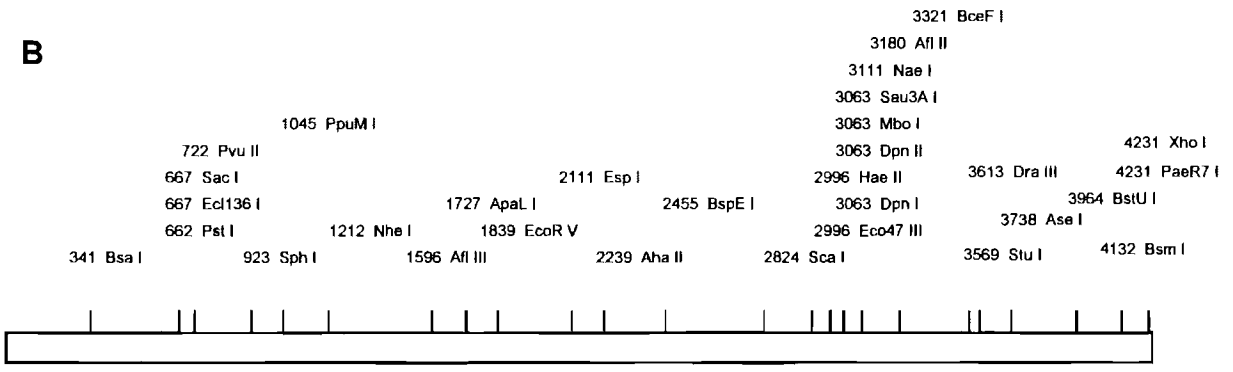
Figure 3.9b: Sequencing strategy (A), restriction map (B) and ORF map (C) for DIF-R6b.

A



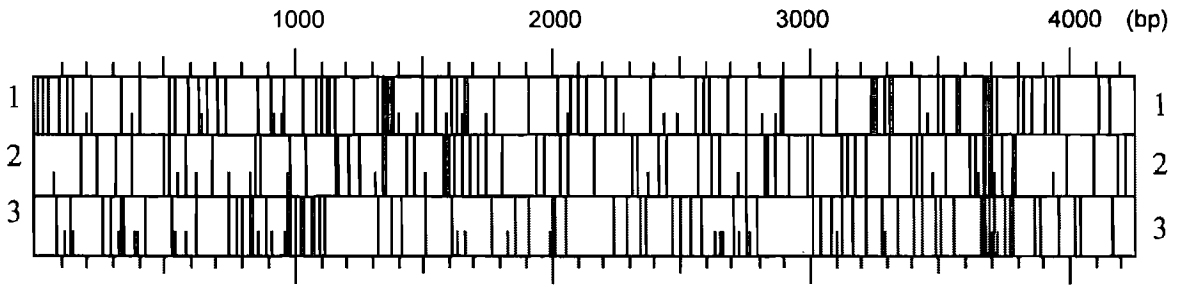
DIF-7 Sequencing strategy

B



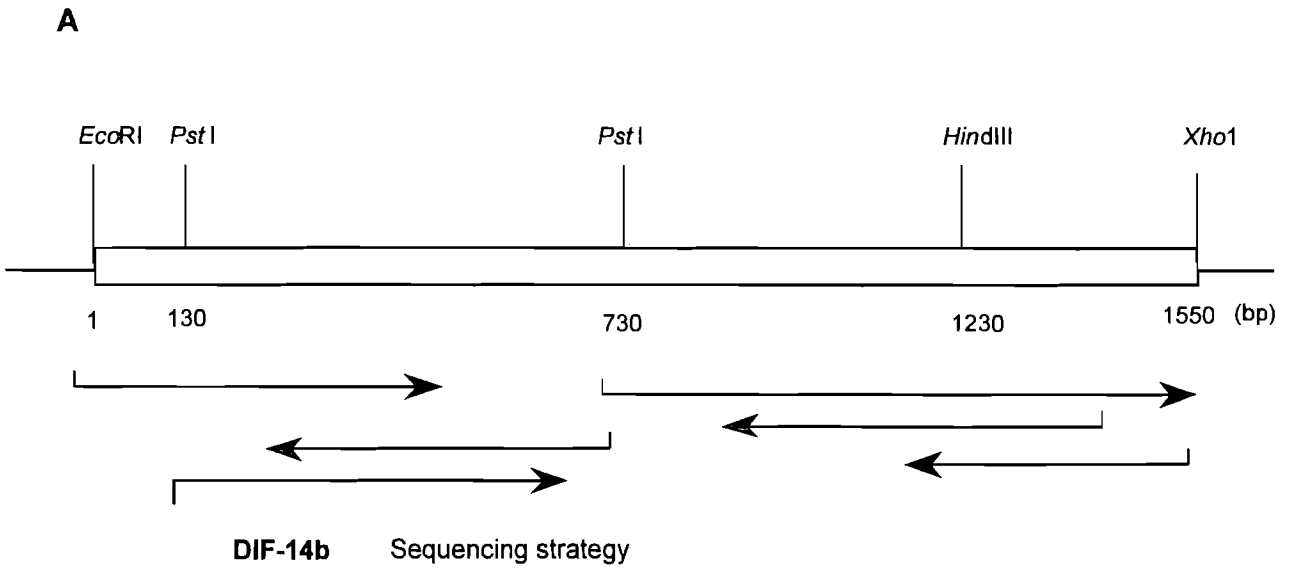
DIF-7 Restriction map Unique sites

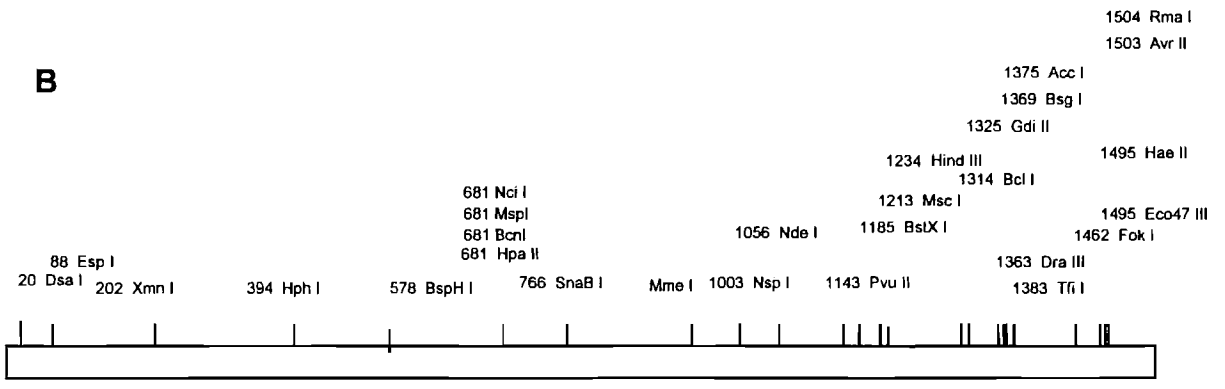
C



DIF-7 ORF map

Figure 3.9c: Sequencing strategy (A), restriction map (B) and ORF map (C) for DIF-7.





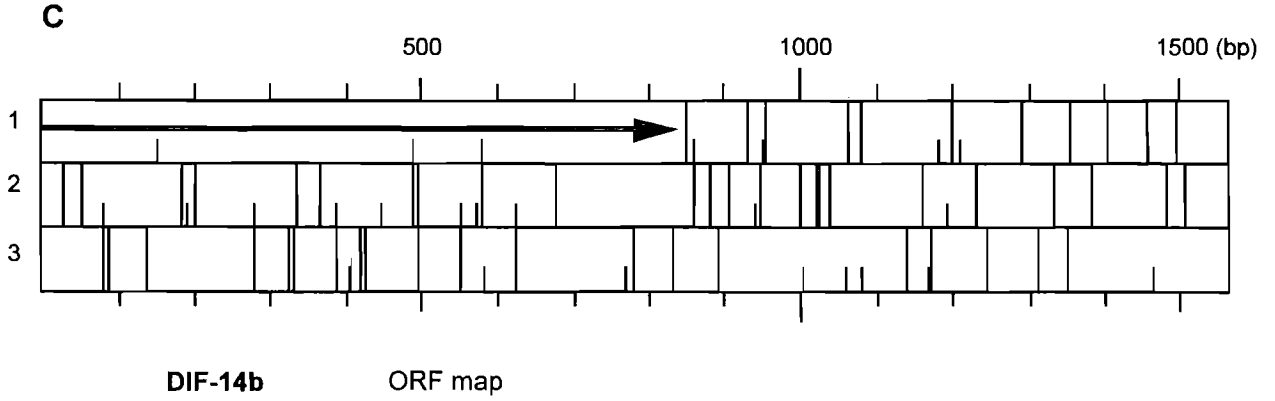
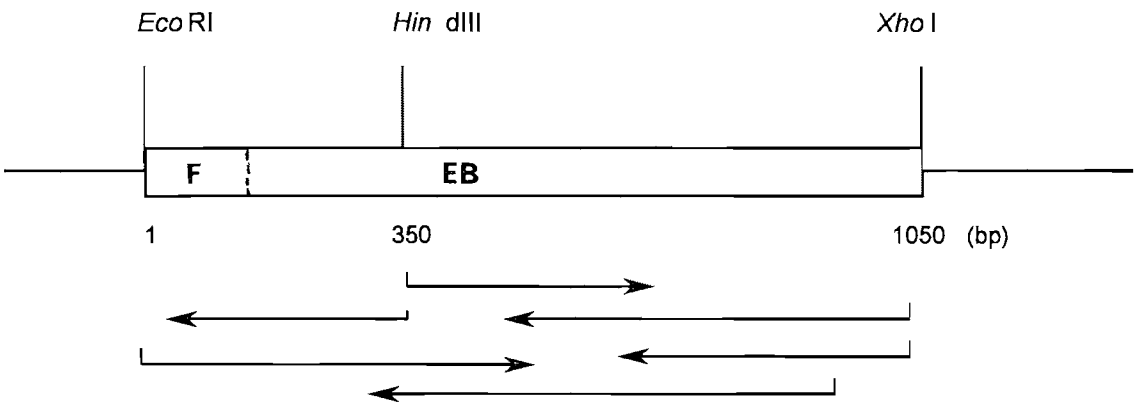


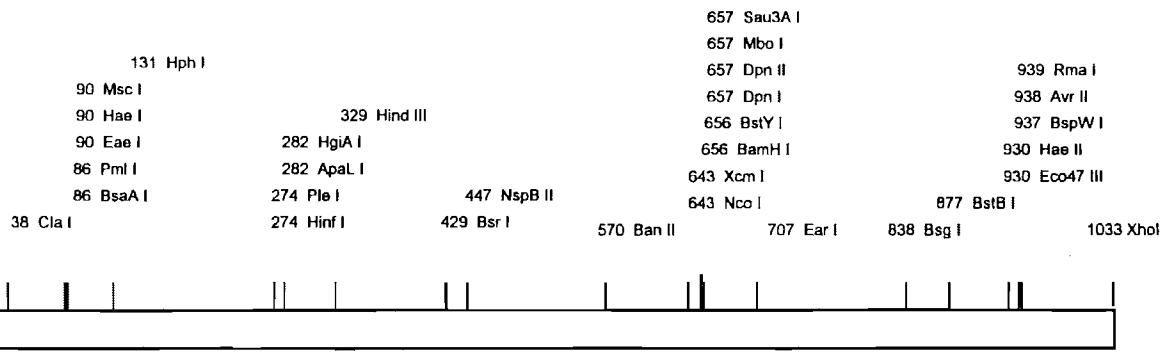
Figure 3.9d: Sequencing strategy (A), restriction map (B) and ORF map (C) for DIF-14b.

A



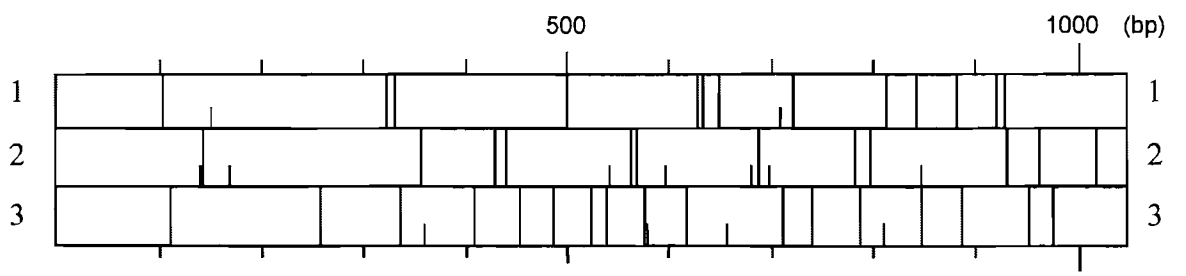
DIF-15 Sequencing strategy

B



DIF-15 Restriction map Unique Sites

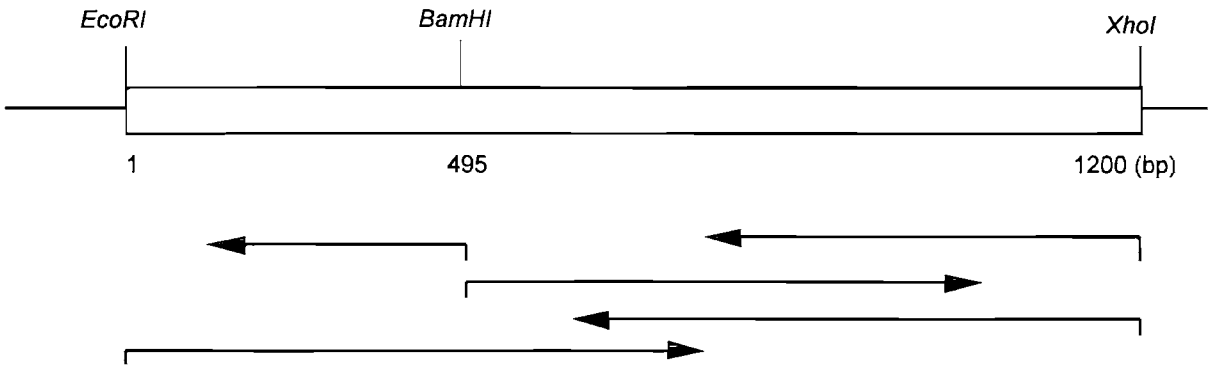
C



DIF-15 ORF map

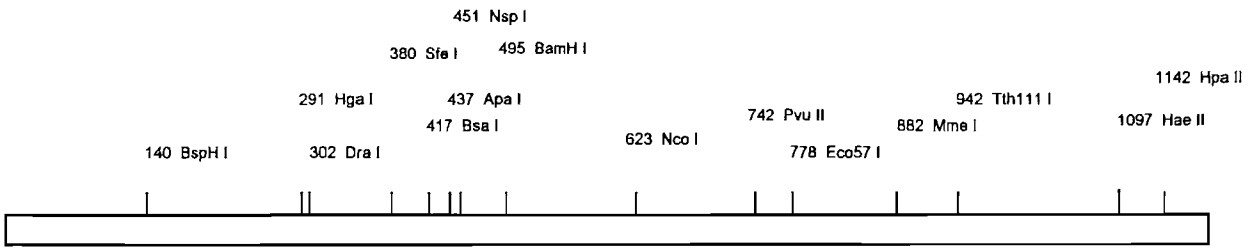
Figure 3.9e: Sequencing strategy (A), restriction map (B) and ORF map (C) for DIF-15.

A



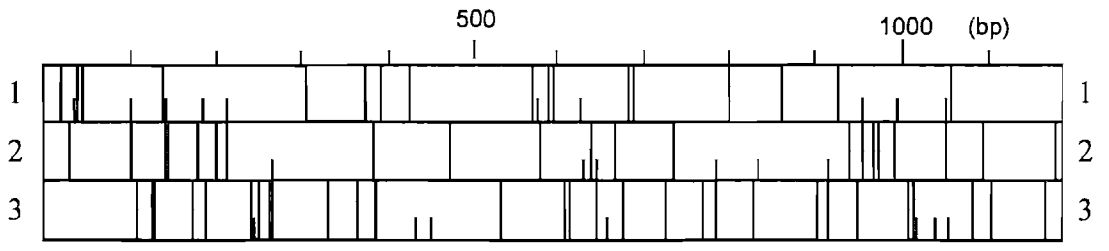
DIF-16 sequencing strategy

B



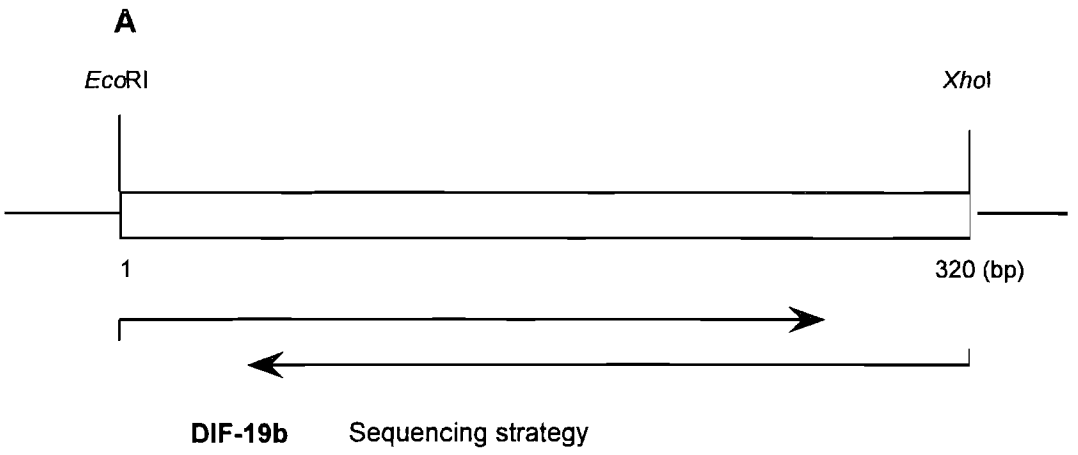
DIF -16 Restriction map Unique sites

C

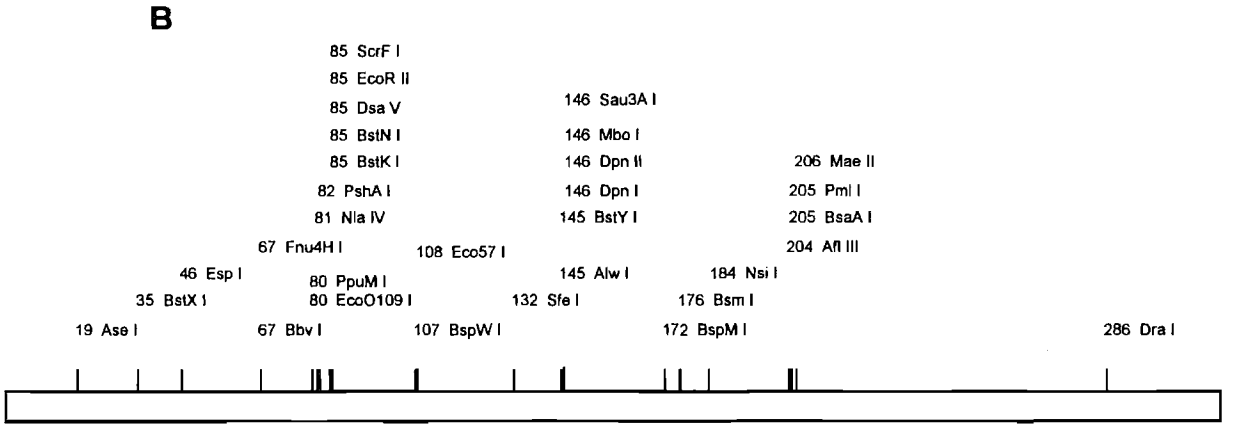


DIF-16 ORF map

Figure 3.9f: Sequencing strategy (A), restriction map (B) and ORF map (C) for DIF-16.



DIF-19b Sequencing strategy



DIF-19b Restriction map Unique sites

DIF-19b ORF map

Figure 3.9g: Sequencing strategy (A), restriction map (B) and ORF map (C) for DIF-19b.

3:1.3 Sequence analysis using the GenBank database

Analysis of the partial sequence data obtained from the putative differentially expressed cDNA clones revealed that two of the eight remaining hybridisation groups corresponded to known genes, a further three were related to sequences in the GenBank and EMBL databases, and the remaining three showed no homology to known sequences. Interestingly, six of the eight clones have a 90bp concensus BC1 repeat element at their 3' ends. The results of the preliminary characterisation are summarised in table 3.5.

Table 3.5: Best homology scores from GenBank and EMBL databases

CLONE	BC1 REPEAT?	HOMOLOGY
DIF-1	YES	No significant homology to cDNA sequences in the databases
DIF-R6b	NO	66.8% identity over 244 bp to FAR-17a, an androgen regulated gene expressed in hamster flank organs.
DIF-7	YES	No significant homology to cDNA sequences in the databases
DIF-14b	YES	68% identity over 795bp to rat calcium independent alpha-latrotoxin receptor.
DIF-15	YES	71% identity over 538bp to mouse mRNA for synaptobrevin-like protein, SYBL-1.
DIF-16	YES	89.9% identity over 433bp to rat phosphatidylinositol 3-kinase regulatory subunit.
DIF-19b	NO	No significant homology to cDNA sequences in the databases
DIF-20	YES	98.1% identity over 485bp to rat mitochondrial rRNA transcript.

The two known genes identified in the differential screen were DIF-20, corresponding to a rat mitochondrial gene transcript, and DIF-16, corresponding to a phosphatidylinositol-3-kinase regulatory subunit (Inukai *et al* 1996). The three clones which showed significant homology to sequences in the GenBank and EMBL databases are; DIF-R6b, having 66.8% identity to an androgen regulated gene expressed in hamster flank organs (Seki *et al* 1991);

DIF-14b, having 59.4% identity to an olfactomedin related protein expressed in rat brain (Danielson *et al.* 1994) and 68% identity to the olfactomedin homology domain of rat calcium independent alpha-latrotoxin receptor (Krasnoperov *et al* 1996, Lelianova *et al* 1997, Sugita *et al* 1998); and DIF-15, having 71% identity to a mouse synaptobrevin-like protein, SYBL-1 (D'Esposito *et al* 1996).

3:1.4 RNase Protection

To confirm that the clones isolated from the differential screen represent mRNA's expressed in adult hair follicles, an RNase protection analysis was performed using a commercially available RNase protection assay system (Promega, RNaseONE system) according to the manufacturers protocol. In each assay, a clone specific ^{32}P labelled antisense riboprobe was used in a hybridisation reaction with 1μg mRNA isolated from rat vibrissa follicles. For each assay, a positive control (purified ^{32}P labelled antisense riboprobe alone) and negative control (RNase treated probe hybridised to target RNA) was included. A positive control assay using a β-actin antisense riboprobe was also performed to check the quality of the target mRNA.

Clones DIF - 16 and DIF - 20 were not used in RNase protection assays as they correspond to previously identified mRNA's, and clone DIF - 17a was not used due to the small size of its cDNA insert. Of the remaining six clones, protected fragments are present in four of the assays, confirming that these four clones represent mRNA's which are expressed in vibrissa follicles (figure 3.10. The protected fragments are always shorter than the probe alone due to a mismatch at the 5' end of the probe which results in digestion and loss of the mismatched region. The mismatch is due to a short vector sequence being present at the end of the probe. Probe sizes are given in table 3.6).

Table 3.6: Probe sizes used for RNase protection assay.

CLONE	PROBE SIZE	PROTECTED FRAGMENT SIZE
β-actin control	350bases	250 bases
DIF-1	350 bases	No protected fragment
DIF-R6b	420 bases	~400 bases
DIF-7	280 bases	No protected fragment
DIF-14b	450 bases	~350 bases
DIF-15	350 bases	~320 bases
DIF-19	300 bases	~250 bases

3:1.5 Non radioactive ISH

Non radioactive *in situ* hybridisation was performed using the clones isolated from the differential screen to show whether they were preferentially expressed in the GE cells. Three of the clones were clearly highly expressed in the GE cells, with the remaining clones being expressed in both the GE and matrix cells at low levels (figure 3.11). This suggested that the differential screening strategy was only partially successful in enriching for GE cell specific clones, since even the clones which were highly expressed in the GE cells were also expressed at varying levels in the epidermal matrix.

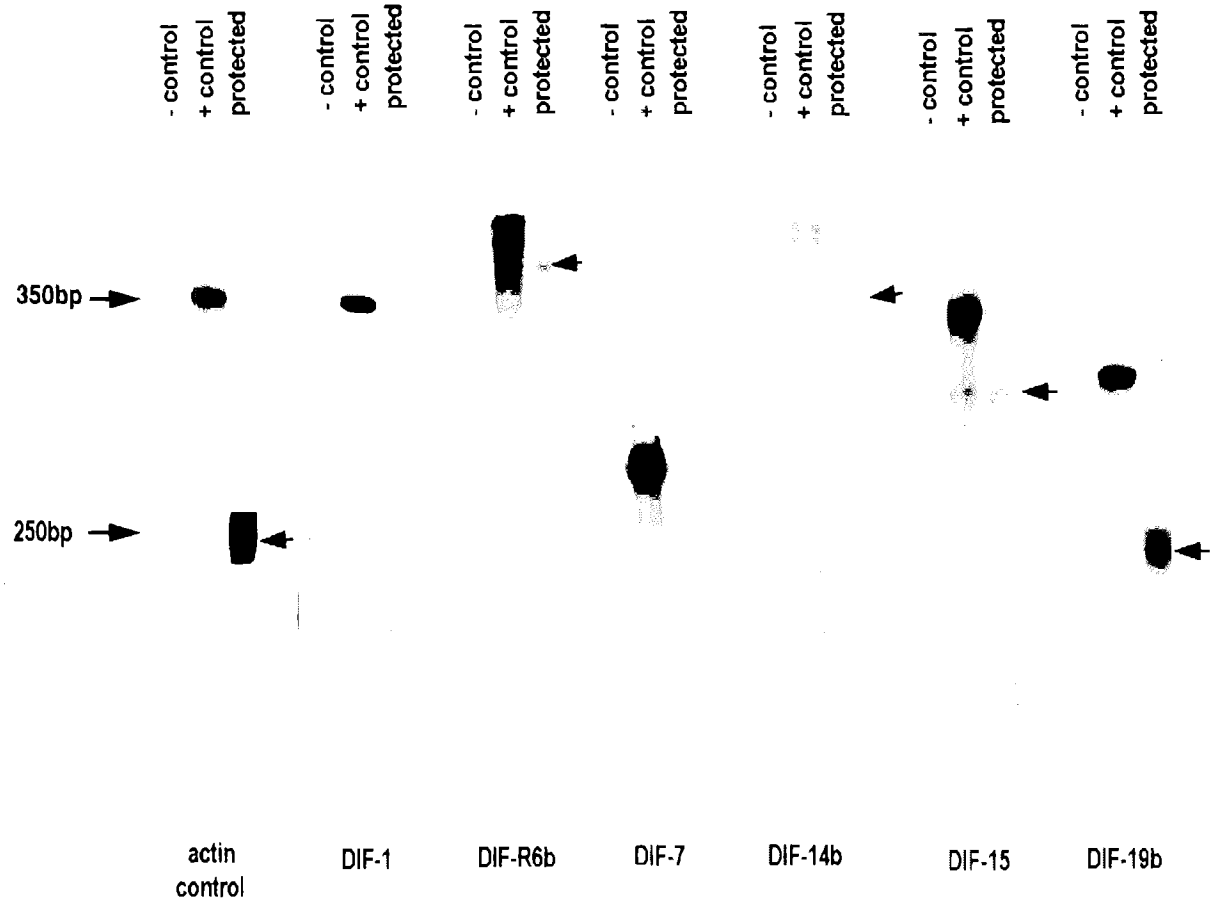


Figure 3.10: RNase protection assays. Rat vibrissa follicle mRNA was hybridised to clone specific antisense RNA probes. Protected fragments are present in four of the assays (DIF-R6b, DIF-14b, DIF-15, DIF-19b), showing that these clones represent genes which are expressed in the hair follicle. The protected fragments are indicated in each case by the arrows.

Table 3.7: Expression of Differential clones in the hair follicle

CLONE	Expression in hair follicle confirmed by	Preferentially expressed in GE?
DIF-1	non radioactive *in situ* hybridisation	NO
DIF-R6b	RNase protection, non radioactive *in situ* hybridisation	NO, appears preferential in bulge
DIF-7	non radioactive *in situ* hybridisation	NO
DIF-14b	RNase protection, non radioactive *in situ* hybridisation	YES
DIF-15	RNase protection, non radioactive *in situ* hybridisation	YES?
DIF-16	non radioactive *in situ* hybridisation	YES?
DIF-19b	RNase protection, non radioactive *in situ* hybridisation	NO
DIF-20	non radioactive *in situ* hybridisation	Strongly expressed throughout

3:2 Further Characterisation of DIF-R6b DIF-14b, DIF-15 and DIF-19b

Of the putative differentially expressed clones isolated in this study, DIF-R6b, DIF-14b, DIF-15 and DIF-19b were selected for further characterisation. The sequence, *in situ* hybridisation and RNase protection data for these clones suggested that they were likely to represent novel genes expressed in specific tissues in the hair follicle. Sequence data showed that three of these clones (DIF-R6b, DIF-15 and DIF-19b) have a concensus polyadenylation signal A(A/T)TAAA at their 3' end, and two clones (DIF-14b, DIF-15) encode a putative partial open reading frame. The results of the ISH and RNase protection assays demonstrated that all of these clones are expressed in the hair follicle, and that DIF-14b and DIF-15 appear to be more strongly expressed in the GE cells.

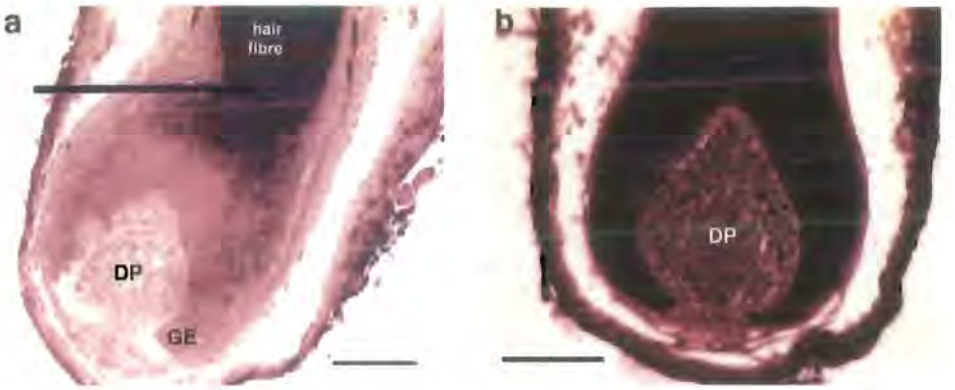


Figure 3.11: Non radioactive *in situ* hybridisation of anagen vibrissa follicle sections with digoxygenin labelled clone specific probes.

a: A mouse hair keratin probe hybridises only to epidermal cells where hair-type differentiation is occurring (arrowed). There is no labelling in either the DP or GE cells. This shows that the hybridisation protocol is specific and effective.(Bar=100um)

b: A section through an anagen rat vibrissa follicle hybridised to a DIF-20 antisense RNA probe. As expected, this probe hybridises strongly to all cells in the end bulb, since DIF-20 is derived from a mitochondrial RNA transcript which is highly expressed in all cell types. (Bar = 100um)

c: A section through an anagen rat vibrissa follicle hybridised to a DIF-1 antisense RNA probe, showing hybridisation to all cells in the epidermal matrix. DIF-1 therefore appears not to be differentially expressed. (Bar = 50um)

d: A section through an anagen rat vibrissa follicle hybridised to a DIF-7 antisense RNA probe. Again, all cells of the epidermis appear to be evenly labelled. However, there appears to be a slightly lower intensity of staining in the outer root sheath (ORS). The dark staining around the dermal papilla (DP) is due to hair fibre pigmentation (melanin). (Bar = 100um)

e: A section through an anagen rat vibrissa follicle hybridised to a DIF-19b antisense RNA probe. Though the staining is less evenly distributed than in (c) and (d), there is no evidence to suggest that DIF-19b is more highly expressed in the GE cells, instead appearing to be most highly expressed in the upper matrix (arrowed). (Bar = 100um)

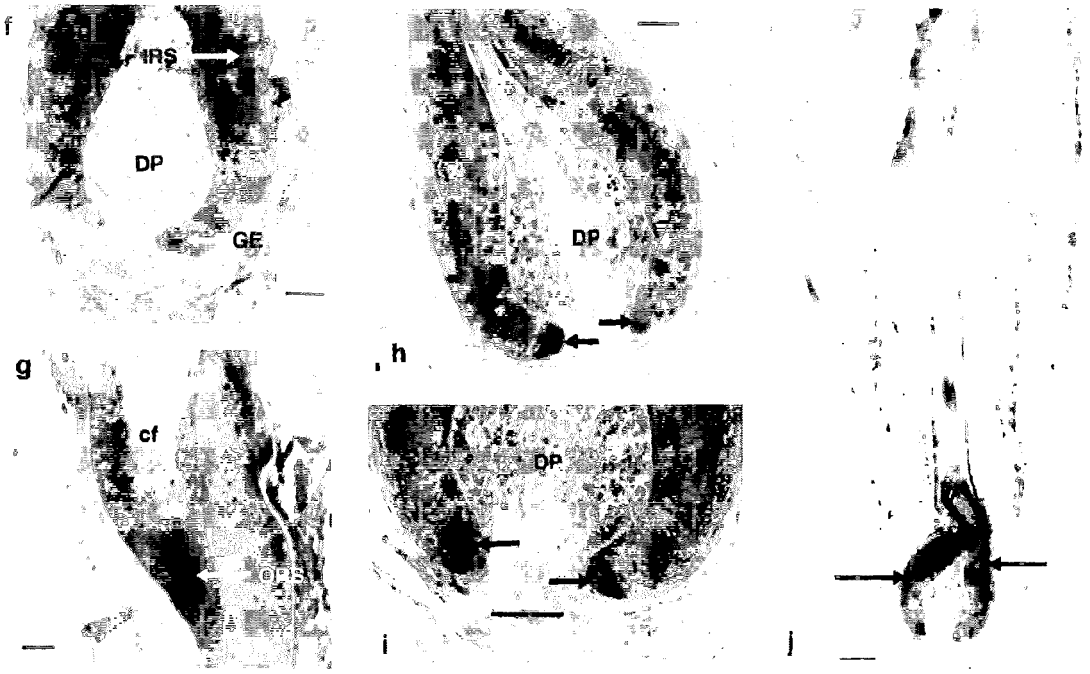


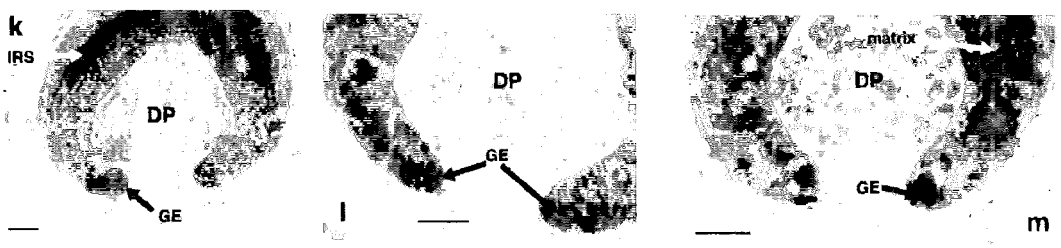
Figure 3.11: Non radioactive *in situ* hybridisation with clone specific probes.

f, g: The DIF-R6b probe shows relatively little hybridisation to cells in the end bulb (f), although there are regions in the GE and IRS that appear to be slightly more intensely stained (arrows). However, this probe hybridises strongly to a region of the outer root sheath at the base of the club fibre (cf), indicated by the arrow (g). (Bars = 50um)

h, i, j: The DIF-14b probe hybridises very strongly to the GE cells, which are intensely stained in both (h) and (i), indicated by the arrows. However, a high level of DIF-14b staining is also evident in the upper end bulb region (j), particularly in the IRS (arrowed). There is very little DIF-14b staining elsewhere in the follicle, although some cells in the DP and matrix also hybridise to this probe (h, i). (Bars = 50um (h, i) and 150um (j))

k, l: DIF-15 also hybridises to the GE cells and IRS, with low levels of hybridisation appearing elsewhere in the follicular epidermis. (Bars = 50um (k) and 100um (l))

m: The level of DIF-16 staining in the end bulb is generally high, particularly in the GE and matrix cells. (Bar = 100um)



3:2.1 cDNA Library Screening by PCR

The end bulb and follicle cDNA libraries were screened by PCR using a vector specific forward primer and clone specific reverse primer to confirm that each of the differentially expressed clones was represented in both libraries. A range of product sizes was obtained in all cases (table 3.8 and figure 3.12)

Table 3.8: Products obtained from library screen PCR's

Clone	Library	Expected PCR Product Size (from known clone)	PCR Product size Range
DIF-R6b	End Bulb	320bp	major bands of 450bp and 300bp
	Follicle		major band of 300bp, smear extending to 750bp.
DIF-14b	End Bulb	390bp	major band of 250bp, smear extending to 1kb
	Follicle		major band of ~400bp, smear extending to 1kb
DIF-15	End Bulb	250bp	major bands of ~250bp and 400bp
	Follicle		major bands of 250bp and 400bp
DIF-19b	End Bulb	175bp	smear up to 750bp
	Follicle		smear up to 750bp

The major band from each PCR reaction performed using follicle cDNA library DNA as the template was isolated, cloned and sequenced. In each case, the sequence data generated was identical to the parent clone isolated from the end bulb cDNA library by differential screening. However, no additional sequence data was generated by this method, except in the case of DIF-15, where an additional 60bp of sequence was obtained (figure 3.13). This sequence data was combined with the known sequence data from the end bulb clone for further analysis.

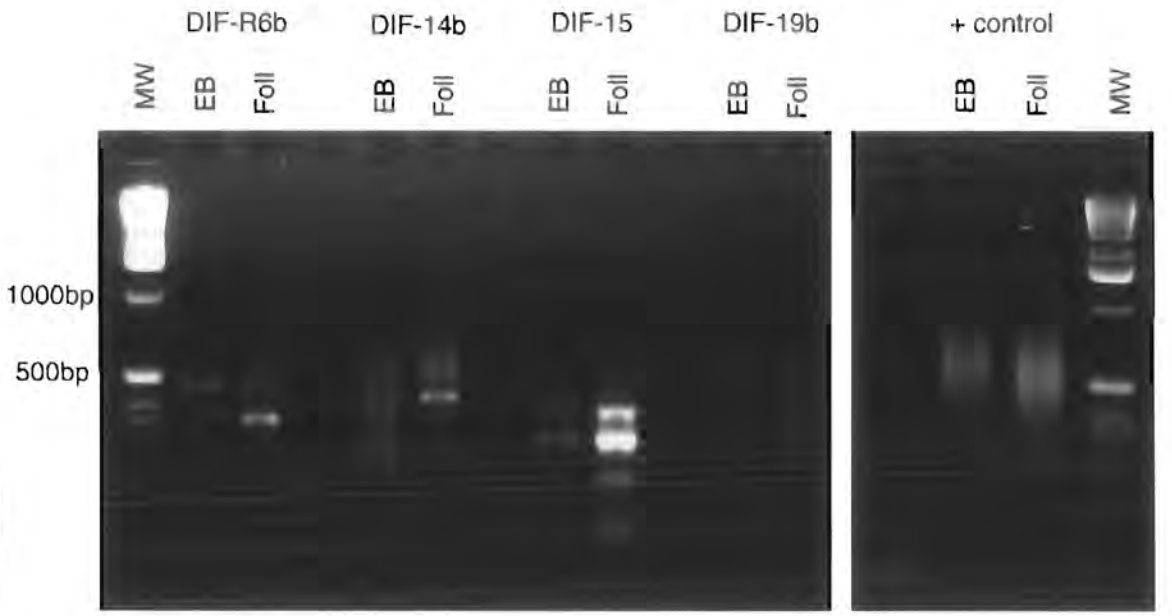


Figure 3.12: Library screening by PCR using a vector specific forward primer and a clone specific reverse primer. Samples of end bulb (EB) and follicle (Foll) library DNA were amplified with each set of primers and analysed by agarose gel electrophoresis. A range of PCR products were obtained from both libraries, confirming that the cDNA clones used in this study were present in both the end bulb and follicle cDNA libraries. The identity of the PCR products obtained from the follicle library was confirmed by sequencing.

The total range of cDNA inserts in each library DNA sample was also amplified using vector specific forward and reverse primers (+ control).

3:2.2 Northern Blotting

The ^{32}P-labelled inserts of the four clones shown to be expressed in hair follicle RNA by RNase protection were used to probe a multiple tissue northern blot to determine the expression pattern in other tissues. Several of the clones hybridised to multiple transcripts in some tissues. Transcript lengths and expression patterns are given in table 3.9 and figure 3.14. When the blot was probed with the insert of DIF-R6b, a high level of background hybridisation was observed in addition to two major bands. This suggests that there is a repeat sequence present in the DIF-R6b insert, even though the sequence data obtained shows that this is one of the clones which does not contain a BC1 repeat.

Table 3.9: Results of MTN blotting

CLONE	TRANSCRIPT LENGTHS (BP)	EXPRESSED IN...
DIF-R6b	3.3kb, high background.	liver, lung, spleen, brain
DIF-14b	4.9kb, may be doublet?	testis, kidney, liver, lung, brain, heart
DIF-15	2.7kb major, 2.4 and 1.3kb minor	testis, kidney, liver (3bands), lung, spleen, brain
DIF-19b	2.7kb	testis, kidney, liver, lung, spleen, brain

3:2.3 Southern Blotting

Southern blotting of each of the putative differentially expressed clones on *Hind*III digested rat genomic DNA showed that two of the four clones were present as single copies in the rat genome. The blots are shown in figure 3.15. The DIF-14b probe hybridised to a single band of 1.3kb, the DIF-15 probe hybridised to two bands (0.6kb and 4.5kb). No obvious band was revealed with the DIF-19b probe, and the DIF-R6b probe revealed a major band of 0.8kb with strong background hybridisation appearing as a smear extending up to around 10kb.

Figure 3.13: DNA sequence alignment of clones isolated from the follicle (FolI) cDNA library with the parental clones from the end bulb (EB) cDNA library. The clone specific primer sequence used in each case is indicated by the arrow.

A DIF-R6b - no extra sequence obtained

```
Foll DIF-R6b                                CTCTGACGAACCTTGGTTAGACCTATTGTT
                                            ||||||| ||||||||||||||||||||||
EB DIF-R6b    GGCAGAGGTTACTATGATTTTGGTGCTCTGACCAACCTTGGTTAGACCTATTGTT

Foll DIF-R6b  GTCTAACAAAGAAATAAGCAAGGCCCTGCCCTTCCAGCTC-GCAGGCTTCTGGCC
              |||||||||||||||||||||||||||||||||||||||| ||||||||||||||
EB DIF-R6b    GTCTAACAAAGAAATAAGCAAGGCCCTGCCCTTCCAGCTCTGCAGGCTTCTGGCC

Foll DIF-R6b  TGAAGAAGGCCTCAGGCCACTTTCCTGAGGTCCTTAGCTGAGCCATTTCCCACCC
              |||||||||||||||||||||||||||||||||||||||||||||||||||||||
EB DIF-R6b    TGAAGAAGGCCTCAGGCCACTTTCCTGAGGTCCTTAGCTGAGCCATTTCCCACCC

Foll DIF-R6b  TTTGACTGAGACAAGTAAACTAGATTTGTCTGTGGGTCCACC
              |||||||||||||||||||||||||||||||||||||||
EB DIF-R6b    TTTGACTGAGACAAGTAAACTAGATTTGTCTGTGGGTCCACC
                                  <---------------------
                                        primer 1214
```

B DIF-14b - no extra sequence obtained

```
Foll DIF-14b     TCTTGTCTTTTTTCCCCCGTGGCGTTAGTTTT-GTGTGTCCTGGAACCTT
                 |||||||||||||||||||||||||||||||| ||||||||||||||||
EB DIF-14b    CTTTCTTGTCTTTTTTCCCCCGTGGCGTTAGTTTTTGTGTGTCCTGGAACCTT

Foll DIF-14b  GAAAGCAATTGTGGACTCTCCAAGTATCTATGAAGCTGAGCAAAAGGCAGGTG
              |||||||||||||||||||||||||||||||||||||||||||||||||||||
EB DIF-14b    GAAAGCAATTGTGGACTCTCCAAGTATCTATGAAGCTGAGCAAAAGGCAGGTG

Foll DIF-14b  CTTGGTCCAAGGACCCCCTTCAGGCTGCAGATAAAATTTATTTTATGCCCTGG
              |||||| ||||||||||||||||||||||||||||||||||||||||||||||
EB DIF-14b    CTTGGTGCAAGGACCCCCTTCAGGCTGCAGATAAAATTTATTTTATGCCCTGG

Foll DIF-14b  ACTCCCTACCGCACCGATACCTTAATAGAATATGCTTCTTTAGAAGATTTTCA
              |||||||||||||||||||||||||||||||||||||||||||||||||||||
EB DIF-14b    ACTCCCTACCGCACCGATACCTTAATAGAATATGCTTCTTTAGAAGATTTTCA

Foll DIF-14b  AAACAGCCGCCAGACAACAACATACAAACTTCCAAACCGAGTGGACGGTACTG
              |||||||||||||||||||||||||||||||||||||||||||||||||||||
EB DIF-14b    AAACAGCCGCCAGACAACAACATACAAACTTCCAAACCGAGTGGACGGTACTG
                                                      <-------------

Foll DIF-14b  GATTTGTG
              ||||||||
EB DIF-14b    GATTTGTG
              ========
              primer 1215
```

C DIF-15 - 60bp extra sequence obtained

```
Foll DIF-15    GGCACGAGGTCAAGCTCACTGCCATCATCGTCGTTGTATCGATTGTGTTCATCTA

Foll DIF-15    CATCATCGTGTCTCCACTGTGCGGTGGCTTCACGTGGCCAAGCTGTGTGAAGAAA
               ||||||||||||||||||||||||||||||||||||||||||||||||||||||
EB DIF-15          TCGTGTCTCCACTGTGCGGTGGCTTCACGTGGCCAAGCTGTGTGAAGAAA

Foll DIF-15    TAAAGGAAAAAAAATCACTGTTCACCAAGGATGTGAGCATGGAAGGAGGAAAAGCC
               ||||||||||||||||||||||||||||||||||||||||||||||||||||||||
EB DIF-15      TAAAGGAAAAAAAATCACTGTTCACCAAGGATGTGAGCATGGAAGGAGGAAAAGCC

Foll DIF-15    AATGCGTGTGGGCCTGGCTTTCACTTCTCTCAGCAAA
               |||||||||||| ||||||||||||||||||||||||
EB DIF-15      AATGCGTGTGGCCCTGGCTTTCACTTCTCTCAGCAAA
                                                    <---------
                                                    primer 15a
```

D DIF-19b - no extra sequence generated

```
Foll DIF-19b                    ATACATAAAATAAATTAATTTTCAAAAAGCCAGTGAAATGGC
                                ||||||||||||||||||||||||||||||||||||||||||
EB DIF-19b     GGCACGAGCTCATATACATAAAATAAATTAATTTTCAAAAAGCCAGTGAAATGGC
                                                                  <-------
                                                                  primer 19b

Foll DIF-19b   TCAGCAGT
               ||||||||
EB DIF-19b     TCAGCAGT
               ========
```

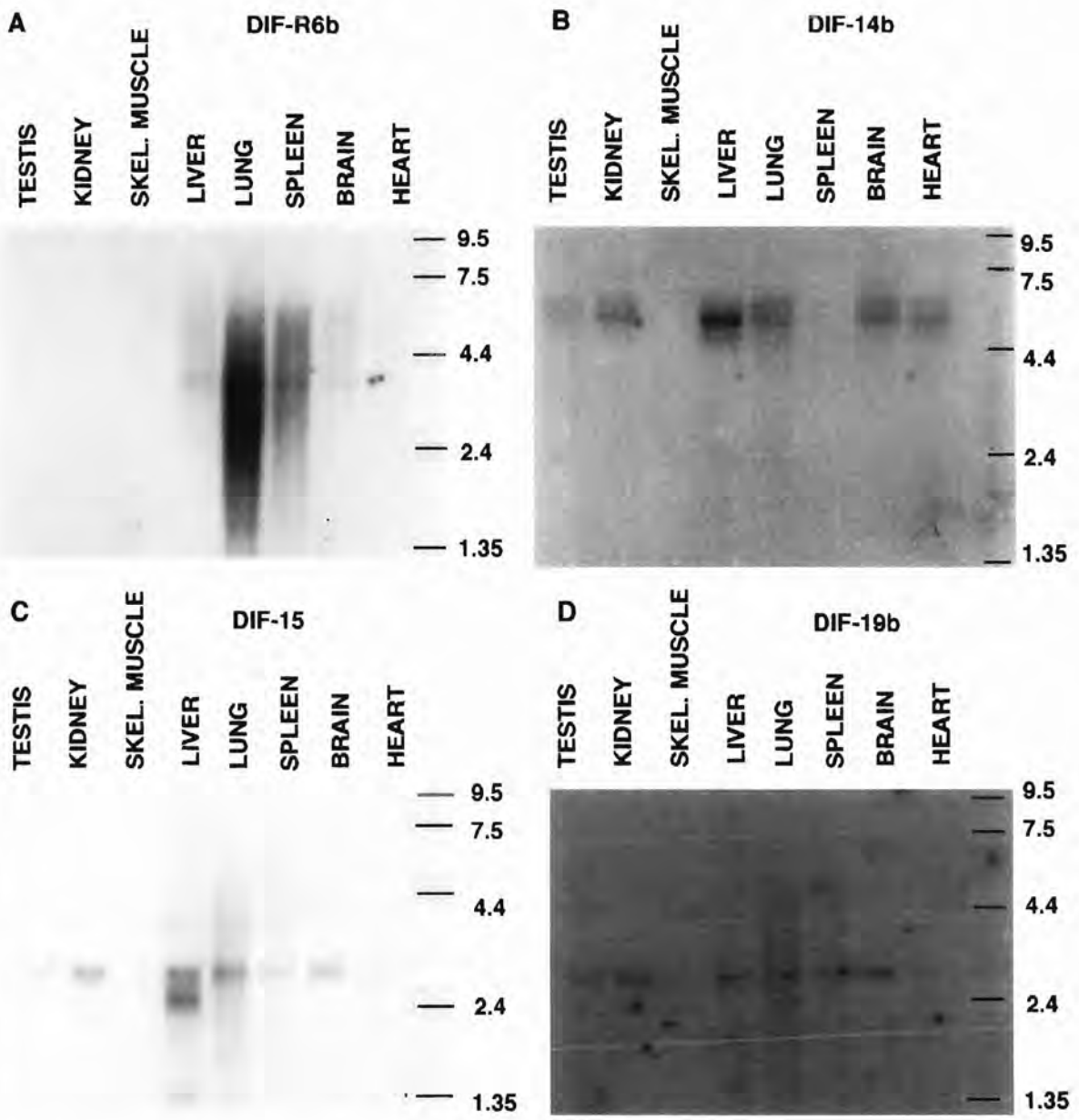


Figure 3.14: A rat multiple tissue northern blot probed with DIF-R6b (A), DIF-14b (B), DIF-15 (C) and DIF-19b (D).

A - The DIF-R6b probe hybridises to a 3.3kb transcript in liver, lung, spleen and brain. The background signal is likely to be due to a repeat element in the probe sequence.

B - The DIF-14b probe hybridises to a band of around 4.9kb in testis, kidney, liver, lung, brain and heart. This may represent a doublet of two related transcripts.

C - The DIF-15 probe hybridises to a 2.7kb transcript in testis, kidney, liver, lung, spleen and brain. Additional transcripts of 1.3kb and 2.4kb are also detected in the liver.

D - The DIF-19b probe hybridises to a 2.7kb transcript in testis, kidney, liver, lung, spleen and brain.

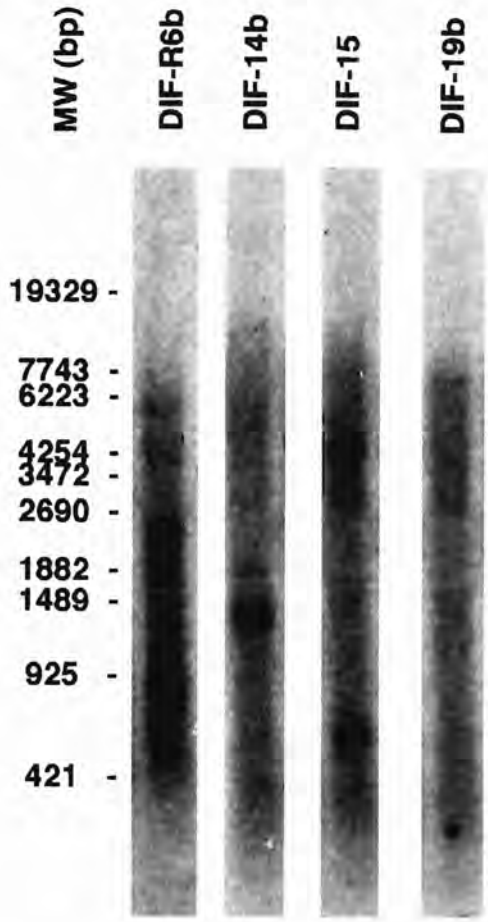


Figure 3.15: A Southern blot of rat HindIII digested genomic DNA probed with DIF-R6b, DIF-14b, DIF-15 and DIF-19b cDNA probes. The hybridisation pattern of DIF-R6b, DIF-14b and DIF-15 indicate that these clones are present as single copies in the rat genome.

3:2.4 Sequence Analysis of DIF-R6b, DIF-14b, DIF-15 and DIF-19b.

Searching of the GenBank and EMBL databases with the sequences of each of these clones revealed several good homology scores with known genes. The best scores for each of the clones are given below.

3:2.4-1 DIF-R6b

There were relatively few high scoring mRNA/cDNA sequences in the database which matched the sequence of the DIF-R6b full length cDNA clone. The best matches are given in table 3.10. These sequences all share a region of homology in the 3' 250bp of DIF-R6b. Since this sequence is common to the 3'UTR of four apparently unrelated cDNA clones isolated from different sources, this suggests that this sequence is a repeat element. This is backed up by the results of the northern blots for DIF-R6b, which indicate that the full length DIF-R6b cDNA probe hybridises to multiple sequences. This putative repeat region is also homologous to many genomic sequences in the databases, both in introns (e.g. in the mouse smooth muscle gamma actin gene U20365, the mouse IgG receptor gene M63159, the rat lysozyme gene L12459, the mouse IgE binding lectin gene M97896) and in promoter regions (e.g. the mouse MHC class I H2-K gene promoter region M11847, the mouse gas-1 promoter Z22668), so it is not surprising that the DIF-R6b Southern blot also showed a high level of background hybridisation.

Table 3.10: Best homology scores for DIF-R6b. The GenBank and EMBL databases were searched using the BLAST algorithm (Altschul *et al* 1997) to compare sequences in the databases with the DIF-R6b full length cDNA sequence. Only high scoring sequences corresponding to mRNA/cDNA are given here.

Homology Score	Gene	GenBank Accession Number	Reference
66.8% over 244bp	Syrian golden hamster androgen dependant expressed protein mRNA, end of 3'UTR.	M80427	Seki *et al* 1991
67.8% over 233bp	Mouse mRNA for P100 serine protease of Ra-reactive factor, middle of 3'UTR.	D16492	Takahashi *et al* 1993
66.3% over 246bp	Rat mRNA for 5E5 antigen, middle of 3'UTR, inverted.	D37934	Suzuki *et al* 1995

Figure 3.16: Alignment of DIF-R6b with the 3' end of hamster FAR-17a (Seki *et al* 1991). The level of homology upstream of this region is relatively low (~33%). The concensus polyadenylation signal present at the 3' end of both transcripts is indicated in bold type.

```
DIF-R6b 211   GTAGTGTTTTGAATATGCTTGGCCCATGGGAAGTGGCACTATTAGGAGGT   260
              || |     |  |   ||||    |    | ||||   | ||| | |
FAR-17a 1375  CTAATAGAAGGTGTGATCTTGTTGGAGTATATGTGGTCTTGTTAAGGGAA  1424

DIF-R6b 261   GTGGTGTTACTGTGGAGGCCAGGCTTTGAGGTCTTCATATATTAGCTCAA   310
              ||  ||| ||||||||||| |||||||||| ||| ||||| ||  ||||||
FAR-17a 1425  GT-TTGTCACTGTGGAGG-CAGGCTTTGAAGTC-TCATACAT--GCTCAA  1469

DIF-R6b 311   GTC-TGACCAGTG-TGAACGGTTCTTCCTCTTGGGCTGCCTCAAGATCAA   358
              | | || ||||||  | |     |||||| || | ||||||    |||||
FAR-17a 1470  GGCATGCCCAGTGAGGTAGACCACTTCCTGTT-GCCTGCCT----ATCAA  1514

DIF-R6b 359   GATGTTAGAACTTTCAGCTTTTTTCCCAGCACCATGTCTGCCTGGACACT   408
              ||| | |||    ||| ||      ||||||||||||||||||  |  |
FAR-17a 1515  GATATAAGA---CTCAACT------CCAGCACCATGTCTGCCTCCATGCC  1555

DIF-R6b 409   GCCATGCTTC----TCGCCATAATGGACTGAGCCTTGAAACTGTAAACCA   454
              || ||   |    |    |||||||||  | |||   || ||||| |||
FAR-17a 1556  ACCGTGTCACACAATGATAATAATGGACCAAACCTCTGAAATGTAAGCCA   1605

DIF-R6b 455   GCCCCATTAAA--TGTTGTCCTTTGTTAAGAAAAAAAAAAAAAAAAAAA   502
              ||| ||||||  | || ||||||
FAR-17a 1606  CCCCAATTAAATTTTTTTCCTTT                             1629
```

3:2.4-2 DIF-14b

A database search using the DIF-14b cDNA sequence gave the best results of the clones under study in terms of the number of relatively high scoring mRNA/cDNA sequence matches obtained. It was necessary to omit the 3' BC1 repeat element from the search, as this gave rise to a large number of BC1 repeat containing sequences and not much else. This is because the homology of the DIF-14b BC1 repeat with those in the database is very high (>90% over 100bp), whereas the remaining sequence gives lower homology scores over larger segments. The best matches in the database are rat calcium independent alpha-latrotoxin receptor (Krasnoperov *et al* 1997), rat neuronal olfactomedin related ER localised protein (Danielson *et al* 1994), and a trabecular meshwork inducible protein (TIGR, Nguyen *et al* 1998), which all share a reasonable identity with the DIF-14b ORF at the amino acid level.

Table 3.11: Best homology scores for DIF-14b. The GenBank and EMBL databases were searched using the BLAST algorithm (Altschul *et al* 1997) to compare sequences in the databases with the DIF-14b cDNA sequence (without the 3' BC1 repeat).

Homology Score	Gene	GenBank Accession Number	Reference
68% over 795bp	Rat calcium independent alpha-latrotoxin receptor mRNA	U72487	Krasnoperov *et al* 1997
68% over 793bp	Rat latrophilin related protein-1 precursor mRNA	U78105	Davletov *et al* 1996
57% over 373bp	Human myocilin mRNA	D88214	Kubota *et al* 1997
57% over 373bp	Human trabecular meshwork inducible glucocorticoid response protein (TIGR) mRNA	U85257	Nguyen *et al* 1998
66.1% over 230bp	Rat lysosomal membrane glycoprotein mRNA	M34959 J03672	Howe *et al* 1988
64.7% over 215bp	Rat mRNA for 107kDa sialoglycoprotein	X14765	Himeno *et al* 1989
66.4% over 146bp	Rat neural receptor protein tyrosine kinase mRNA	M55293	Middlemas *et al* 1991
54.7% over 358bp	Rat olfactomedin related ER localised protein mRNA	U03417 U03416	Danielson *et al* 1994

This is discussed further in section 3:3. The alignment of the DIF-14b cDNA sequence with rat alpha-latrotoxin receptor is given in figure 3.17.

Figure 3.17: Alignment of DIF-14b ORF (1-879bp) with rat alpha-latrotoxin receptor mRNA sequence (Latro) (from Krasnoperov *et al* 1997).

```
DIF-14b  30   AGTTTTTGTGTGTCCTGGAACCTTGAAAGCAATTGTGGACTCTCCAAGTA  79
              ||| || ||||| || || ||  || |     |  ||||  |  |    |
Latro   492   AGTCTTCGTGTGCCCAGGGACACTGCAGAAGGTGCTGGAGCCCACCTCCA  541

DIF-14b  80   TCTATGAAGCTGAGCAAAAGGCAGGTGCTTGGTGCAAGGACCCCCTTCAG  129
                 ||||| | |||||  || | || || ||||||||||||||| || |||
Latro   542   CACATGAATCGGAGCACCAGTCTGGCGCATGGTGCAAGGACCCACTGCAG  591

DIF-14b 130   GCTGCAGATAAAATTTATTTTATGCCCTGGACTCCCTACCGCACCGATAC  179
              || |  ||    || ||  |||||||||||  |||||||||| || ||
Latro   592   GCAGGTGACCGTATCTACGTTATGCCCTGGATCCCCTACCGCACGGACAC  641

DIF-14b 180   CTTAATAGAATATGCTTCTTTAGAAGATTTTCAAAACAGCCGCCAGACAA  229
                | |  || |||||||| |  || || | |         ||||| || |
Latro   642   ACTGACCGAGTATGCTTCCTGGGAGGACTATGTGGCTGCACGCCACACCA  691

DIF-14b 230   CAACATACAAACTTCCAAACCGAGTGGACGGTACTGGATTTGTGGTGTAT  279
              | || |||| ||| || ||||| || || || ||||| ||||||||  |||
Latro   692   CCACGTACAGACTGCCCAACCGTGTAGATGGCACTGGCTTTGTGGTATAT  741

DIF-14b 280   GACGGGGCAGTCTTCTTCAACAAAGAAAGAACGAGAAACATTGTTAAATT  329
              || || || |||||||  ||||| ||| | ||  | ||||||| ||||
Latro   742   GATGGTGCCGTCTTCTATAACAAGGAACGTACTCGCAACATTGTCAAATA  791

DIF-14b 330   TGACTTGAGGACTACAATCAAGAGTGGGGAGGCCATAATCAACTACGCCA  379
              |||| || ||||    |||||||| || ||  |  | || |||   ||||
Latro   792   TGACCTGCGGACCCGCATCAAGAGCGGAGAAACAGTCATAAACACAGCCA  841

DIF-14b 380   ACTACCATGACACTTCACCCTACAGATGGGGGGGGAAGACTGACATTGAC  429
              ||||||| ||||| ||||| ||  | ||||| || || || |||||||||
Latro   842   ACTACCACGACACCTCACCTTATCGCTGGGGAGGCAAAACCGACATTGAC  891

DIF-14b 430   CTGGCAGTGGACGAAAATGGCCTGTGGGTCATTTACGCCACCGAGCAGAA  479
              |||||||||||| || || || |||||||||||| || |||||||||  |||
Latro   892   CTGGCAGTGGATGAGAACGGGCTGTGGGTCATCTATGCCACCGAGGGGAA  941

DIF-14b 480   CAACGGAATGATCGTGATTAGCCAGCTCAATCCGTACATTTTCCGATTGG  529
              ||||||     | ||| | |||||||||||| || ||||   | || || |
Latro   942   CAACGGGCGTCTGGTGGTGAGCCAGCTCAACCCCTACACACTGCGTTTCG  991

DIF-14b 530   AAGCAACCTGGGAGACGACGTATGACAAGCGTGCGGCGTCCAATGCTTTC  579
              | |  |||||||| ||    |||||||||||  | || |||||||| |||
Latro   992   AGGGCACCTGGGAAACAGGCTATGACAAGCGCTCAGCCTCCAATGCCTTC  1041

DIF-14b 580   ATGATATGCGGGGTCCTATACGTGGTCAGGTCAGTGTACCAAGACAATGA  629
              ||| | || || ||||| || ||| |  | || || ||    ||  | ||
Latro  1042   ATGGTGTGTGGTGTCCTCTATGTGCTGCGCTCTGTTTATGTGGATGACGA  1091
```

```
DIF-14b  630  AAGCGAAGCTGGCAAGAACGTCATCGACTACATTTACAACACAAGGTTGA  679
              || || || |     ||| | | |||||    | |||||  |     |
Latro   1092  CAGTGAGGCAGCAGGCAACCGCGTGGACTATGCCTTTAACACCAATGCAA  1141

DIF-14b  680  GCCGGGGAGAGCACGTGGACGTTCCCTTCCCCAACCAGTACCAGTACATC  729
              ||| |  |||| |||     |  ||||||||||||  |||||||   |
Latro   1142  ACCGAGAGGAGCCCGTCAGTCTCGCCTTCCCCAACCCCTACCAGTTTGTA  1191

DIF-14b  730  GCTGCAGTGGATTACAACCCAAGAGACAACCAACTCTACGTATGGAACAA  779
              || | || || ||||| ||  | ||||||| || || || ||||||||
Latro   1192  TCTTCTGTTGACTACAATCCCCGGGACAACCAGCTGTATGTGTGGAACAA  1241

DIF-14b  780  TAACTTTATCTTACGGTATTCTCTGGAGTTTGGTCCACCCGACCC       824
                | ||  |  | || ||    ||||||||||| || || || ||
Latro   1242  CTATTTCGTGGTGCGCTACAGCCTGGAGTTTGGACCCCCAGATCC      1286
```

3:2.4-3 DIF-15

The BC1 repeat was removed from the 3' end of the DIF-15 cDNA sequence for the database search, for the reason outlined in the last section. The remaining sequence produced a high homology score when compared with mouse and human homologues of a cDNA encoding a synaptobrevin-like protein SYBL-1, both at the cDNA and amino acid level. This is shown in figure 3.18. The high score at the cDNA level indicates that DIF-15 is likely to represent a gene closely related to SYBL-1. The mouse SYBL-1 transcript is around 1.6kb long, and the human transcript is 2.6kb long (D'Esposito *et al* 1996), whereas rat DIF-15 hybridises to a 2.7kb transcript in most tissues studied (figure 3.14).

Table 3.12: Best homology scores for DIF-15. The GenBank and EMBL databases were searched using the BLAST algorithm (Altschul *et al* 1997) to compare sequences in the databases with the DIF-15 cDNA sequence (without the 3' BC1 repeat).

Homology Score	Gene	Accession Number	Reference
71% over 538bp	mRNA for mouse synaptobrevin-like protein SYBL-1	X96737	D'Esposito *et al* 1996, GenBank submission
70.9% over 265bp	mRNA for novel gene from human Xq28 region	X92396	D'Esposito *et al* 1996
71% over 52bp	mRNA for mouse gut enriched Kruppel-like factor GKLF.	U20344	Shields *et al* 1996

Figure 3.18 : A - Alignment of DIF-15 with mouse synaptobrevin-like protein 1 (SYBL-1, D'Esposito *et al* 1996). The two sequences are 71% identical over the first 538bp of DIF-15. The SYBL-1 ORF stop codon, common to both sequences, is indicated by the shaded box. A putative polyadenylation signal sequence in DIF-15 (upstream of the one present at the 3' end of the cDNA clone) is indicated in bold type.

B - Translation of the DIF-15 putative partial ORF corresponding to the region upstream of the TAA stop codon present in both DIF-15 and SYBL-1. The amino acid sequence is given in single letter code below the cDNA sequence.

A

```
DIF-15     1  GGCACGAGGTCAAGCTCACTGCCATCATCGTCGTTGTATCGATTGTGTTC  50
SYBL-1   682  TGAAGAATATCAAGCTCACTATCATCATCATCATTGTATCAATTGTGTTC  731

DIF-15    51  ATCTACATCATCGTGTCTCCACTGTGCGGTGGCTTCACGTGGCCAAGCTG  100
SYBL-1   732  ATCTATATCATTGTGTCACTTCTCTGTGGTGGGTTTACATGGCCAAACTG  781

DIF-15   101  TGTGAAGAAATAAAGGAAAAAAATCACTGTTCACC..AAGGATGTGAGCA  148
SYBL-1   782  TGTGAAGAAATAAGAAAGAAAAAAGTTACCATTAACTAAGGATATGAGAG  831

DIF-15   149  TGGAAGGAGGAAAAGCCAATGCGTGTGGCCCTGGCTTT.CACTTCTCTCA  197
SYBL-1   832  TGAAAGGAGATAAAGGAAATTCATGTGACTATGTCTTTTCACTACTCTCA  881

DIF-15   198  GCAAACGCCA.............TTTCTTTCATCCTCTTTCCTTTTTCT  233
SYBL-1   882  GAAAATGCCAGTTTCTTTAATCTTTTTTTTCTCTTTTTCTTGTTTTAAT  931

DIF-15   234  CTTTGCCCATCACACGGTTTGTGTTAACGAATTTCACATCGAGTCGTTGT  283
SYBL-1   932  CTTTGCCTGTCATATAGTTTGTGTTAATTAATTTCACATTGAATCATTAT  981

DIF-15   284  GCACAGGAGAAAGGACATCG.TTTGTTCCTCCGATTTGTTAAGTATAAGC  332
SYBL-1   982  TCACATGAGAAAGGGCATTTATTTGTTTCTTTAGTTTAAGTAGTACCTTC  1031

DIF-15   333  TTCT.AGAAAGACATCTTTGCTTCTAACATGC.AAGCTTTTGCCAAAGTG  380
SYBL-1  1032  TACTTAGGAAGACATTTTTGCTCTTAATATACAAAGCCTTTGCCAAGGTG  1081

DIF-15   381  GAAACGGCTGCGCTCACGACCTGTCTGTGAACTCACGACCTGTCTGCTAA  430
SYBL-1  1082  GAAACCACCAAACTTATGACCTGTCTGTTAA......AACTGTCTAGGA.  1124

DIF-15   431  ACCTGTCTGTGACGTTGTGTGTTGACACAAAGCACTGGGAAACGGGTTGC  480
SYBL-1  1125  ...........ACTTTGTATGTTCACACAAAGTGCTAGGAAATGGATTGT  1163
```

```
DIF-15    481 ACCGCTGACATCTCACTTTAGTCTCTTTCGTTGTGGGAGAATTTTGAGTG  530
              |    | |||||||| |||||||||||| ||| ||||||||||||  |||
SYBL-1   1164 ATTATTAACATCTCATTTTAGTCTCTTTTGTTATGGGAGAATTTTAGGTG  1213

DIF-15    531 TTTCAACAA..TAATGGAGAAATTTGGGGAATTATAATAAATTACAATAA  578
               ||||| |   |||      ||||| | ||||| ||||||  |   | |
SYBL-1   1214 CTTCAAAATAATAAATAGAGAATTTTGAGAATTGTAATAATTGTTAGTGC  1263
```

B

```
DIF-15 ORF    GG CAC GAG GTC AAG CTC ACT GCC ATC ATC GTC GTT GTA TCG
Translation      H   E   V   K   L   T   A   I   I   V   V   V   S

DIF-15 ORF    ATT GTG TTC ATC TAC ATC ATC GTG TCT CCA CTG TGC GGT GGC
Translation    I   V   F   I   Y   I   I   V   S   P   L   C   G   G

DIF-15 ORF    TTC ACG TGG CCA AGC TGT GTG AAG AAA TAA
Translation    F   T   W   P   S   C   V   K   K   *
```

3:2.4-4 DIF-19b

DIF-19b is the shortest of the clones described here, and according to the database searches it appears to share no significant homology with any mRNA sequences currently in the databases.

Table 3.13: Best homology scores for DIF-19b.

Homology Score	Gene	Accession Number	Reference
58.4% over 245bp	Mouse ACL gene (genomic sequence)	Z24722	van Eck *et al* 1994
58.1% over 236bp	Mouse osteopontin gene (genomic sequence)	X51834	Miyazaki *et al* 1990
60.7% over 191bp	Mouse ribosomal protein processed pseudogene containing B2 repeat sequences (genomic sequence)	K02929	Wiedemann and Perry 1984

3:3 Further Characterisation of DIF-14b

DIF-14b was the only clone isolated in this study which had a putative partial open reading frame. The predicted protein sequence is given in figure 3.19a. Figure 3.19b shows the hydrophobicity, surface probability and structural plots of this sequence (performed by Severn Biotech using DNA Strider).

3:3.1 Northern blotting

A northern blot of end bulb total RNA revealed that DIF-14b was present as a single transcript of around 4-5kb, using the ribosomal RNA bands as size markers. This is shown in figure 3.20a.

Total RNA was extracted from whole 10dpc, 13dpc, 14dpc and 15dpc rat embryos, and skin from 14dpc and 20dpc embryos. A northern blot of 3μg this RNA was probed with a ^{32}P labelled DIF-14b ORF probe. The ribosomal RNA bands were used as size markers. This revealed a single transcript of about 4-5kb in all lanes (figure 3.20b). A GAPDH probe was used as the loading control for this blot, revealing a single transcript of around 1.7kb in all lanes. A comparison of the relative intensity of the signals obtained for GAPDH and DIF-14b in each of the lanes suggests that DIF-14b may be most highly expressed at around 14dpc, although this is inconclusive.

Figure 3.19a: Translation of DIF-14b putative open reading frame. The amino acid sequence is given below the cDNA sequence in single letter code.

DIF-14b ORF CTTTCTTGTCTTTTTTCCCCCGTGGCGTTAGTTTTTGTGTGTCCTGGAACCTTG
translation L C S L F S P V A L V F V C P G T L

DIF-14b ORF AAAGCAATTGTGGACTCTCCAAGTATCTATGAAGCTGAGCAAAAGGCAGGTGCT
translation K A I V D S P S I Y E A E Q K A G A

DIF 14b ORF TGGTGCAAGGACCCCCTTCAGGCTGCAGATAAAATTTATTTTATGCCCTGGACT
translation W C K D P L Q A A D K I Y F M P W T

DIF-14b ORF CCCTACCGCACCGATACCTTAATAGAATATGCTTCTTTAGAAGATTTTCAAAAC
translation P Y R T D T L I E Y A S L E D F Q N

DIF-14b ORF AGCCGCCAGACAACAACATACAAACTTCCAAACCGAGTGGACGGTACTGGATTT
translation S R Q T T T Y K L P N R V D G T G F

DIF-14b ORF GTGGTGTATGACGGGGCAGTCTTCTTCAACAAAGAAAGAACGAGAAACATTGTT
translation V V Y D G A V F F N K E R T R N I V

DIF-14b ORF AAATTTGACTTGAGGACTACAATCAAGAGTGGGGAGGCCATAATCAACTACGCC
translation K F D L R T T I K S G E A I I N Y A

DIF-14b ORF AACTACCATGACACTTCACCCTACAGATGGGGGGGGAAGACTGACATTGACCTG
translation N Y H D T S P Y R W G G K T D I D L

DIF-14b ORF GCAGTGGACGAAAATGGCCTGTGGGTCATTTACGCCACCGAGCAGAACAACGGA
translation A V D E N G L W V I Y A T E Q N N G

DIF-14b ORF ATGATCGTGATTAGCCAGCTCAATCCGTACATTTTCCGATTGGAAGCAACCTGG
translation M I V I S Q L N P Y I F R L E A T W

DIF-14b ORF GAGACGACGTATGACAAGCGTGCGGCGTCCAATGCTTTCATGATATGCGGGGTC
translation E T T Y D K R A A S N A F M I C G V

DIF-14b ORF CTATACGTGGTCAGGTCAGTGTACCAAGACAATGAAAGCGAAGCTGGCAAGAAC
translation L Y V V R S V Y Q D N E S E A G K N

DIF-14b ORF GTCATCGACTACATTTACAACACAAGGTTGAGCCGGGGAGAGCACGTGGACGTT
translation V I D Y I Y N T R L S R G E H V D V

DIF-14b ORF CCCTTCCCCAACCAGTACCAGTACATCGCTGCAGTGGATTACAACCCAAGAGAC
translation P F P N Q Y Q Y I A A V D Y N P R D

DIF-14b ORF AACCAACTCTACGTATGGAACAATAACTTTATCTTACGGTATTCTCTGGAGTTT
translation N Q L Y V W N N N F I L R Y S L E F

DIF-14b ORF GGTCCACCCGACCCTGCCCAAGGTAAGAGTGTTCGCTGTTAGTTCTCCATGACT
translation G P P D P A Q G K S V R C *

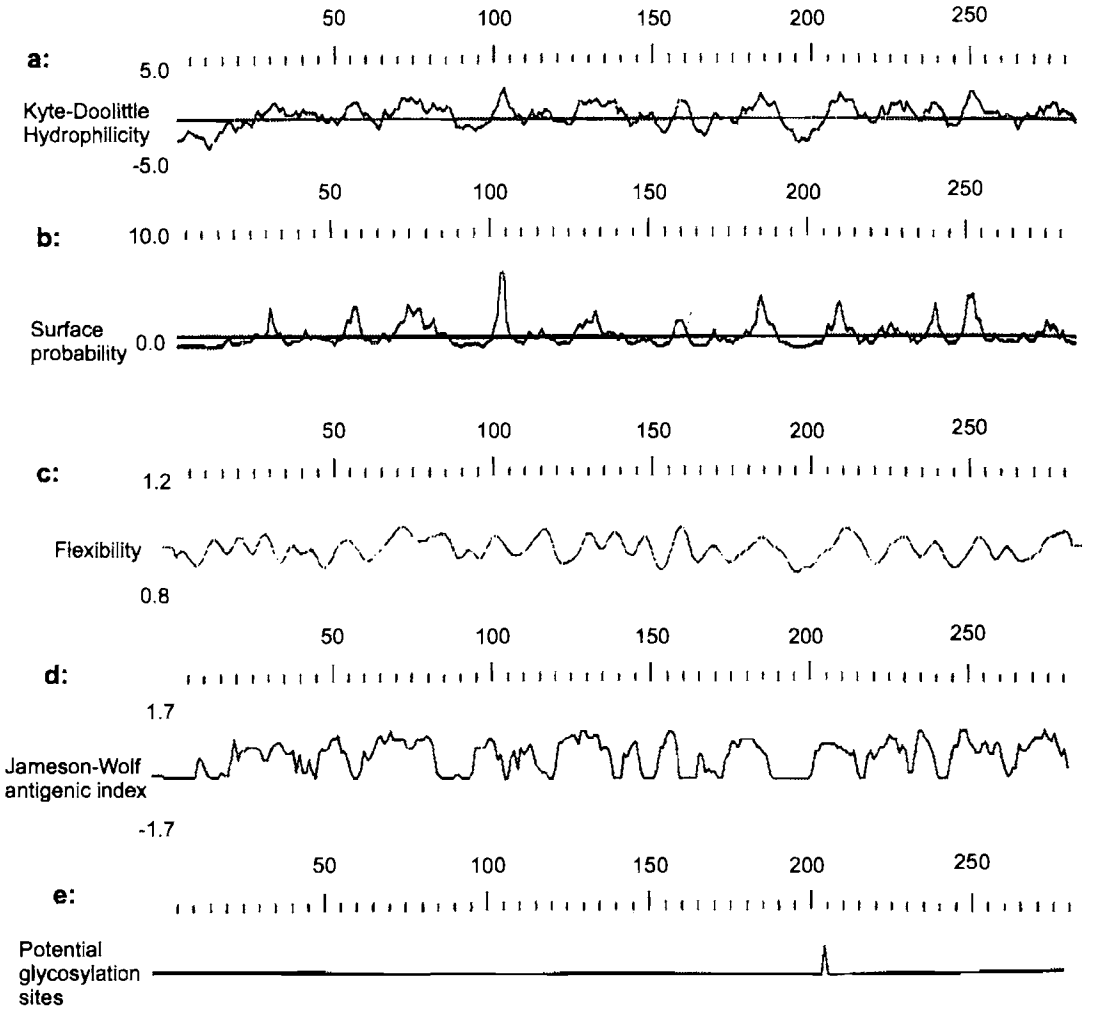


Figure 3.19b: Structural plots of the DIF-14b predicted peptide sequence. The sequence was analysed according to the method of Kyte and Doolittle (1982) to produce the hydropathy plot shown in (a). This shows that the predicted peptide has hydrophilic regions at amino acids 65-85, 100-105, 125-140, 175-190 and 205-215, with strongly hydrophobic regions between amino acids 0-20 and 190-205. This closely corresponds with the surface probability (b), flexibility (c) and antigenicity (d) plots. Regions that are particularly antigenic using the Jameson-Wolf antigenic index algorithm are present at amino acids 60-80, 120-140 and around 250. There is a potential glycosylation site around amino acids 205-210 (e), suggesting that the peptide is likely to be post-translationally modified in this region.

3:3.2 Genomic Zooblot

A Southern blot of 10μg *Hin*dIII digested rat, mouse, chicken, *Xenopus borealis*, *Carassius auratus* and human genomic DNA probed with a 600bp DIF-14b probe corresponding to the DIF-14b ORF revealed a single band in rat (1.3kb), mouse (8kb) and human (8kb) genomic DNA, indicating that DIF-14b is a single gene in these organisms. The probe hybridised to three major bands in chicken (4.2kb major, 6kb and 12kb minor) and *Xenopus borealis* (2.4kb, 3kb and 6kb) DNA, and four in *Carassius auratus* (0.6kb, 0.9kb, 2kb and 10kb). This is shown in figure 3.21.

3:3.3 5' RACE-PCR and LAPCR

Four different DIF-14b antisense primers were used for 5' RACE-PCR. These are shown in figure 3.22. RNA prepared from whole follicles was reverse transcribed using each of these primers and either *Tth* DNA polymerase at 70°C in the presence of Mn^{2+}, or M-MuLV reverse transcriptase at 42°C. However, in all cases, no further sequence was generated. The consistent isolation of good quality RNA from hair follicles was inefficient, generally resulting in isolation of sufficient template for only a few reactions. RNA was therefore extracted from adult rat liver, since this tissue expresses DIF-14b according to the multiple tissue northern blot, is easier to handle, and generates larger quantities of RNA per extraction. Liver RNA was used as the template in a set of RACE-PCR reactions using all four antisense DIF-14b primers and both reverse transcriptases. However, once again, no cDNA extending past the 5' end of the parental clone was produced. This may be due to strong secondary structure in the DIF-14b mRNA immediately upstream of the 5' end of the parental clone. It is also possible that both reverse transcriptases used were unable to process the long tract of A's in the template RNA. A second strategy was then used in an attempt to gain further DIF-14b sequence data from rat genomic DNA. This involved digestion of rat genomic DNA with *Hin*dIII, ligation of the mixed fragments to a *Hin*dIII digested pBluescript anchor, followed by PCR using one of the DIF-14b RACE primers and a pBluescript anchor primer. This resulted in the production of a PCR product containing 175bp of DIF-14b sequence, of which the 5'

140bp were not present in the parental clone. The DIF-14b restriction map shows that there is a *Hin*dIII site 1230bp from the 5' end of the clone, and the Southern blot indicates that the DIF-14b ORF probe hybridises to a band of around 1.3kb. The results of the LAPCR therefore suggest that the *Hin*dIII restriction fragment of the DIF-14b gene that is recognised by the ORF probe used in this study is 1370bp long. However, the sequence of the LAPCR clone generated from *Hin*dIII digested DNA indicates that there is a stop codon (TAA) just 4 amino acids upstream of the start of the partial ORF of the parental clone. This may indicate that there is an intron present immediately upstream of the start of the DIF-14b cDNA clone (although there does not appear to be a 3' splice acceptor site present anywhere in the LAPCR sequence), or it may be due to a point mutation (nonsense/frameshift) introduced during the PCR. It is also possible that the LAPCR is picking up a gene related to DIF-14b, since the region of homology between the LAPCR and DIF-14b parental sequence is only 35bp, of which ~21 are PCR primer. The results of the LAPCR are shown in figure 3.22.

3:3.4 Analysis of DIF-14b Predicted Protein

A striking feature of the DIF-14b protein sequence is the olfactomedin homology domain. This motif is common to several proteins. Sequence alignment is given in figure 3.23.

The only sequences in the database which show homology to DIF-14b outside the olfactomedin domain are the rat latrotoxin family members. The C-terminal end of DIF-14b is over 70% identical at the amino acid level to a region of the alpha-latrotoxin receptor close to its N-terminal end. This suggests that DIF-14b is a closely related member of this gene family. The alignment of the DIF-14b predicted protein with the alpha-latrotoxin receptor is given in figure 3.24.

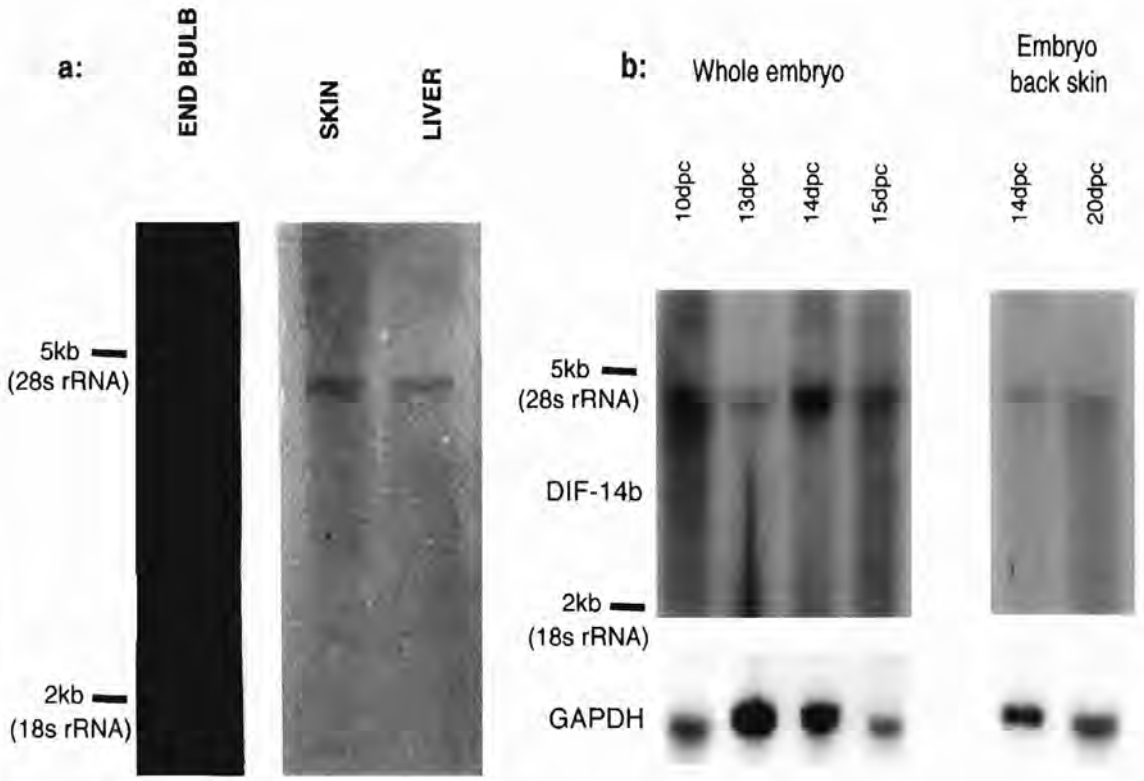


Figure 3.20: Northern blots of adult (a) and embryonic(b) total RNA hybridised with a DIF-14b probe.

a: Northern blots of 5ug end bulb mRNA and 10ug skin and liver total RNA probed with a DIF-14b probe. This hybridises to a single transcript of around 4.5kb, using the ribosomal RNA bands as size markers.

b: A northern blot of 3.5ug total RNA from whole embryos (10dpc, 13dpc, 14dpc and 15dpc), and embryonic skin (14dpc and 20dpc) probed with a DIF-14b probe. Again, this hybridises to a single transcript of around 4-5kb. A comparison with the hybridisation signal produced with a GAPDH probe suggests that DIF-14b is most highly expressed in whole embryos at around 14dpc.

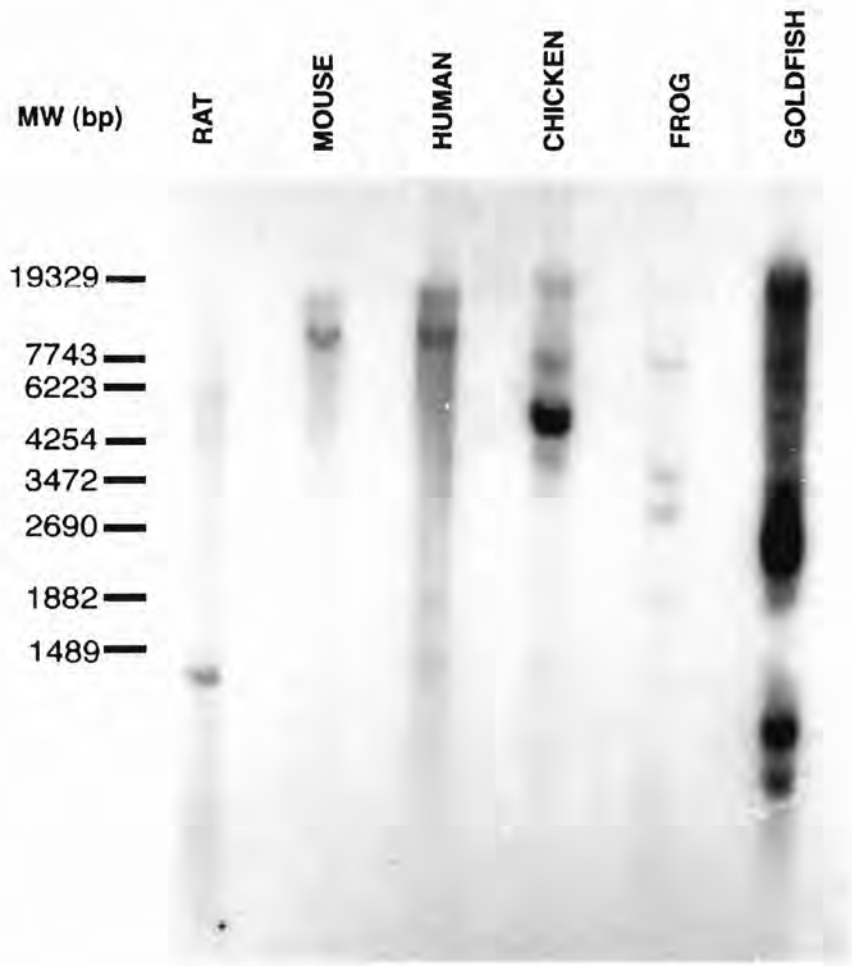
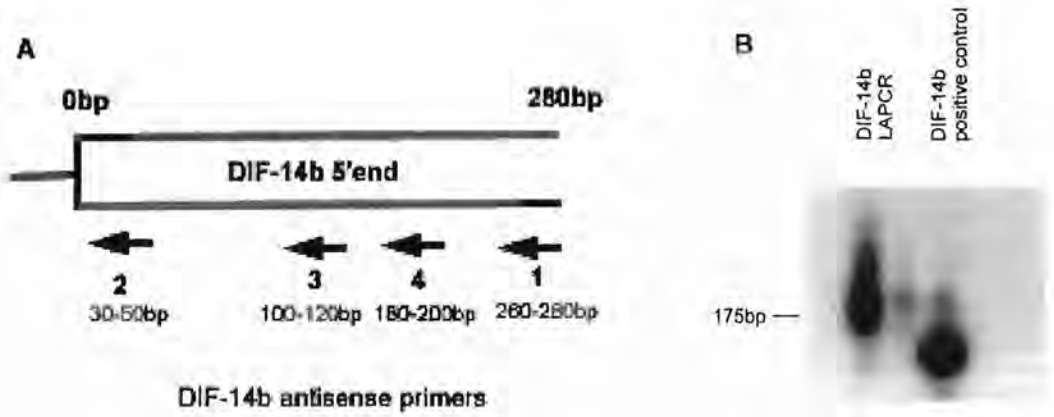


Figure 3.21: A genomic zooblot (*Hin d* III digested rat, mouse, human, chicken, frog and goldfish DNA) probed with a 600bp DIF-14b ORF probe. The probe hybridises to a single major band in rat, mouse and human genomic DNA, indicating that DIF-14b is a conserved single gene in these organisms. The presence of multiple hybridising species in chicken, frog and goldfish DNA may indicate that a gene related to DIF-14b is present with one or more *Hin d* III sites present in the region homologous to the rat DIF-14b ORF probe. Alternatively, several genes or pseudogenes may be present which are related to DIF-14b. Since the DIF-14b ORF probe contains the olfactomedin homology domain which is highly conserved between species, it is likely that this probe is detecting other olfactomedin related sequences, in addition to sequences related to DIF-14b itself. This is supported by the presence of weak bands in the rat, mouse and human samples, since DIF-14b is known to share 60-70% homology with olfactomedin containing genes in these species.



C

*Hin*dIII

LAPCR **AAGCTT**TGGAAAACAAGGGTAGTACGTACAAAGTATACATTTTTAAAAGA

LAPCR TGGAATGCTGAGTAAGATAGCTTTTCCTCCTTTGACTTGTGATCAATTAA

DIF-14b CTTTCTTGTC

LAPCR ATGCGTGATTACCGGTGTCATTTTCTTTTAACTTTATTCCCTTTCTTGTC

DIF-14b TTTTTTCCCCCGTGGCGTTAGTTTTTGTGTGTCCTGGAACCTTG

LAPCR TTTTTTCCCCCGTGGCGTTAGTTTT

Figure 3.22: Results of the LAPCR strategy to obtain 5' sequence for DIF-14b.

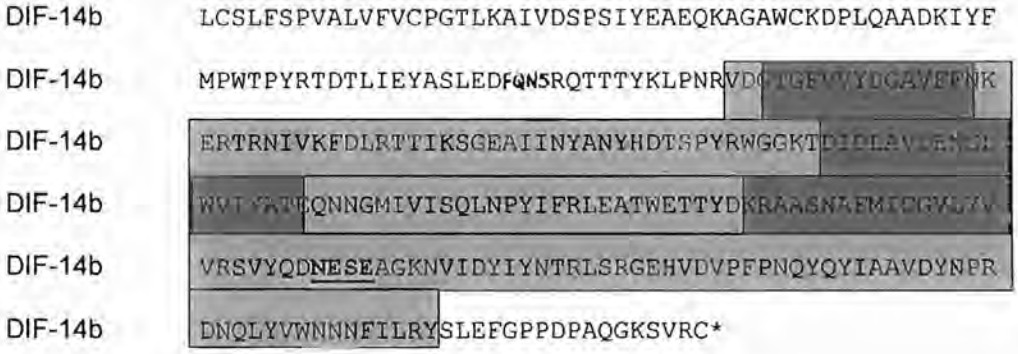
A: Schematic diagram of the original DIF-14b clone showing the position of the four antisense primers used in this study. The primer sequences are given in appendix B.

B: Southern blot of DIF-14b PCR products obtained by LAPCR from genomic DNA and 5'RACE from the original clone (control) using DIF-14b primer 2 and a vector specific forward primer. The blot was probed with a ^{32}P-labelled DIF-14b probe, showing that the LAPCR product is around 175bp.

C: Sequence of the LAPCR clone obtained by this strategy (LAPCR) compared to the original clone sequence (DIF-14b).

Figure 3.23: A - Sequence of DIF-14b predicted protein showing the olfactomedin homology domain (shaded box) The regions of greatest homology are indicated with darker shading, and the single potential N-linked glycosylation site is underlined in bold type.
B - Homology of 14b in the olfactomedin domain (adapted from Nguyen *et al* 1998 and Adam *et al* 1997). The conserved cysteine is shown in bold type, and the three regions where the similarity between all the related proteins is greatest are boxed, with the similarity and identity scores given above the alignment. DIF-14b; A clone isolated from the hair follicle end bulb library as a result of this study, Latro; Rat calcium independent alpha-latrotoxin receptor, TIGR; Trabecular meshwork inducible glucocorticoid response, NOEL-AMZ; rat neuronal olfactomedin related glycoprotein, Z region, OLFACTO(f); bullfrog olfactory olfactomedin, OLFACTO(m); mouse olfactomedin, PANC3; mouse pancortin 3, C.elegans; an olfactomedin related gene from *C. elegans*, Human EST; an EST sequence from human brain.

A



B

64.3% sim, 50% ident

DIF-14b	GTGFVVYDGA	VFFNKERTRN	IVKFDLRTTI	KSGEA-----	------IINY
Latro	GTGFVVYDGA	VFYNKERTRN	IVKYDLRTRI	KSGET-----	------VINT
TIGR	STGAVVYSGS	LYFQGAESRT	VIRYELNTET	VKAEK-----	------EIPG
NOEL-AMZ	GTGQVVYNGS	IYFNKFQSHI	IIRFDLKTET	ILKTR-----	------SLDY
OLFACTO(f)	GAGVVVHNNN	LYYNCFNSHD	MCRASL-TSG	VYQKK-----	------PLLN
OLFACTO(m)	GTGQVVYNGS	IYFNKFQSHI	IIRFDLKTEM	ILKTR-----	------SLDY
PANC3	GTGQVVYNGS	IYFNKFQSHI	IIRFDLKTEA	ILKTR-----	------SLDY
C.elegans	GTGNVVYNGS	YYYHKHGTTS	LVRYELETGV	QVEAELDPEM	SHIDCGRLPD
HUMAN EST	----------	----------	--RFDLKTET	ILKTR-----	------SLDY
Concensus	GTG.VVY...	~Y.~......	~~R..L.T..	....	.......~..

55.6% sim, 44.4% ident

```
DIF-14b      ANYHD----- --TSPYRWGG KTDIDLAVDE NGLWVIYATE QNNGMIVISQ
Latro        ANYHD----- --TSPYRWGG KTDIDLAVDE NGLWVIYATE GNNGRLVVSQ
TIGR         AGYHG----- --QFPYSWGG YTDIDLAVDE AGLWVIYSTD EAKGAIVLSK
NOEL-AMZ     AGYNN----- --MYHYAWGG HSDIDLMVDE NGLWAVYATN QNAGNIVISK
OLFACTO(f)   ALFNN----- --RFSYAGTM FQDMDFSSDE KGLWVIFTTE KSAGKIVVGK
OLFACTO(m)   AGYNN----- --MYHYAWGG HSDIDLMVDE NGLWAVYATN QNAGNIVISK
PANC3        AGYNN----- --MYHYAWGG HSDIDLMVDE NGLWAVYATN QNAGNIVISK
C.elegans    HTFEECNATD RHVWLYDRP- HNYVDFAVDE NGLWAIYAGA DSE-TMRMAK
HUMAN EST    AGYNN----- --MYHYAWGG HSDIDLMVDE SGLWAVYATN QNAGNIVVSR
Concensus    A......... ....Y...G ..D~D..VDE .GLW~IY.T. ...G.~V~.~
```

50% sim, 43.8% ident

```
DIF-14b      LNPYIFRLEA TWETTYDKRA ASNAFMICGV LYVVRSVYQD NESEAGKNVI
Latro        LNPYTLRFEG TWETGYDKRS ASNAFMVCGV LYVLRSVYVD DDSEAAGNRV
TIGR         LNPENLELEQ TWETNIRKQS VANAFIICGT LYTVSSYTSA DATVNFAYDT
NOEL-AMZ     LDPVSLQILQ TWNTSYPKRS AGEAFIICGT LYVTNGYSGG -TKVHYAYQT
OLFACTO(f)   VNVATFTVDN IWITTQNKSD ASNAFMICGV LYVTRSLGPK MEEVFYMFDT
OLFACTO(m)   LDPVSLQILQ TWNTSYPKRS AGEAFIICGT LYVTNGYSGG -TKVHYAYRT
PANC3        LDPVSLQILQ TWNTSYPKRS AGEAFIICGT LYVTNGYSGG -TKVHYAYQT
C.elegans    IEP-SLFVVN IWNVEVNTTE IADSFIMCGV WYGLKSANNL QTQITHAYDL
HUMAN EST    LDPVSLQTLQ TWNTSYPKRX PGXAFIICGT CYVTNGY-SG GTKVHYAYQT
Concensus    ~.P....~.. .W.T...K.. ~..AF.ICG. .Y...S.... ..........
```

```
DIF-14b      DYIYNTRLSR GEHVDVPFPN QYQYIAAVDY NPRDNQLYVW NNNFILRYSL
Latro        DYAFNTNANR EEPVSLAFPN PYQFVSSVDY NPRDNQLYVW NNYFVVRYSL
TIGR         GTGISKTLTI PFKNRYKYSS MIDYNPLEKK LFAWDNLNMV TYDIKLSKM
NOEL-AMZ     NASTYEYIDI PFQNKYSHIS MLDYNPKDRA LYAWNNGHQT LYNVTLFHVI
OLFACTO(f)   KTGKEGHLSI MMEKMAEKVH SLSYNSNDRK LYMFSEGYLL HYDIAL-KP
OLFACTO(m)   NASTYEYIDI PFQNKYSHIS MLDYNPKDRA LYAWNNGHQT LYNVTLFHVI
PANC3        NASTYEYIDI PFQNKYSHIS MLDYNPKDRA LYAWNNGHQT LYNVTLFHVI
C.elegans    FSPVFSFLSF V*NPYQGLT MLHYNPLDAR LYFFDNSSLL SVNVRI----
HUMAN EST    NAST------ -----YEY-- -IDI-PFQNK LXP------- HFPC------
Concensus    .......... .......... .......... L......... ...~.L....
```

Overall similarity in the olfactomedin domain = 28.5% over 200 amino acids, overall identity = 21.5% over 200 amino acids.

Figure 3.24: DIF-14b is over 70% identical to rat alpha-latrotoxin receptor (Latro) at the amino acid level. Identical residues are indicated in bold type (concensus), and conservative substitutions are indicated by ~.

```
DIF-14b   1  L C S L F S P V A L V F V C P G T L K A I V D  23
Latro   126  D C V P Y K V E Q K V F V C P G T L Q K V L E  149
Concensus    C     ~           V F V C P G T L     ~ ~

DIF-14b  24  S P S I Y E A E Q K A G A W C K D P L Q A A D  46
Latro   150  P T S T H E S E H Q S G A W C K D P L Q A G D  172
Concensus      S     E   E       G A W C K D P L Q A ~ D

DIF-14b  47  K I Y F M P W T P Y R T D T L I E Y A S L E D  69
Latro   173  R I Y V M P W I P Y R T D T L T E Y A S W E D  195
Concensus    ~ I Y   M P W   P Y R T D T L   E Y A S   E D

DIF-14b  70  F Q N S R Q T T T Y K L P N R V D G T G F V V  91
Latro   196  Y V A A R H T T T Y R L P N R V D G T G F V V  218
Concensus    ~       R   T T T Y ~ L P N R V D G T G F V V

DIF-14b  92  Y D G A V F F N K E R T R N I V K F D L R T T  114
Latro   219  Y D G A V F Y N K E R T R N I V K Y D L R T R  241
Concensus    Y D G A V F ~ N K E R T R N I V K ~ D L R T

DIF-14b 115  I K S G E A I I N Y A N Y H D T S P Y R W G G  137
Latro   242  I K S G E T V I N T A N Y H D T S P Y R W G G  264
Concensus    I K S G E   ~ I N   A N Y H D T S P Y R W G G

DIF-14b 138  K T D I D L A V D E N G L W V I Y A T E Q N N  160
Latro   265  K T D I D L A V D E N G L W V I Y A T E G N N  287
Concensus    K T D I D L A V D E N G L W V I Y A T E   N N

DIF-14b 161  G M I V I S Q L N P Y I F R L E A T W E T T Y  183
Latro   288  G R L V V S Q L N P Y T L R F E G T W E T G Y  310
Concensus    G   ~ V ~ S Q L N P Y   ~ R ~ E ~ T W E T   Y

DIF-14b 184  D K R A A S N A F M I C G V L Y V V R S V Y Q  206
Latro   311  D K R S A S N A F M V C G V L Y V L R S V Y V  333
Concensus    D K R   A S N A F M ~ C G V L Y V ~ R S V Y

DIF-14b 207  D N E S E A G K N V I D Y I Y N T R L S R G E  229
Latro   334  D D D S E A A G N R V D Y A F N T N A N R E E  356
Concensus    D     S E A ~   N   ~ D Y   ~ N T       R ~ E
```

```
DIF-14b   230 H V D V P F P N Q Y Q Y I A A V D Y N P R D N 252
Latro     357 P V S L A F P N P Y Q F V S S V D Y N P R D N 379
Concensus     V   ~   F P N   Y Q ~ ~     V D Y N P R D N

DIF-14b   253 Q L Y V W N N N F I L R Y S L E F G P P D P A 275
Latro     380 Q L Y V W N N Y F V V R Y S L E F G P P D P S 402
Concensus   Q L Y V W N N   F ~ ~ R Y S L E F G P P D P

DIF-14b   276 Q G K S V R C *
Latro     403 A G P A T S P
Concensus     G
```

70% id, 79.5% sim

4: DISCUSSION

Several cDNA clones, which appear to be preferentially expressed in the germinative epidermis, were isolated from a hair follicle end bulb cDNA library using a differential screening technique. Of nine putative differentially expressed clones identified, sequence data obtained for each of these clones reveals that four have no homology to previously identified genes. However, we do not have full-length cDNA sequences for any of these four clones, so that they may yet turn out to encode proteins with recognisable sequence motifs which will provide clues to their function. Of the remaining five clones, two correspond to known genes, and three are related to sequences in the GenBank database.

4:1 DIFFERENTIAL SCREENING OF A VIBRISSA FOLLICLE END BULB cDNA LIBRARY

A problem with any approach based on screening of cDNA populations is that information obtained in such studies does not usually permit functional characterisation of novel genes isolated. However, identification of genes which are differentially expressed under very specific conditions will provide clues to functional significance. Techniques based on PCR such as DDRT-PCR, are potentially the fastest methods for identification of differentially expressed genes. However, it has been shown that such techniques give a high level of false positives and artefacts, with variable reproducibility, and they share the limitations of differential screening approaches in that they are biased towards isolation of highly expressed clones (Sompayrac *et al* 1995, Bertioli *et al* 1995, Wan *et al* 1996). A major disadvantage of subtractive techniques is that a relatively large amount of mRNA is required for each subtraction, and multiple controls and subtraction steps reduce the level of reproducibility that can be obtained.

Since this study was initiated, many variations of subtractive and differential screening approaches have been reported. A method based on suppression subtractive hybridisation (SSH) to identify differentially expressed genes of low abundance, first reported by Diatchenko *et al* (1996) has been used by Kuang *et al* (1998) to isolate differentially expressed genes from an estrogen receptor positive breast carcinoma cell line. SSH combines subtractive hybridisation with PCR to generate a pool of PCR products enriched for differentially expressed sequences using mRNA from target and driver populations. The method involves a normalisation step which equalises wide differences in abundance of different mRNA species, thus allowing isolation of low abundance differentially expressed genes, as well as those which are more highly expressed, without generating a large number of false positives or having to screen huge numbers of clones. Ordinarily, differential screening approaches tend to result in isolation of highly expressed clones at the expense of those which are expressed at a low level, though the more clones that are screened, the less of a problem this becomes. Even so, we found that over half of the differential clones obtained from the end bulb differential screen were derived from the same gene.

A novel approach has been reported by Perret *et al* (1998) to analyse transcriptional variations in organised cDNA libraries. A rat brain cDNA library in DH5α was organised into 1536 well dishes using a FACS to deposit a single cell into each well. Filters were then prepared from duplicated plates, and probed with a series of complex radiolabelled probes. Computerised densitometric analysis was then performed to isolate differentially expressed clones, after normalisation of the amount of target DNA present in each spot. This automated and computerised approach was shown to be a rapid and reproducible method of differential screening of a cDNA library, limited only by the quality of the initial library.

The major advantage of any differential or subtractive approach in which the first step is the construction of a cDNA library is the ready availability of a stable, reproducible cDNA stock. However, the success of subsequent experiments is very dependent on the quality and quantity of cDNA clones present in the starting library. It is for this reason that we have used a library of known quality as the reference library for this study, rather than PCR-generated libraries where the quality is not as good.

A dual labelling differential screening method based on that described by Olszewski *et al* (1989) was used for this study. Although at that time, several similar methods had been described, this method was preferred for several reasons:-

- It was not possible to create a cDNA library from GE cells by conventional methods due to the very limited quantity of material available.

- Though it was possible to generate a library from this tissue by PCR, it was felt that this would result in disproportionate representation of short cDNA's of limited use for direct screening. Furthermore, such a library would contain a large number of "non-relevant" clones in addition to the potentially important GE specific clones.

- It was not possible to culture GE cells in order to amplify the available starting material. Furthermore, systems involving cultured cells were not considered due to the rapid changes in cell behaviour which can occur when the cell type under study is isolated and grown under artificial conditions. It was felt that information gained from systems using cultured cells was likely to be of limited use because the behaviour of the hair follicle components is likely to be dependent on their anatomical and functional integration *in vivo*.

- To eliminate or reduce the number of "non-relevant" clones isolated in the study, a differential or subtractive method was preferred, using cDNA from a closely related tissue to remove cDNA's which were common to both tissues, thus enriching for GE specific clones.

- This method was chosen in preference to DDRT-PCR due to the difficulty of obtaining quantities of RNA from the GE and non germinative tissues. The production of a PCR-generated library relied on a single set of PCR reactions from a single round of dissection and RNA isolation, whereas DDRT-PCR requires multiple PCR reactions and dissections. Also we did not want to rely on a PCR based method alone for the reasons outlined above.

- Since a good quality end bulb cDNA library was available (chapter 2), it was decided to use this as the basis of the differential screen to isolate longer clones of more reliable quality. This library has previously been successfully used as the basis of a differential screen using cultured dermal papilla cells at different stages of growth (Sleeman 1995).

- Single labelling differential screening was not used due to the difficulty of obtaining reliable results from sequential screening of the same filters. It was felt that stripping and

reprobing the same filters with positive and negative probes was likely to introduce artefacts, even if the conditions for each round of probing and autoradiography were exactly reproduced.

- The dual labelling method used in which the positive and negative probes were hybridised to the same filter simultaneously was chosen as the most reliable method given the starting materials and expertise available in the lab at the time of the study. It was felt that this method gave a better chance of picking up small differences in the level of gene expression in closely related tissues.

4:2 ANALYSIS OF THE CLONES ISOLATED BY DIFFERENTIAL SCREENING OF THE END BULB cDNA LIBRARY

Nine putative differentially expressed cDNA clones were isolated from the end bulb cDNA library by differential screening. The insert sizes of these clones ranged from 0.1 to 4.2kb. The smallest clone (DIF-17, 100bp) was not included in further studies. Much of the work described in this chapter was performed to confirm that the remaining eight clones did in fact represent mRNA's expressed in the germinative epidermis. Although the end bulb cDNA library was known to be of good quality (see chapter 2 and Sleeman 1995), it was likely that the methods used for generation of the + and - probes used in the differential screen could result in the isolation of "false positives". In fact, eleven of the 53 clones initially isolated were immediately discarded as further rounds of differential screening could not confirm that these eleven were differentially expressed. This may be due to differences in the efficiency of the original reverse transcription reactions and subsequent disproportionate amplification of particular cDNA's from each of the original populations. It was therefore prudent to demonstrate that each of the clones isolated by the differential screening protocol, firstly originated from mRNA present in the hair follicle, and secondly was preferentially expressed in the germinative epidermis. RNase protection assays were performed to address the first point, and non-radioactive *in situ* hybridisation addressed the second. Once the validity of the clones had been established they could be further characterised.

4:2.1 Repeat Elements

Seven of the eight differentially expressed clones described in this study contain a repeat element in their 3'UTR. Differential or subtractive hybridisation protocols generally result in the elimination of sequences containing repeat elements, unless the repeat elements are specific to mRNA's expressed in the + population. However, since only three of these clones are clearly preferentially expressed in the GE cells, the appearance of repeat elements in the clones isolated from the screen may indicate that the ratio of - to + probes used in the primary hybridisation was suboptimal.

4:2.1-1 BC1

Six of the putative differentially expressed cDNA clones isolated from the end bulb library have a concensus rat BC1 repeat sequence in their 3'UTR's. Originally, these were thought to be brain specific identifier sequences, but since then it has been postulated that these sequences are involved in growth and transformation dependent regulation and may be a marker of differentiation. It appears that such BC1 elements are necessary for the growth dependent expression of genes containing them, along with further upstream regulatory sequences which appear to confer tissue specific expression. Repeated sequences such as this have been reported to accumulate in a tissue specific manner, and to be expressed as a function of cell differentiation, embryonic development and cell proliferation and transformation (Glaichenhaus and Cuzin 1987, Vidal *et al* 1993). Many transcribed sequences contain a BC1 repeat in their 3'UTR - some examples are given in table 3.14.

Table 3.14 : Examples of some rat mRNA sequences in the GenBank and EMBL databases containing a BC1 repeat.

GENE	Position of BC1 repeat	Accession No.
Rat alternative brain Ca2+ ATPase mRNA	3' end of 3'UTR	J04024
Rat T-cell receptor zeta chain mRNA	70bp upstream of 3' end of 3'UTR	D13555
Rat growth and transformation dependent mRNA	3' end of 3'UTR	M17412
Rat sialoglycoprotein mRNA	3' end of 3'UTR	X14765
Rat lysosomal membrane glycoprotein mRNA	3' end of 3'UTR	M34959 J03672
Rat cysteine sulfinic acid decarboxylase mRNA	3' end of 3'UTR	M64755
Rat androgen regulated dorsal protein 1 mRNA	120-230bp downstream of 5' end of 3'UTR	M90310
Rat EIF-2a kinase mRNA	3' end of 3'UTR	L27707
Rat 5' nucleotidase mRNA	3' end of 3'UTR	J05214

BC1 is a very abundant ID-related transcript which is transcribed from a conserved single copy gene. ID elements are a major class of short interspersed repetitive DNA elements found in rodent genomes. They are generally 85-105bp long and have a core region of around 75 bases with a 10-40 base oligo(dA) tail. The core region shares sequence homology with alanine tRNA, suggesting that ID elements were ancestrally derived from tRNA's (Daniels and Deininger 1985). ID elements are transcribed by RNA polymerase III, and their amplification occurs by retroposition of these RNA polymerase III-derived transcripts (Weiner *et al* 1986). Various rodent genomes contain different numbers of ID elements, with the rat genome having the largest number, around 130,000 copies- suggesting that ID elements are amplified at different rates in different rodent species, possibly due to the different levels of germ-line BC1 expression in different rodent species (Kim *et al* 1994).

An interesting feature of the BC1 repeat sequence with reference to this study is the presence of the oligo(dA) rich region at the 3' end of the repeat element. In some of the BC1 containing clones isolated in this study (DIF-1, DIF-14b, DIF-16), polyadenylation signals were not present at the 3' end of the cDNA sequences. This indicates that the oligo(dA) present at the 3' end of these clones is the result of priming at a BC1 repeat upstream of the 3' end of the mRNA, so that these three clones may be truncated at the 3' end. It is likely that this will be the case in any method of cDNA production involving priming with oligo(dT), and that such cDNA populations will also contain clones resulting from oligo(dT) priming of adenine rich regions in ribosomal and transfer RNA's.

A BC1 master gene is highly expressed in neural tissue, where the 160 base BC1 transcript complexes with proteins to form a ribonucleoprotein particle (RNP). However, a study by Sutcliffe *et al* (1984) showed that 62% of gene transcripts synthesised *in vitro* from brain chromatin contained 80-90bp BC1 elements. It was suggested that many of these were present in regions of the transcripts that were spliced out, and brain specific cDNA clones reported in the literature which did not contain a BC1 repeat in the mRNA sequence had BC1 elements present in one or more introns of the corresponding genomic clone (Milner *et al* 1984). This work led to the suggestion that these repeat elements may control tissue specific gene expression (Sutcliffe *et al* 1982, 1984, Milner *et al* 1984), either at the level of transcription or at the level of mRNA maturation. It was subsequently shown that BC1 repeat elements were not restricted specifically to the brain, also being present in liver, kidney and muscle mRNA populations (Owens *et al* 1985, Lone *et al* 1986, Sapienza and St-Jaques 1986).

A study by Glaichenhaus and Cuzin (1987) involving genes shown to be highly expressed in rat FR3T3 cells, either following transformation by polyoma virus, or following serum stimulation of cells arrested in G0 by serum starvation (Glaichenhaus *et al* 1986) showed that these growth dependent mRNA's contained a BC1 repeat element. It was suggested that the bulk of rat fibroblast mRNA's containing BC1 repeat sequences may be expressed in a similar growth dependent manner, as clones isolated from a polyoma transformed FR3T3 library using a BC1 probe were expressed with the same kinetics as those isolated from the original screen (Glaichenhaus and Cuzin 1987). In order to test this theory, a chimeric rabbit β-globin gene containing a rat BC1 repeat in the 3' non coding region was

transfected into FR3T3 cells. It was shown that transcripts of this gene also accumulated in a growth dependent manner following stimulation of arrested cells with serum, while control constructs containing the repeat sequence in reverse orientation, or not containing the repeat sequence, showed no accumulation following serum stimulation. Further studies suggested that this increase was likely to be due to changes in the rate of processing or degradation of the mRNA, rather than an upregulation of mRNA synthesis. Sequences at the 3' end of a variety of mRNA's have been shown to be important for mRNA stability (Clemens 1987), although BC1 repeats are also commonly found in introns. This suggested that regulation might occur at the level of RNA splicing. An interesting point noted by several groups studying expression of genes containing BC1 repeats is that such genes tend to encode proteins expressed during terminal differentiation (Sutcliffe *et al* 1984, Herget *et al* 1986, Glaichenhaus and Cuzin 1987).

A study by Vidal *et al* (1993) suggested that transcribed regions including a repetitive sequence such as BC1 in rat and B1 in mouse were important in post-transcriptional regulation of gene expression. It was suggested that the regulatory element included both the core repeat sequence and the 5' flanking region, which may contain short repeats specific for mRNA species under the same regulation. A study of the 70bp upstream of the start of the BC1 repeat in two of the differentially expressed clones reported in this study reveals that five common 4 - 7bp sequences are present in similar positions relative to the BC1 repeat in both of these clones (figure 3.25). This appears similar to the putative regulatory elements reported by Vidal *et al* (1993), in which there are three to five common 5- or 6-bp nucleotide blocks present within 100bp of sequence at the 5' end of the repeat element in five rat and mouse mRNA's which are differentially expressed in arrested and growing cells. It may therefore be possible that DIF-14b and DIF-15 may be subject to some common regulation at the post-translational level in rat follicles, although this remains to be demonstrated.

It has already been shown that the 160bp BC1 RNA transcribed by RNA polymerase III in neural tissue is able to bind to several ID sequence binding proteins to constitute a ribonucleoprotein particle, and that these proteins may be involved in transport of BC1 transcripts out of the nucleus, or in regulation of transcription of BC1 by RNA polymerase III (Kobayashi *et al* 1992, Kobayashi and Anzai 1997). It is therefore possible that BC1 elements

present in the untranslated regions of other mRNA's may also bind proteins which may be responsible for their transport to specific locations.

Figure 3.25: Nucleotide sequence flanking the BC1 repeat in two of the differentially expressed clones isolated from the vibrissa follicle end bulb library. Bold type indicates nucleotide blocks present in both sequences in similar positions relative to the BC1 repeat, which is indicated by the boxed region.

DIF-14b GCTA**ACTCTGC**TCACT**CACT**CCCGCTTGC**TCCATT**GGCGTCC

DIF-15 TC**ACTCTGC**ACATAGTTTAA**CACT**TTCTGTTTA**TCCATT**ACC

DIF-14b CCCACAGGTATAT**TTAAGAAGGA**TGGCA-**BC1 REPEAT**

DIF-15 AATTCGAACTGAGA**TTAAG**TA**AAGGA**GT-**BC1 REPEAT**

A study by Muslimov *et al* (1997) used a microinjection protocol in cultured neurons to investigate transport of neuronal BC1 RNA. They discovered that BC1 RNA was selectively transported to dendritic targets, and that chimeric non-neuronal RNA's containing an inserted BC1 sequence were also similarly transported. It has been shown that RNA localisation signals may be associated with 3'UTR's, particularly in *Drosophila* (St. Johnston 1995), although it is not clear whether the function of such a signal is dependent on its position within the mRNA. Although most reported studies concentrate on neuronal cells or embryonic systems, RNA targeting may play an important role in the management of microgeometry in various eukaryotic cell systems.

4:2.1-2 Other Repeat Elements

Two of the differential clones isolated in this study showed no homology to known repeated elements in the GenBank and EMBL databases. However, Southern and northern blots hybridised to a DIF-R6b probe show that this clone shares homology with a wide range

of sequences, apparently through a 250bp repeat element at the 3'UTR. This sequence is present in several cDNA clones isolated from different rodent species (see table 3.10), and also in many rodent genomic sequences (sections 3:2.4-1, 4:4.1-1). When DIF-R6b was used for *in situ* hybridisation, it was apparently not highly expressed in the GE cells of anagen vibrissa follicles, instead appearing preferentially expressed around the bulge region. It may therefore be possible that this repeat element is involved in regulation of mRNA expression or its localisation to specific sites, in a similar manner to that proposed for the BC1 repeat.

4.3 ISOLATION OF KNOWN GENES

Two of the clones described in this study, DIF-16 and DIF-20, correspond to sequences previously reported in the databases. It is not surprising that DIF-20, a mitochondrial rRNA transcript, should be preferentially expressed in the germinative epidermis, as the GE cells are very rich in mitochondria and free ribosomes (Reynolds and Jahoda 1991b). *In situ* hybridisation data suggests that there is in fact not a great deal of difference in the levels of expression of DIF-20 in the GE and matrix cells, although this may be an artefact due to the long development times used in the study, which would result in "overexposure" of highly expressed genes. It is possible that a shorter development time would reveal a much clearer difference in expression levels between the GE cells and other parts of the follicle.

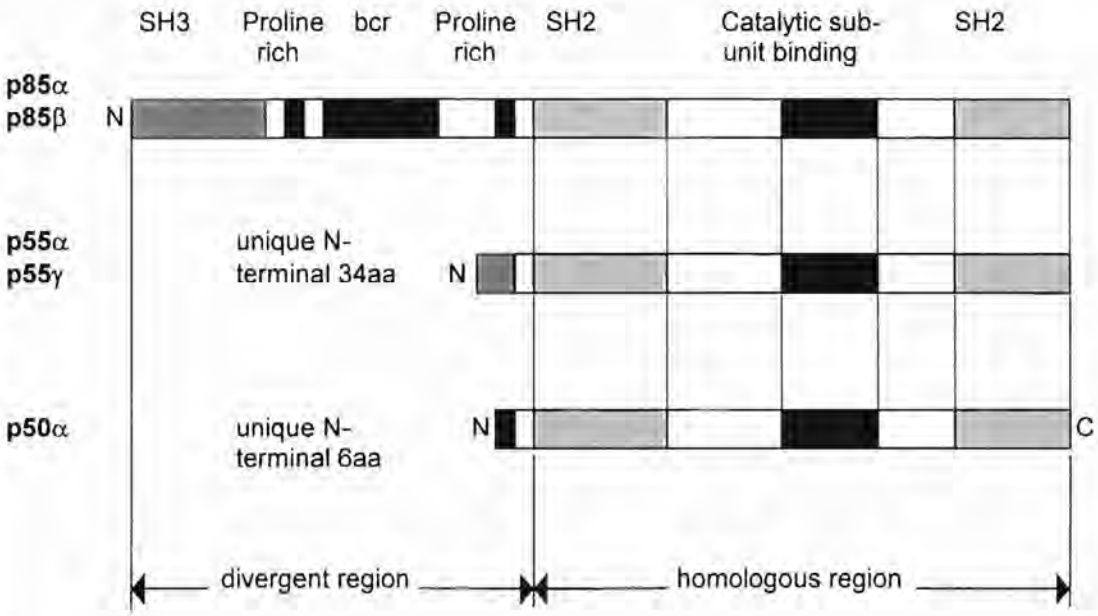
The function of the other previously identified gene, DIF-16, a phosphatidylinositol-3-kinase regulatory subunit, in the germinative epidermis, is not clear. Although phosphatidylinositol-3-kinase (PI 3-kinase) itself has been implicated in proliferation (Cheatham *et al* 1994, Gold *et al* 1994), differentiation (Kimura *et al* 1994), and prevention of apoptosis (Yao and Cooper 1995), the role of its various regulatory subunits is not well characterised. However, the role of the various regulatory subunits of PI 3-kinase in other tissues is discussed in section 4:3.1.

4:3.1 DIF-16: A Phosphatidylinositol-3-kinase Regulatory Subunit

The differential screen of the end bulb cDNA library resulted in the isolation of two independent cDNA clones homologous to the rat phosphatidylinositol-3-kinase regulatory subunit p55γ (previously known as p55PIK). *In situ* hybridisation analysis suggested that p55γ was more highly expressed in the germinative epidermis than in other tissues of the hair follicle.

Phosphatidylinositol-3-kinase (PI 3-kinase) is a lipid kinase consisting of a catalytic subunit of 110kDa (Shibasaki *et al* 1991, Hu *et al* 1993), and a regulatory subunit, of which five forms have currently been identified (Inukai *et al* 1996, Inukai *et al* 1997, Shin *et al* 1998). Although the exact function of PI 3-kinase remains to be determined, it has been implicated in the regulation of a variety of cellular activities, including proliferation (Cheatham *et al* 1994, Gold *et al* 1994) and differentiation (Kimura *et al* 1994, Ptasznik *et al* 1997). PI 3-kinase catalyses the formation of PI 3 phosphates by phosphorylation of the inositol ring in response to stimulation with a variety of growth factors and hormones (Cantley *et al* 1991), and it has been suggested that activation of PI 3-kinase leads to the activation of a variety of cellular protein kinases (Ettinger *et al* 1996, Dahl *et al* 1996). PI 3-kinase itself is activated by interaction between src homology-2 (SH2) domains in the regulatory subunit and phosphorylated YxxM motifs present in activated cell surface receptors possessing tyrosine kinase activity (Otsu *et al* 1991, Skolnik *et al* 1991, Songyang *et al* 1993, White and Kahn 1994). The five regulatory isoforms so far identified for PI 3-kinase are the 85kDa p85α and p85β, the 55 kDa p55α and p55γ, and the 50kDa p50α. The 85kDa forms have two proline rich motifs, two SH2 domains, a breakpoint cluster region (bcr) homology domain, an SH3 domain and a region between the SH2 domains responsible for binding to the catalytic subunit. The 55 kDa forms lack the bcr and SH3 domains, and have unique 34 amino acid N-terminal sequence, and the 50kDa form appears to be a truncated splice variant of the p85α gene (Inukai *et al* 1997). A schematic comparison of the regulatory subunit isoforms is shown in figure 3.26.

Figure 3.26: A schematic comparison of the five PI 3-kinase regulatory subunit isoforms. (Adapted from Inukai *et al* 1997).



A study by Inukai *et al* (1996) isolated four cDNA clones corresponding to PI 3-kinase regulatory subunits from a rat brain cDNA library. Three of these clones were the rat homologues of the previously identified p85α, p85β and p55γ. The fourth clone had the N-terminal SH2 domain of p85α, with a previously undocumented 166bp sequence 5' to this, and a putative open reading frame of 1362bp. The putative protein was designated p55α on the basis of its predicted molecular weight and its similarity to p55γ. The p55α subunit contains two SH2 domains and a catalytic subunit binding domain which are identical to those of p85α, but it lacks the bcr and SH3 domains of p85α, instead having a unique 34 amino acid N-terminal region similar to that of p55γ (Pons *et al* 1995). It therefore appears that p55α is transcribed by alternative splicing from the p85α gene, and that the unique N-terminal region has a specific functional role shared with p55γ. Northern blotting using a p55α specific cDNA probe revealed a major 4.2kb transcript in all tissues examined, with additional transcripts of 2.8 and 6.0kb present in the brain. Analysis of northern blots using a p85α specific probe, a p55α specific probe and a probe common to both p55α and p85α suggested that p85α is

expressed more abundantly than p55α in all tissues examined, with the exception of brain and skeletal muscle. This was confirmed by western blotting using specific antibodies. Northern blotting using a p55γ specific probe identified a single transcript of 5.8kb, highly expressed in rat brain, and present at lower levels in most rat tissues with the exception of liver and skeletal muscle (Inukai *et al* 1996). It was suggested that the p55 subunits have a different role in the brain to the p85 subunits as a function of their divergent N-terminal domains (Inukai *et al* 1996). The functional role of the SH3 domain of the p85 subunits is not clear, but SH3 has been shown to interact with proline rich regions in various signalling proteins (Gout *et al* 1993, Weng *et al* 1993, Kapeller *et al* 1995). Therefore the differences in the N-terminal regions of the PI 3-kinase regulatory subunits may contribute to differences in subcellular distribution of PI 3-kinase, or to varying degrees of activation in response to different signals. The localisation of all five regulatory isoforms of PI 3-kinase in the rat brain was investigated further by Inukai *et al* (1997) and Shin *et al* (1998), using RNase protection assays and *in situ* hybridisation. It was shown that p50α, p55α and p85β mRNA was abundant in the cerebral cortex, p85α was detected in all parts of the brain, and p55γ was particularly highly expressed in the cerebellum. Western blotting of various rat tissues to localise p85α and its two splice variants at the protein level showed that p85α is abundantly expressed in many rat tissues, whereas p55α is only detected in brain and muscle and p50α is present in liver, brain and kidney.

PI 3-kinase has been proposed as an important signal transduction enzyme in processes of induction between cell types and vesicle trafficking within cells. Induction is the process by which one group of cells signals to a second group in such a way as to determine the developmental fate of the second, and it requires a signalling ability in the first set of cells and a responsive ability in the second. Any system involving induction between tissue layers, be it in the hair follicle or in the earliest stages of mesoderm induction in the embryo, is controlled by growth factor mediated cell-cell communication. As early as 1992, the phosphoinositide signal transduction pathway was implicated in this process, since inhibitors of this signal transduction pathway disrupted axis formation and mesoderm induction in early *Xenopus* embryos (Maslanski *et al* 1992). Activation of the phosphoinositide pathway by

growth factors leads to hydrolysis of plasma membrane phospholipids, activation of PI 3-kinase and synthesis of inositol triphosphates, triggering release of Ca^{2+} from the endoplasmic reticulum (ER). An increase in the level of inositol triphosphate in *Xenopus* embryos is detectable coincident with the onset of mesoderm induction, supporting the argument that the PI cycle is part of a complex suite of signal transducers involved in induction (Maslanski *et al* 1992).

PI 3-kinases and their lipid products have also been implicated in several aspects of intracellular membrane trafficking over the past few years. Less than 0.25% of the total of inositol containing lipids in a cell are phosphorylated at the 3-position, suggesting that such lipids exert a very specific function within the cell, and are not merely structural (reviewed by Rameh and Cantley 1999). The process of eukaryotic membrane homeostasis depends on an interplay of membrane budding and fusion with vesicle transport events which must be collectively and individually regulated to maintain cell integrity and function. Endocytosis occurs in most eukaryotic cells, and is an important process in the uptake of nutrients and control of the level of cell surface receptors by internalisation of endosomal vesicles. These vesicles are then fused into a population of early endosomes, from which the contents are sorted to a variety of cellular locations. The endosomal compartment plays a central role in the intracellular transport and sorting of endocytosed ligands, membrane proteins and lysosomal enzymes, both from the cell surface and the *trans*-Golgi network (TGN). PI 3-kinase has been shown to be involved in IgE mediated histamine secretion (Yano *et al* 1993), insulin regulated glucose transport (Gould *et al* 1994, Okada *et al* 1994), and downregulation of activated PDGF receptor (Joly *et al* 1994). Many studies have shown that PI 3-kinase activity is required prior to activation of GTPases during signal transduction through the G protein-coupled receptor signalling pathway (for example, Li *et al* 1995, Akasaki *et al* 1999). The study by Li *et al* (1995) demonstrated that PI 3-kinase plays an important role in early endosome fusion, probably via activation of the small GTPase Rab5. Treatment of cells with the specific PI 3-kinase inhibitor, wortmannin, resulted in inhibition of constitutive endocytosis. A similar study by Hansen *et al* (1995) demonstrated that wortmannin inhibited apical to basolateral and basolateral to apical transcytosis in polarised epithelial cells, suggesting that PI 3-kinase is important in specific postendocytic vesicular trafficking pathways in epidermal cells.

How PI 3-kinase products function in membrane trafficking is not clearly understood, but it has been suggested that the negative charge of the lipid products of this kinase may be important in membrane invagination or recruitment of adaptor proteins (Panayotou and Waterfield 1992). PI 3-kinase activity has also been shown to mediate transfer of lysosomal membrane glycoproteins from late endosomal structures to lysosomes (Reaves *et al* 1996). Therefore PI 3-kinase is clearly very important in diverse aspects of vesicular trafficking. PI 3-kinase activity is also known to be a critical factor in the control of cell proliferation and survival (Thakker *et al* 1999).

Although PI 3-kinase homologues are ubiquitously expressed signal transduction molecules which are expressed as one or more isoforms in almost all eukaryotic cells, it is interesting to speculate that it may be specifically involved in transduction of an induction pathway in the follicular germinative epidermis, as the GE cells are directed along one of several differentiation pathways. Studies by Reynolds and Jahoda (Reynolds 1989, Reynolds and Jahoda 1991a, 1991b, 1993) demonstrate that the inductive signal arises from the dermal papilla, with the GE cells responding to this signal. The potential role of PI 3-kinase in vesicle trafficking in the germinative epidermis is discussed in section 4:6.

4:4 ISOLATION OF NOVEL GENES

The six putative differentially expressed clones described in this study which do not correspond to known genes showed varying degrees of homology to sequences reported in the databases. In some cases, for example, DIF-1, DIF-R6b, DIF-7, the homology was confined to a repeat element at the 3' end of the clone. One of the clones, DIF-19b, showed no significant homology to any previously reported sequences, and the remaining clones, DIF-14b and DIF-15, were related to rat or mouse neural mRNA's.

4:4.1 DIF-R6b

4:4.1-1 The Repeat Element Present in DIF-R6b is Homologous to a Similar Repeat Element at the 3' End of FAR-17a, an Androgen Regulated Gene.

A sequence of 250bp at the 3' end of DIF-R6b shows some homology to the 3'UTR of FAR-17a, an androgen regulated gene differentially expressed in hamster flank organs (Seki *et al* 1991). This was originally isolated from a male Golden Syrian hamster flank organ cDNA library, using a differential screening technique involving probes made from RNA isolated from normal male flank organs and the flank organs of 3-week postcastrated males. The FAR-17a mRNA was shown to be 1.8kb long by northern blotting, and its expression was shown to be strongly upregulated by androgens.

A probe containing the 3' end of FAR-17a was shown to produce a smear when hybridised to a Southern blot of mouse genomic DNA, in the same way that DIF-R6b hybridises to a range of rat genomic DNA fragments. This is not surprising, since many genomic sequences contain this repeat element in either forward or reverse orientation (for example, the genes for mouse or rat smooth muscle gamma actin, histocompatibility antigen H-2k, IgG receptor, erythrocyte membrane protein, IgE binding lectin, T-cell receptor, glucagon receptor, dopamine transporter, gamma crystallin, aspartate aminotransferase, lysozyme, vitamin D binding protein, TRPM-2, cytochrome c oxidase subunit Vb, cytochrome P-450 IVA1, fibronectin, 5E5 antigen, and those given in table 3.10). The repeat sequence is generally present in intron or promoter regions, and is usually spliced out of the mature mRNA (similarly to BC1). However, the DIF-R6b multiple tissue northern blot (figure 3.14) shows a high level of background hybridisation with the full length DIF-R6b probe, as well as revealing a single transcript of 3.3kb. This suggests that the repeat sequence is fairly common in mRNA species, though less abundant than BC1. It is interesting to note that DIF-R6b has the most restricted expression pattern in terms of tissue type, appearing in only liver, lung, spleen and brain, and background hybridisation is not seen in other tissue types, suggesting that this repeat element may be associated with a more restricted pattern of expression than BC1.

It was shown that FAR-17a expression in hamster flank organ hair follicles was localised to the outer root sheath by Puy *et al* (1996). This is similar to the localisation of DIF-R6b transcripts primarily in the region of the outer root sheath bulge, as shown by *in situ* hybridisation in this study. It is interesting to note that DIF-R6b was the only clone isolated from the differential screen having this 250bp repeat element, and it was the only clone that clearly showed a different hybridisation pattern to the BC1 repeat containing clones. Further, the fact that FAR-17a mRNA appears not to expressed in other epidermal components of the hair follicle supports the suggestion that the repeat element may be involved in localisation/control of mRNA expression. It would be even more interesting to speculate that this repeat element may be involved in some form of androgen regulated control, but this appears unlikely given the range of mRNA species known to contain this element, most of which are unaffected by androgens.

The region of DIF-R6b upstream of the repeat element shows no homology to sequences reported in the databases. However, the multiple tissue northern blotting and Southern blotting analysis shows that DIF-R6b appears to represent a 3.3kb mRNA, derived from a single copy genomic sequence, which is expressed in several rat tissues. Given the sequence data currently available for DIF-R6b, it is impossible to speculate what its role in the hair follicle might be.

4:4.2 DIF-14b

4:4.2-1 DIF-14b is Related to the Rat Alpha-Latrotoxin Receptor Family, and Shows Homology to Proteins Having an Olfactomedin Domain.

The only sequences in the databases which show significant homology to DIF-14b are those containing an olfactomedin domain, particularly the rat latrotoxin receptor family members. The C-terminal end of DIF-14b is over 70% identical at the amino acid level to a region of the alpha-latrotoxin receptor close to its N-terminal end, suggesting that DIF-14b may be a related member of this gene family.

Alpha-latrotoxin (α-LTX) is a neurotoxin produced by the black widow spider, *Latrodectus tredecimguttatus*, which binds specifically to presynaptic neuronal membranes causing neurotransmitter release. Identification of α-LTX binding partners led to the discovery of a highly polymorphic family of neurexins, which are neuronal cell surface proteins (Ushkaryov *et al* 1992). Later studies identified a novel α-LTX binding protein, latrophilin, which differs from the neurexins in that it does not require Ca^{2+} for its interaction with the toxin (Davletov *et al* 1996, Krasnoperov *et al* 1996). Latrophilin has been cloned and shown to be a G-protein coupled receptor (GPCR) of the secretin family (Krasnoperov *et al* 1997, Lelianova *et al* 1997).

A large number of structurally related GTPases act as regulators of endo- and exocytic protein transport reactions in a wide variety of cells. It has been shown that secretion of peptide hormones such as insulin shares features in common with neuroexocytosis, and indeed, latrophilin has been shown to be present in the membranes of insulin secreting β-cells, and well differentiated β-cell derived cell lines (Lang *et al* 1998).

The region of latrophilin which shares homology to the C-terminal of DIF-14b is the N-terminal extracellular domain. Recently, a family of related latrophilins CL-1, CL-2 and CL-3 have been described (Sugita *et al* 1998). These all share a high degree of identity in a region of their N-terminal extracellular domain which is homologous to olfactomedin (figure 3.27). The N - terminal of CL1, 2 and 3 contains both lectin and olfactomedin homology domains, and may be involved in the coupling of cell adhesion through these domains to cell signalling (Sugita *et al* 1998).

In recent years, many proteins have been described which have homology at the C-terminal to olfactomedin, a protein which is highly conserved in fish, frog, rat, mouse and human (Karavanich and Anholt 1997). Olfactomedin is thought to oligomerise through cysteine-cysteine interactions, and a common feature of many proteins having olfactomedin domains, including the DIF-14b predicted protein, are one or more conserved cysteines (as shown in figure 3.23b).

Figure 3.27: Sequence alignment of DIF-14b with the N-terminal olfactomedin domain of CL1, 2 and 3 (Sugita *et al* 1998). Amino acids which are conserved in three of the four protein sequences are indicated below the alignment (CON). Those which are identical in all four proteins are shown in bold type.

```
DIF-14b   1  L C S L F S P V A L V F V C P G T L K A I V D  23
CL1     127  D C V P Y - - - - - V F V C P G T L Q K V L E 144
CL2     128  E C V P Y - M E Q K V F V C P G T L K A I V D 149
CL3     189  E C V P Y K V E Q K V F L C P G L L K G V Y Q 211
CON          C                   V F   C P G   L

DIF-14b  24  S P S I Y E A E Q K A G A W C K D P L Q A A D  46
CL1     145  P T S T H E S E H Q S G A W C K D P L Q A G D 167
CL2     150  S P S I Y E A E Q K A G A W C K D P L Q A A D 172
CL3     212  S E H L F E S D H Q S G A W C K D P L Q A S D 234
CON          S   S     E   E       G A W C K D P L Q A   D

DIF-14b  47  K I Y F M P W T P Y R T D T L I E Y A S L E D  69
CL1     168  R I Y V M P W I P Y R T D T L T E Y A S W E D 190
CL2     173  K I Y F M P W T P Y R T D T L I E Y A S L E D 195
CL3     235  K I Y Y M P W T P Y R T D T L T E Y S S K D D 257
CON          K I Y   M P W T P Y R T D T L   E Y A S   E D

DIF-14b  70  F Q N S R Q T T T Y K L P N R V D G T G F V V  91
CL1     191  Y V A A R H T T T Y R L P N R V D G T G F V V 213
CL2     196  F Q N S R Q T T T Y K L P N R V D G T G F V V 218
CL3     258  F I A G R P T T T Y K L P H R V D G T G F V V 280
CON          F       R   T T T Y K L P N R V D G T G F V V

DIF-14b  92  Y D G A V F F N K E R T R N I V K F D L R T T 114
CL1     214  Y D G A V F Y N K E R T R N I V K Y D L R T R 236
CL2     219  Y D G A V F F N K E R T R N I V K F D L R T R 241
CL3     281  Y D G A L F F N K E R T R N I V K F D L R T R 303
CON          Y D G A V F F N K E R T R N I V K F D L R T R

DIF-14b 115  I K S G E A I I N Y A N Y H D T S P Y R W G G 137
CL1     237  I K S G E T V I N T A N Y H D T S P Y R W G G 259
CL2     242  I K S G E A I I N Y A N Y H D T S P Y R W G G 264
CL3     304  I K S G E A I I A N A N Y H D T S P Y R W G G 326
CON          I K S G E A I I N   A N Y H D T S P Y R W G G

DIF-14b 138  K T D I D L A V D E N G L W V I Y A T E Q N N 160
CL1     260  K T D I D L A V D E N G L W V I Y A T E G N N 282
CL2     265  K T D I D L A V D E N G L W V I Y A T E Q N N 287
CL3     327  K S D I D L A V D E N G L W V I Y A T E Q N N 349
CON          K T D I D L A V D E N G L W V I Y A T E Q N N
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DIF-14b  161 G M I V I S Q L N P Y I F R L E A T W E T T Y 183
CL1      283 G R L V V S Q L N P Y T L R F E G T W E T G Y 305
CL2      288 G M I V I S Q L N P Y T L R F E A T W E T T Y 310
CL3      350 G K I V I S Q L N P Y T L R I E G T W D T A Y 372
CON          G   I V I S Q L N P Y T L R   E   T W E T   Y

DIF-14b  184 D K R A A S N A F M I C G V L Y V V R S V Y Q 206
CL1      306 D K R S A S N A F M V C G V L Y V L R S V Y V 328
CL2      311 D K R A A S N A F M I C G V L Y V V R S V Y Q 333
CL3      373 D K R S A S N A F M I C G I L Y V V K S V Y E 395
CON          D K R   A S N A F M I C G V L Y V V R S V Y

DIF-14b  207 D N E S E A G K N V I D Y I Y N T R L S R G E 229
CL1      329 D D D S E A A G N R V D Y A F N T N A N R E E 351
CL2      334 D N E S E A G K N V I D Y I Y N T R L S R G E 356
CL3      396 D D D N E A T G N K I D Y I Y N T D Q S K D S 418
CON          D     S E A     N   I D Y I Y N T     S R   E

DIF-14b  230 H V D V P F P N Q Y Q Y I A A V D Y N P R D N 252
CL1      352 P V S L A F P N P Y Q F V S S V D Y N P R D N 374
CL2      357 H V D V P F P N Q Y Q Y I A A V D Y N P R D N 379
CL3      419 L V D V P F P N S Y Q Y I A A V D Y N P R D N 441
CON          V D V P F P N   Y Q Y I A A V D Y N P R D N

DIF-14b  253 Q L Y V W N N N F I L R Y S L E F G P P D P A 275
CL1      375 Q L Y V W N N Y F V V R Y S L E F G P P D P S 397
CL2      380 Q L Y V W N N N F I L R Y S L E F G P P D P A 402
CL3      442 L L Y V W N N Y H V V K Y S L D F G P L D S R 464
CON          Q L Y V W N N   F     R Y S L E F G P P D P

DIF-14b  276 Q G K S V R C *
CL1      398 A G P A T - - - - - - - S P P L S - - - - - - 407
CL2      403 Q V P T T - - - - - - - - - - - - - - - - - - 407
CL3      465 S G P V H H G Q V S Y I S P P I H L D S D L E 487
```

Given the data currently available, it seems likely that DIF-14b is located in the GE cell membrane, and it may be that the olfactomedin domain of this protein is partly responsible for the adhesive nature of these cells. As described in chapter 1, these cells actually appear "sticky", and the extracellular material surrounding freshly isolated GE cells will adhere to many substrates. In view of the fact that there are few hemidesmosomes and other anchoring structures between the DP and follicular epidermis (Nutbrown and Randall 1995) some other factor must be responsible for maintaining the tight association between the papilla and GE cells *in vivo*.

DIF-14b is probably the most clearly differential of the clones isolated in this study (figure 3.11), and is also the best characterised of these clones. However, further studies must be performed before it will be possible to determine whether this clone is a G-protein coupled receptor involved in both cell adhesion and cell signalling, similar to latrophilin, or an integral membrane protein involved in vesicular trafficking within the GE cells. This is discussed further in section 4:6.

4:4.3 DIF-15

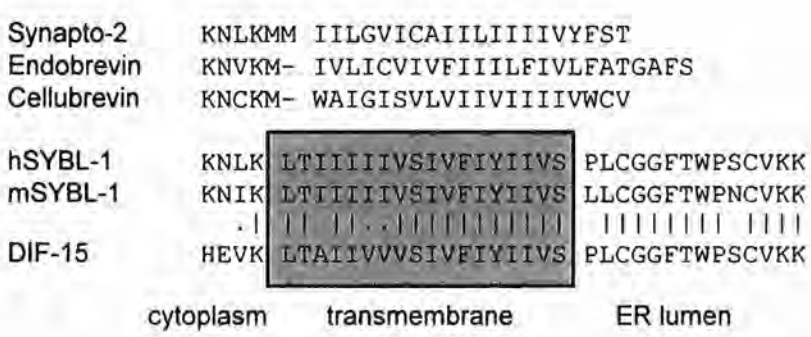
4:4.3-1 DIF-15 is Related to SYBL-1

The putative C-terminal 36 amino acids encoded by the DIF-15 cDNA clone show 96% similarity to the C-terminal of a human synaptobrevin-like protein, SYBL-1. A similar C-terminal hydrophobic domain is common to all members of the synaptobrevin family, acting as a membrane anchor for the protein.

Human and mouse SYBL-1 are 91.7% identical at the amino acid level over their C-terminal 36 amino acids, and DIF-15 is 77.8% identical to mouse SYBL-1 and 83.3% identical to human SYBL-1 over the same region. The transmembrane domains of mouse and human SYBL-1 are identical, comprising amino acids 189-206 (of 220). The putative transmembrane domain of rat DIF-15 is 83.3% identical to that of SYBL-1, and shares similarity with the transmembrane domains of other members of the synaptobrevin family. This is shown in figure 3.28.

A human SYBL-1 cDNA clone was isolated by D'Esposito *et al* (1996). A probe derived from this clone hybridised to a 2.6kb transcript in all tissues tested by northern blotting. This is similar to the major 2.7kb transcript hybridising to the DIF-15 probe in all tissues except heart and skeletal muscle tested by northern blotting in this study (figure 3.14). Furthermore, the fact that DIF-15 is 71% identical at the cDNA level to both mouse and human SYBL-1 suggests that this clone is a member of the synaptobrevin family closely related to SYBL-1.

Figure 3.28: Alignment of the mouse SYBL-1 (mSYBL-1) and human SYBL-1 (hSYBL-1) C terminal 36 amino acids with the putative DIF-15 peptide. DIF-15 and mSYBL-1 have 77.8% identity in this region. The C-terminal amino acid sequence of other members of the synaptobrevin family is given for comparison (adapted from Wong *et al* 1998). The hydrophobic transmembrane domain is indicated by the shaded box.



The synaptobrevin family is a large class of proteins acting to provide specificity in targeting pathways within cells. Synaptobrevins insert into a target membrane such that the N-terminal of the protein remains in the cytoplasm with the C-terminal in the lumen of the endoplasmic reticulum. The absolute conservation between species of the two cysteines in the C-terminal region of SYBL-1 supports the suggestion that these may be involved in intermolecular disulphide bonding (D'Esposito *et al* 1997).

The specificity in intracellular vesicle targeting and fusion can be explained by the SNARE hypothesis. According to this hypothesis, vesicle bound v-SNARE's pair with t-SNARE's in the target membrane to ensure correct direction of vesicles to the right docking site. Most membrane proteins located in the compartments of the mammalian secretory pathway or in organelles derived from it contain signal sequences which direct them to a translocation apparatus of the ER membrane which is also used by secretory proteins. Generally, the signal sequence of a ribosome bound polypeptide chain (generally at the N-terminus) is recognised by a signal recognition particle SRP which binds the signal sequence, and is then targeted to the ER membrane by interaction of the SRP with its membrane receptor (docking). The insertion of the protein into the ER membrane is then triggered by a

hydrophobic segment that may follow the signal or coincide with it (signal-anchor sequence). SNARE's do not possess signal sequences, instead having a hydrophobic C-terminal region which serves to anchor them in the target membrane (tail anchored). This translocation must occur post translationally, since while the peptide chain is still bound to the ribosome, at least part of the C-terminal hydrophobic region must be masked. This process is therefore not likely to involve the SRP since this appears only to interact with signal sequences of proteins which are ribosome bound (Kutay *et al* 1995).

DIF-15 apparently belongs to this class of tail anchored membrane proteins which lack a signal sequence. The insertion of synaptobrevin family membrane proteins into the ER membrane is dependent on ATP and requires ER proteins different from those required to translocate proteins with signal sequences into the ER membranes, thus suggesting that such membrane proteins have a novel method of insertion (Kutay *et al* 1995). It has been shown that the C-terminal membrane anchors of the synaptobrevin family have targeting information and may be involved in directing the different proteins to different cellular compartments via the ER through a secretory pathway (Ossig *et al* 1995). It was suggested that proteins with such C-terminal tags are then transported to their final destination via the ER, as shown by Ossig *et al* (1995).

Synaptobrevin family members are likely to perform a receptor like function, acting as v-SNAREs to pair with specific target membrane proteins (t-SNAREs), and the v-SNARE is likely to be initially directed to its correct membrane location by its C-terminal. The study by Kutay *et al* (1995) reports a novel mechanism of membrane insertion of synaptobrevin type molecules. They showed that synaptobrevin must first be inserted into the RER, from where it proceeds to the Golgi, and then to its final destination. Presumably, other tail anchored SNARE's follow the same pathway. A consequence of this is that there must be signals and mechanisms for their retention at various points of the secretory pathway. Both v- and t-SNARE's must be kept inactive when their function is not required, perhaps through interactions with other proteins and regions of the cytoplasmic domain.

4:4.4 DIF-19b

Analysis of the DIF-19b clone sequence showed no homology to known cDNA sequences in the GenBank and EMBL databases. However, a DIF-19b probe hybridised to a single transcript of 2.7kb in rat testis, kidney, liver, lung, spleen and brain by northern blotting. A comparison of DIF-19b with genomic sequences in the databases revealed that this clone is related to some extent to sequences containing B2 like elements. DIF-19b may therefore represent a third repeat containing element isolated from the differential screen. However, this element is clearly not as common as the others described in this study, as the level of background hybridisation to a DIF-19b probe is very low both by Southern and northern blotting.

4:4.5 Other Clones

The remaining two characterised clones reported in this study, DIF-1 and DIF-7, did not show protected fragments in the RNase protection assay, and were not shown to be highly expressed in the GE cells by *in situ* hybridisation. Further, they had no features typical of mRNA sequences such as a polyadenylation signal or open reading frame, although both contained BC1 repeats. The full sequence of the 4.2kb DIF-7 suggests that there may be an element of genomic contamination present in the EB library, since one would expect some hint of an ORF to be present in an mRNA of this size. These two clones were therefore not characterised further due to the limited time available.

4:6 SUMMARY

This chapter describes the production of PCR-generated tissue specific cDNA libraries from particular regions of the hair follicle, for use in a dual labelling differential screening technique designed to identify genes which are preferentially expressed in the germinative epidermis of the hair follicle. This resulted in the isolation of nine putative differentially expressed cDNA clones, the preliminary characterisation of which is reported in

this chapter. Three of these clones are related to proteins which are known to be involved in the eukaryotic vesicular trafficking system.

Generally, a feature of differential or subtractive screening approaches is that any sequences containing common repeat elements are significantly reduced in the resulting enriched population of clones. However, all the clones isolated from this study potentially contained a repeat element in the 3'UTR. In the absence of any other data, this would suggest that the differential screen had been suboptimal, since it would be expected that the number of repeat containing cDNA's in the upper end bulb cDNA library would be at least equivalent to the number present in the GE cDNA library, and the excess of UEB used would be expected to give greater hybridisation of repeat containing EB plaques to the - than the + probe. However, the technique resulted in the isolation of some novel sequences which appeared to be preferentially expressed in the germinative epidermis.

4:6.1 A Potential Role for DIF-14b, DIF-15 and DIF-16 in Vesicular Trafficking in the Germinative Epidermis.

DIF-15 has been shown to be closely related to SYBL-1 (section 4:4.3), a protein which is known to be essential for directing transport or fusion of vesicles to the plasma membrane. Historically, morphological analysis of eukaryotic cells led to the hypothesis that small vesicles mediate the directional transport of proteins between successive organelles in the secretory pathway (Palade 1975). The characterisation of vesicles that mediate intercompartmental protein transport addresses the role of the integral membrane proteins, such as homologues of SYBL-1, that function in vesicular traffic. Since vesicular transport is the primary means of shuttling proteins to the cell surface either for secretion or inclusion into the plasma membrane, a range of molecules must be involved in this process to provide the necessary specificity and directionality for regulated transport and secretion. A study by Rexach *et al* (1994) demonstrated that synaptobrevin like proteins were highly enriched in ER derived vesicles which were competent to fuse with Golgi but not ER membranes in yeast. This supports the hypothesis that SYBL related proteins are functionally involved with vectorial vesicle transport, rather than being merely structural components of vesicles. It therefore

seems likely that DIF-15 is part of the machinery that catalyses vesicular transport in the germinative epidermis (figure 3.29).

DIF-14b is related to the α-latrophilins CL1, CL2 and CL3 (Sugita *et al* 1998). Of these, it is most closely related (94% identical at the amino acid level) to CL2, which was shown to be constitutively expressed at low levels in all tissues studied (Krasnoperov *et al* 1999). CL2 is proteolytically processed into two heterologous subunits, a 120kDa extracellular domain and an 85kDa transmembrane/intracellular domain. It is the olfactomedin containing extracellular domain of CL2 which shares the high level of identity with DIF-14b. Olfactomedin itself is a major structural protein in the extracellular matrix of olfactory epithelium. Therefore, in the simplest case, DIF-14b is likely to be an extracellular matrix protein which is involved in GE cell adhesion. However, due to the high level of identity with latrophilin, it is possible to speculate that DIF-14b may similarly be a large G-protein coupled receptor with similar functions to the latrophilins. Latrophilin itself is known to be involved in regulation of exocytosis, and DIF-14b may function in a similar manner with a C-terminal extracellular domain (containing the olfactomedin homology domain) modulating secretion or signalling through direct physical contact between cells.

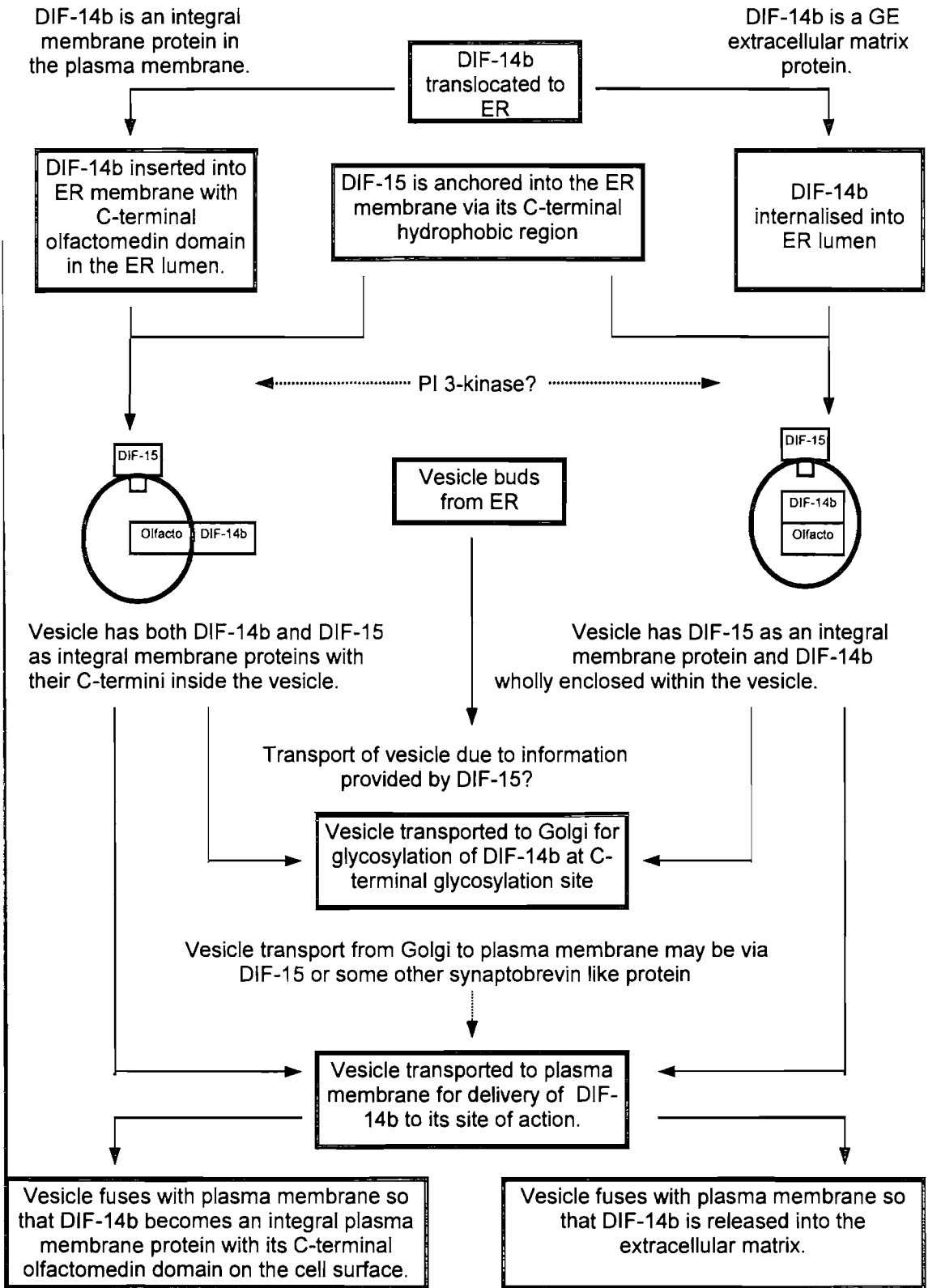
DIF-14b and DIF-15 are clearly not hair specific genes, as probes derived from these clones hybridise to multiple tissues by northern blotting (section 3:2.2). In fact, of the clones characterised in the study, DIF-15 is the most ubiquitous, appearing in all tissues tested except skeletal muscle and heart. It is interesting to note that a study by Krasnoperov *et al* (1999) found that the latrophilin CL2 copurified with the synaptobrevin-like molecule syntaxin, suggesting that these two proteins may act as v- and t- SNARE's, and that the docking or fusion of vesicles carrying syntaxin with the plasma membrane may be mediated by an interaction between CL2 and syntaxin. It is therefore similarly possible that DIF-14b and DIF-15 may act in a similar pathway. The 3'UTR of each of these clones contains a BC1 repeat flanked by five 4-7bp elements common to both clones, in approximately the same position relative to the start of the BC1 repeat. According to the theory of Vidal *et al* (1993), this suggests that these two clones may be regulated in the same way at the RNA level.

It is interesting to note that GE cells can only interact with DP cells in co-culture when the cells are actually in physical contact. The presence of DIF-14b in the plasma membrane or ECM may therefore be indicative of a role in transduction of some signal from the DP cells to the GE cells. If DIF-14b is acting as a novel G-protein coupled receptor, it may be responsible for activating PI 3-kinase in response to an inductive signal from the dermal papilla. This provides a potential link between the putative high levels of DIF-14b and DIF-16 in the GE cells. A second potential link is suggested by the interaction of PI 3-kinase and lysosomal membrane glycoproteins in the late endocytic pathway as described by Reaves *et al* (1996), since DIF-14b shares homology with lysosomal membrane glycoproteins through the olfactomedin domain. Most of the PI 3-kinase activity described in induction and endocytosis in the literature is ascribable to the catalytic subunit of PI 3-kinase coupled to the p85 regulatory subunit. A role for the p55 subunits in such pathways has not been clearly demonstrated; however, it has been suggested that the different regulatory subunits of PI 3-kinase may merely be responsible for the different subcellular location of the enzyme, or different levels of its activity (section 4:3.1). There are therefore several pathways in which DIF-14b, DIF-15 and DIF-16 could act coordinately. These are described in figure 3.29.

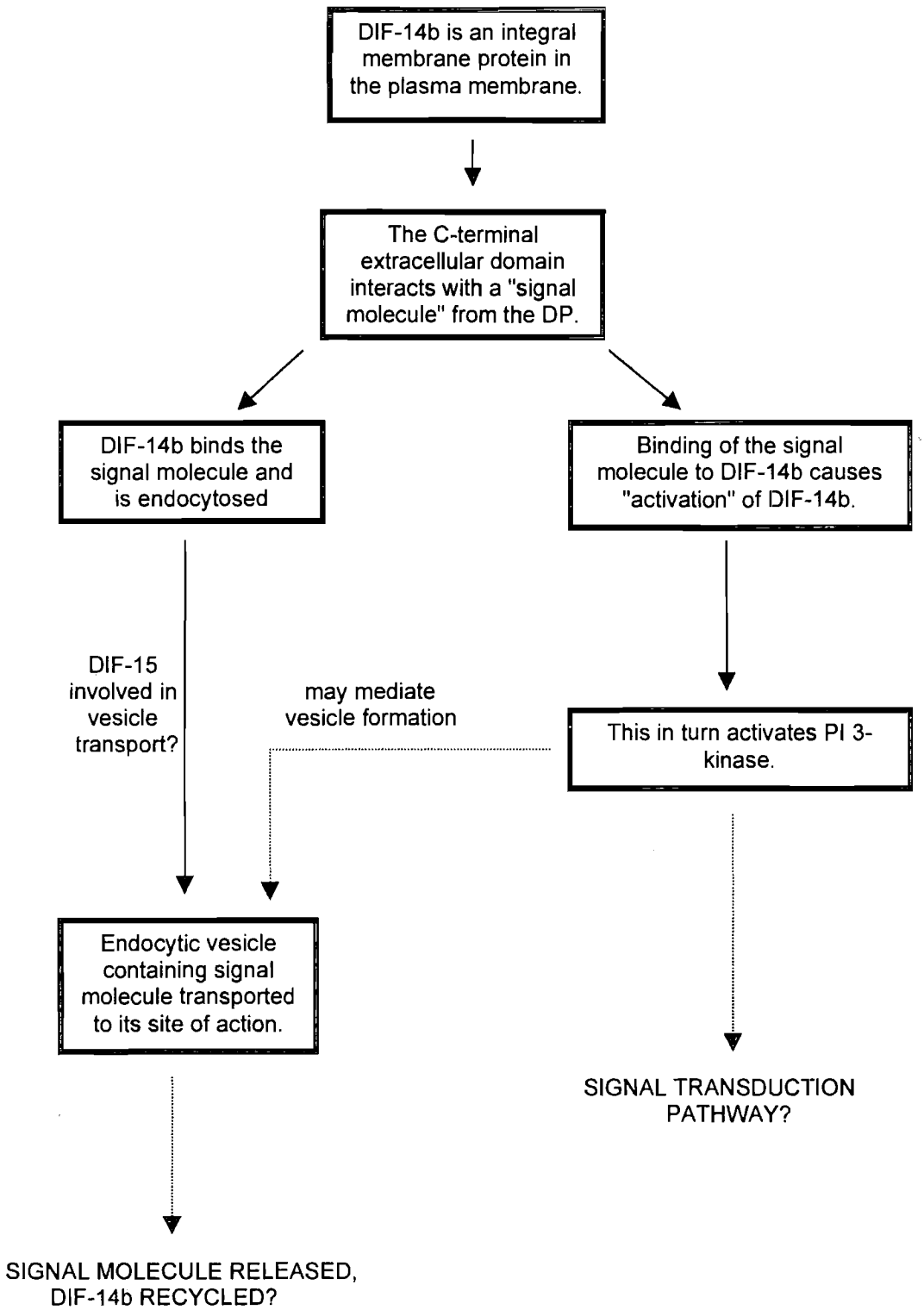
Vesicular transport between cells in the germinative epidermis has been observed in coculture with dermal papilla cells (Reynolds and Jahoda 1991b). The fact that the GE cells remain quiescent unless they are in contact with DP cells, unlike other epidermal cell types isolated from the hair follicle, suggests that GE cells must "wait for orders", and are dependent on dermal influences for induction and direction of hair fibre production. The clones described in this study may therefore make up part of the pathway by which such signals are received and translated. This is discussed further in chapter 4.

Figure 3.29: Potential roles of DIF-14b, DIF-15 and DIF-16 in vesicular trafficking in the germinative epidermis.

A: DIF-15 is responsible for transport of DIF-14b to the plasma membrane?



B: DIF-14b acts to transduce an inductive signal received from the dermal papilla?



CHAPTER 4

CONCLUSIONS AND

FURTHER WORK

1: CONCLUSIONS

The aim of this study was to begin the molecular characterisation of hair follicle germinative epidermis, a specialised population of epidermal cells at the base of the hair follicle matrix. Many molecular studies of genes expressed in hair follicles have shown that a wide spectrum of known genes can be localised to various regions of the hair follicle (reviewed in Stenn *et al* 1996). Though this is interesting in itself, it would be more useful to characterise those genes that are specifically expressed in the population of interest, rather than "fishing" through known genes in the hope that they may prove to be important in the tissue under investigation. This study was therefore designed with this in mind, with the aim of isolating and characterising known or unknown genes which are more highly expressed in the GE cells than other regions of the follicle epidermis.

Though none of the clones isolated in this study could be shown to be hair follicle specific, it is likely that specifying the production of hair type differentiation products occurs at a later stage in epidermal differentiation, with the preliminary events being the establishment of cells enabled to produce the layers of epidermal differentiation products. The GE cells may therefore act as an information "clearing house" transducing an inductive signal from the dermal tissue and "streaming" the information, thus rendering them capable of differentiation into the multiple layers of the hair follicle epidermis. In this context, it is interesting that the clones isolated in this study appear to be involved in the transfer and translation of information. Though the suggested roles for these genes presented in this study is largely speculative, further experiments of the kind described in section 3 could be performed to address the specific role in vesicular trafficking of each of the clones described here.

At the beginning of this study, it was expected that the clones isolated would include a range of known growth factors and other molecules which have already been shown to be important in hair growth. Many examples of primary signalling molecules in the hair follicle have already been characterised (see chapter 2); however, details of the mechanics of signal transduction in the follicle remain to be elucidated. The work reported in this thesis represents

the preliminary characterisation of molecules expressed in the germinative epidermis which are potentially important in signal transduction pathways in the earliest stages of epidermal differentiation in the hair follicle.

Since this study was initiated in 1992, many improvements in differential and subtractive screening approaches have been reported. Much of this recent work is already covered in chapter 3, however, global RT-PCR from single cells (Brady and Iscove 1993, Trumper *et al* 1993, Brady *et al* 1995) could prove to be a particularly useful starting point for the isolation of novel genes from small amounts of germinative epidermis.

2: OTHER WORK PERFORMED IN PARALLEL WITH THIS STUDY

Several other strategies were attempted during the course of this study to further characterise the GE cells at the molecular and biochemical level. These are briefly described below.

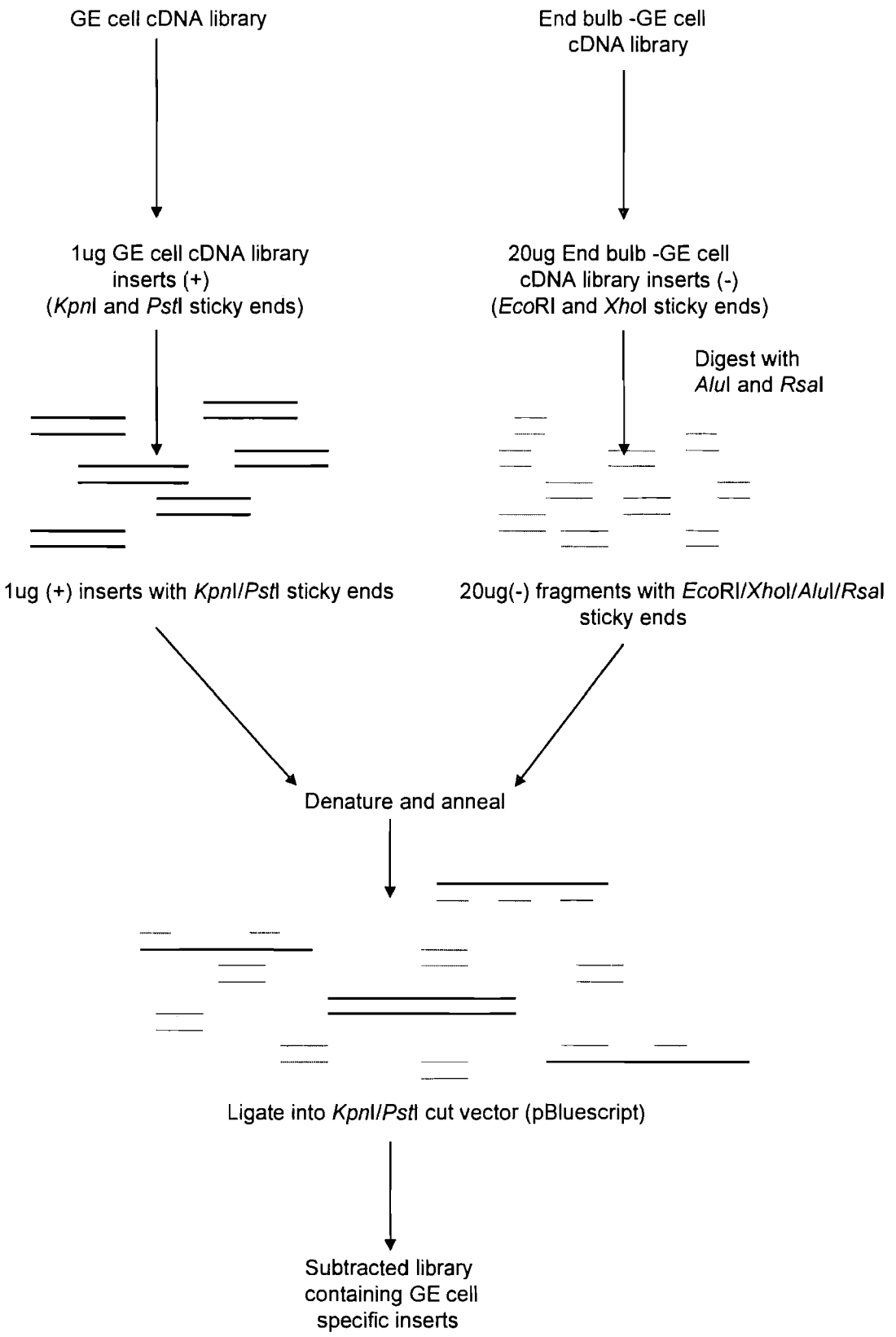
2:1 Production of a Subtractive Library

As well as the differential screening approach reported in this study, a subtractive library was constructed using the cDNA inserts from the GE and upper end bulb PCR generated libraries as the starting material for the subtraction. Briefly, insert DNA was prepared from 10^7pfu of both of these cDNA libraries, using a different pair of restriction enzymes to cut out the insert from each library cDNA pool (*Pst*I/*Kpn*I for the GE library, *Eco*RI/*Xho*I for the upper end bulb library). The upper end bulb library cDNA insert pool was then further digested with *Alu*I and *Rsa*I (restriction enzymes with 4-mer recognition sequences, cutting approximately every 260bp on average), to give fragments of 50-300bp. The GE library insert cDNA was then mixed with a fifty-fold excess of the digested upper end bulb library cDNA fragments (200ng GE library insert + 10μg upper end bulb library fragments), denatured by boiling and allowed to reanneal. Only those GE library inserts which were not also represented in the upper end bulb library could reanneal in such a way to

enable their ligation into *PstI*/*KpnI* digested pBluescript. This is summarised in figure 4.1.The plasmids produced were transformed into competent *E. coli*, and recombinant transformants selected by Blue/White selection. This resulted in the isolation of 268 recombinant clones. Plasmids isolated from a random sample of 18 of these were digested with *PstI* and *KpnI*, and their insert sizes determined by agarose gel electrophoresis on a 2.0% agarose/TAE gel (figure 4.2). This showed that the subtractive library had inserts of between 100-600bp, with an average insert size of around 250bp. Duplicate Southern blots of these library inserts were then probed with ^{32}P-labelled total GE and upper end bulb library probes. This showed that most of the 18 cDNA inserts hybridised strongly to the GE probe, but only two hybridised strongly to the upper end bulb probe, suggesting that the subtraction strategy was successful. To confirm this, a total subtracted library probe was prepared from all 268 clones by PCR using vector specific primers, and this was used to probe filters of GE and upper end bulb library, again showing that the enrichment strategy was successful. This is shown in figure 4.2. Further tests to determine the quality of the subtractive library could include probing colony filters with a BC1 repeat probe to determine whether the subtraction strategy had successfully removed repeat-containing sequences.

Although these tests showed that the subtraction protocol was reasonably effective, perhaps more so than the differential screening strategy reported in chapter 3, this work was not continued further. It was felt that the starting material for the subtraction was not ideal, as the GE library cDNA inserts used were generated by PCR from a very limited quantity of tissue, potentially resulting in contamination from various sources (genomic DNA, mitochondrial transcripts, rRNA's etc). Also, the very short inserts present in the subtracted library were felt to be of limited use, since they are likely to consist almost exclusively of 3'UTR sequences. Further characterisation of this library was therefore suspended due to the limited time and resources available for the study. However, the pooled inserts of this library could be used to probe the EB library to obtain longer potentially GE specific clones for further characterisation.

Figure 4.1: Strategy for the production of a subtracted cDNA library enriched for GE specific clones.



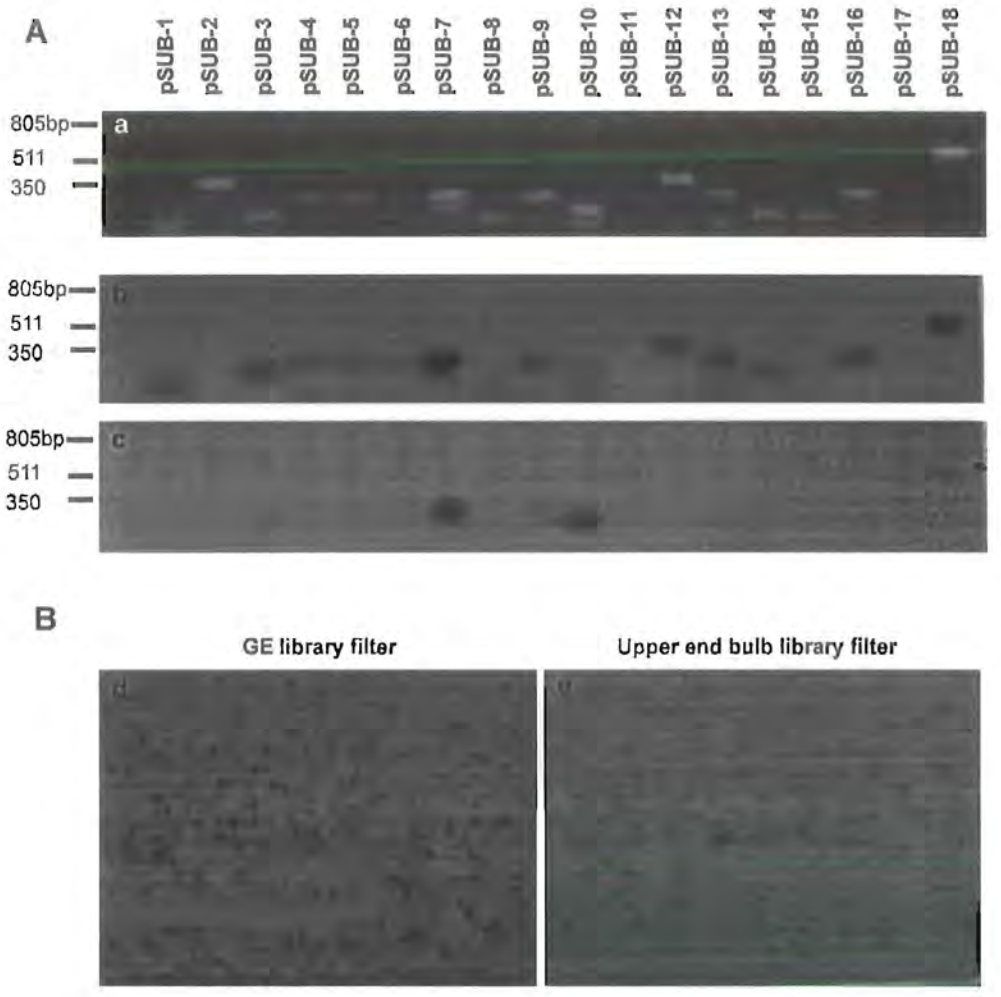


Figure 4.2: Testing the subtracted library.

A: 18 clones were selected at random from the subtracted library, and the cDNA insert sizes determined by agarose gel electrophoresis (a). This shows that in this sample, the inserts ranged from 100-650bp, with an average size of around 250bp. Duplicate Southern blots of these inserts probed with a total GE library probe (b) and a total upper end bulb probe (c) showed that most of the inserts hybridised strongly to the GE probe, but only two hybridised strongly to the upper end bulb probe, suggesting that the subtraction strategy was successful.

B: Filters of GE library plaques (d) and upper end bulb library plaques(e) probed with a total subtracted library probe. This shows that the subtracted library is much more highly represented in the GE library background, as expected.

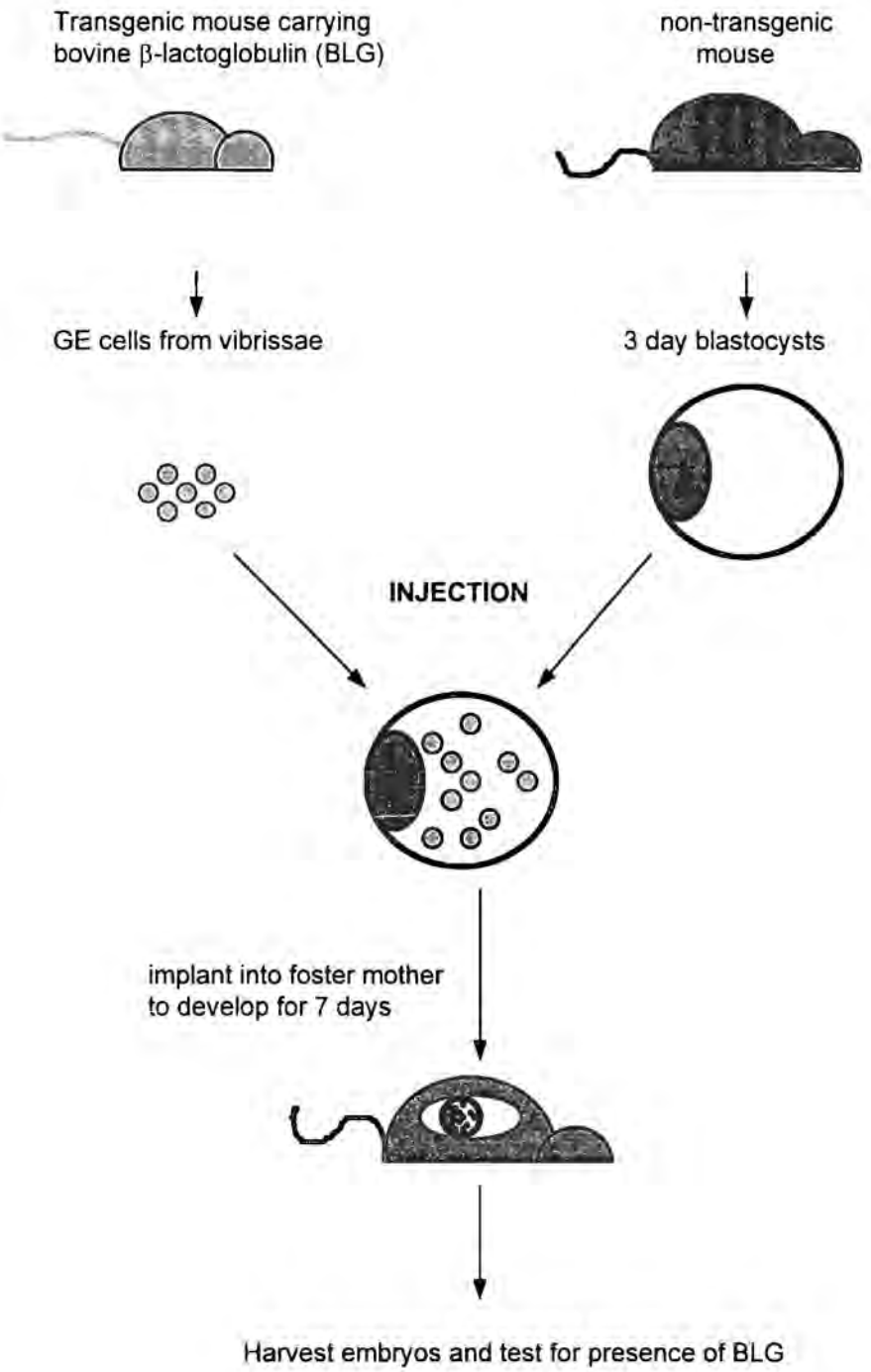
2:2 Blastocyst Injection Work

In order to study the potential of GE cells to contribute to other cell lines, blastocyst injection experiments were performed using GE cells taken from a transgenic mouse carrying the 5' region of the ovine β-lactoglobulin gene, an ideal transgene for such a study, since rodents do not themselves have an endogenous BLG gene. Therefore, assays by PCR and blotting should not give rise to false positive results due to interference by the native copy of this gene. GE cells were injected into 3 day blastocysts taken from wild-type mice and allowed to develop following implantation into a foster mother. The methods used are briefly described below.

Developing embryos were flushed from the mother at the morula stage and allowed to develop in culture for 48 hours to allow condensation of the inner cell mass (ICM). GE cells were obtained from the vibrissa follicles of freshly killed mice carrying the 5' end of the ovine β–lactoglobulin (BLG) gene as a transgenic marker. The end bulbs were removed and transferred to a dish of E-MEM on ice. GE cells were dissected free as described by Reynolds and Jahoda (1991b) and transferred to Ca^{2+}/Mg^{2+} free PBS. When sufficient material had been accumulated, the tissue was subjected to a brief dispase/elastase/ collagenase digestion to separate the cells into a loose suspension.

GE cells were injected into the space between the ICM and blastocyst wall until the space was full. Treated blastocysts were then implanted into a foster mother and allowed to develop for seven days, after which the mother was killed and the embryos and extraembryonic membranes dissected free, transferred to microcentrifuge tubes and snap frozen in liquid nitrogen. The embryos were then processed in order to determine whether any cells carrying the transgenic marker were still present (strategy shown in figure 4.3). Genomic DNA was extracted from the embryonic tissue by proteinase K digestion and phenol extraction followed by ethanol precipitation. This DNA was then used as the template for PCR reactions using test primers specific for regions of the BLG gene, and control primers for HGPRT (hypoxanthine-guanine phosphoribosyl transferase).

Figure 4.3: Blastocyst injection protocol to test GE cell potential - can the GE cells be "re-directed" to contribute to the developing embryo?



This work was performed in collaboration with Ian Wilmut and Bill Ritchie at AFRC Roslin, where the necessary equipment and expertise was available. In total, 24 ten day old embryos produced from several repeats of the injection and implantation procedure were processed in this way. However, optimisation of the PCR reaction conditions to reliably amplify BLG and HGPRT together proved very difficult, and this work was suspended.

2:3 Cell Culture Work

Although no cell culture is reported in this study, some was done in collaboration with Dr. A. J. Reynolds and Mr. M. Robinson. This work will be reported fully elsewhere, however, a brief description is provided below.

2:3.1 End Bulb Culture

All of the model systems currently reported depend on the culture of intact hair follicles, assuming that all the follicular components are necessary in an *in vitro* system for normal fibre growth to continue. However, if continued growth depends only on the fundamental interactions between the dermal papilla and epidermal matrix, then it should be possible to show that normal fibre production continues in culture when the portion of the follicle above the end bulb, including the putative stem cell site in the upper ORS, is removed. Such a model system has advantages over whole follicle culture, in that the cell systems operating are more accessible to topical agents that may be added to study the basis of cell-cell and tissue-tissue interactions involved in the process of fibre production at the site of the dermal-epidermal interactions. Also, the interactions between the papilla and the germinative epidermis can be studied more informatively when influences from the rest of the follicle are removed. Retaining the spatial organisation of the tissues present in the follicle has obvious advantages over the use of combined populations of cultured cells.

Many organ culture systems have been reported in which antisense oligonucleotides are used to study the mechanisms of dermal-epidermal interactions at the molecular level. A 46% inhibition of amelogenin translation by an antisense oligonucleotide blocking the AUG start codon has been reported in a mouse embryonic molar organ culture system (Diekwisch *et al* 1993), and NRG and HGF activity in murine mammary gland organ culture has been shown to be blocked by antisense oligonucleotides (Yang *et al* 1995). A potential problem in the use of such a method in hair growth models relates to the ability of the antisense oligonucleotide to penetrate the collagen capsule and gain access to the cells within. However, in a culture system using only the follicle end bulb, in which most of the collagen capsule is removed, access of topical agents to the cells at the base of the follicle is greatly enhanced. Combined with the greater simplicity of an end bulb model over a whole follicle culture system in terms of cell types involved, this method has great potential for the study of the molecular basis of the fundamental dermal-epidermal interactions occurring at the base of the hair follicle.

It has been shown that all the tissues of the end bulb are accessible to oligonucleotides of 30-40 bases in length using a "ubiquitous" poly-T oligo complementary to the poly-A tail of cellular mRNA's (M. Robinson, unpublished). The continued growth of the end bulbs used in the antisense experiments shows that the presence of the oligonucleotide in itself does not affect the growth of the fibre. Therefore, using oligonucleotides designed to be complementary to the 3'UTR or the 3'end of the ORF of specific mRNA's, their expression in the tissues of the end bulb can be accurately pinpointed without significantly affecting the behaviour of the model system. We have successfully used this strategy to confirm the site of expression of macrophage migration inhibitory factor (MIF) in the end bulb, using a 30-mer oligonucleotide complementary to the 3' end of the MIF ORF (unpublished). With careful oligonucleotide design, it will be possible to block the translation of specific mRNA's and therefore study the action of particular molecules operating in this culture system.

3: FURTHER WORK

3:1 Genomic Library Screening

The obvious next step in the characterisation of the cDNA clones reported in this study is the screening of a rat genomic library to obtain the corresponding genomic clones. It will be important to use probes derived from the 5' end of all the clones described in chapter 3 since including the 3' BC1 repeat in the probe would give a high level of background hybridisation. Genomic clones have several advantages over cDNA clones -

- mRNA secondary structure and the efficiency of the reverse transcriptase reaction frequently results in 5' truncation of cDNA clones produced by priming from the polyA tail. Since this is not a problem encountered in genomic library production, "missing" 5' sequence information can be readily obtained, and the full sequence of the open reading frame can be deduced from the transcribed region. An attempt was made in this study to obtain genomic sequence information for DIF-14b by LAPCR, since resources for making and screening a genomic library were not available. Though this was successful in principle, a more exhaustive trial using several different enzymes to digest the genomic DNA could have provided more information.

- Genomic sequence information for a particular clone provides information outside the transcribed region which is not available from a full length cDNA clone. In particular, the upstream regulatory sequences can be analysed to determine what proteins are likely to activate or repress transcription of the gene in question.

3:2 Protein Studies

The only clones described in this study for which any protein sequence information was available were DIF-14b and DIF-15. The DIF-15 partial ORF encodes only the C-terminal

36 amino acids, and is therefore of limited use for protein studies. However, the 283 amino acid sequence of the DIF-14b partial ORF would potentially be useful for several further experimental approaches.

- Subcloning the ORF into an expression vector for expression of the C-terminal end of the protein in bacteria. It would be particularly useful to express DIF-14b as a fusion protein with an N-terminal sequence (for example, an N-terminal histidine tag) which could be used to purify the recombinant protein.

- Purified recombinant protein could then be used to produce a polyclonal antibody to the C-terminal end of DIF-14b. Antibodies can also be produced from synthetic peptide sequences when a purified recombinant protein is not available. However, this is not the preferred strategy in this case because an antibody generated from a short peptide sequence could be less likely to pick up the native protein *in vivo*, as the conformation of the protein may hide the antigenic site. This is less of a problem when using a long protein sequence generated by bacterial expression, as multiple antigenic sites are present.

- A specific DIF-14b antibody could be used to identify the size of the native protein by western blotting, and to precisely localise its expression in the hair follicle and other tissues. This strategy could be used to confirm that DIF-14b is an extracellular/membrane protein highly expressed in the germinative epidermis.

- Such an antibody could also be used for immunoprecipitation experiments to purify native DIF-14b from a follicle protein extract, and potentially to identify some of its binding partners.

3:3 Cell and Organ Culture Experiments

The strategies described in this section could be the most useful to confirm the theories proposed in chapter 3. The behaviour of the GE cells in recombination with DP cells *in vitro* has already been reported (Reynolds and Jahoda 1991b), and this culture system

provides a useful starting point for characterisation of the fundamental interactions occurring between these two cell types.

- Now that techniques are available for studying the differential expression of genes from very small amounts of tissue, it may be useful to compare differences in gene expression between a GE/DP recombination where profound interactions occur *in vitro*, and a GE/DS recombination where such interactions do not occur.

- As a starting point to determine whether PI 3-kinase is required for transduction of an inductive signal from the papilla in the GE cells, the behaviour of cocultures of these two cell types could be compared in the presence and absence of the specific PI 3-kinase inhibitor, wortmannin. A similar study could be performed using end bulb cultures to determine whether the presence of wortmannin has an effect on epidermal proliferation and differentiation.

- The DP/GE coculture system could also be useful to determine the potential of GE cells to respond to different inductive signals. It has been shown that interacting GE/DP cocultures can produce hair specific epidermal differentiation products (Dr. A. J. Reynolds, unpublished), and it would be interesting to see if hair GE cells could produce, for example, tooth specific epidermal differentiation products when grown in coculture with tooth DP cells. This would support the theory that the GE cells are acting as an information "clearing house", with the behaviour of their progeny determined by signals received from the dermal tissue.

4: THE STEM CELL QUESTION

Though a great deal of information has been accumulated on the behaviour of skin basal epidermal stem cells, characterisation of follicular epidermal stem cells is still in its infancy. Though this study was not designed primarily to determine whether GE cells are an epidermal stem cell population, it is interesting to note that none of the known epidermal stem cell markers were isolated in the differential screen. A comparison of the properties of skin stem cells and GE cells reveals more differences than similarities. One of the most fundamental differences relates to the behaviour of these cell types in culture. GE cells cannot

be induced to divide when cultured in isolation regardless of the media, substrates and supplements used, whereas skin basal cells form colonies very rapidly *in vitro*. The fact that stem cells cycle very slowly unless they are stimulated to proliferate is one of the most basic characteristics of a stem cell. If this were an intrinsic property of a stem cell, one would expect that this behaviour would continue in culture. Therefore, it seems strange that both skin basal cells and follicular ORS cells proliferate very readily in culture, while GE cells do not. The GE cells behave in a very different way, in that they are quiescent when removed from the influence of the DP *in vivo*, and can only be stimulated to proliferate *in vitro* when DP cells are also present.

This seems to suggest not that skin basal cells cycle slowly unless they are stimulated to proliferate, but rather that they cycle rapidly unless they are stimulated not to. It is well established that mammalian epithelia are critically dependent on interactions with components in the basement membrane for correct morphogenesis and function. If a cell is not attached to the basal lamina *in vivo*, it begins to differentiate. Many studies of epidermal stem cells have noted that they adhere very readily to culture substrates. This is thought to be due to their high level of β-integrin (particularly β-1 and β-4) expression, and indeed, a high surface β-1 integrin level is frequently used as an epidermal stem cell marker . A study by Zhu *et al* (1999) proposed that β-1 integrin and MAP kinase cooperate to maintain the epidermal stem cell compartment *in vitro*, and a study by Jensen *et al* (1999) showed that cells from the skin basal layer expressing a high level of β-1 integrin behaved differently *in vitro* than cells expressing lower levels of β-1 integrin. However, a study by Bickenbach and Chism (1998) could not demonstrate that the rapid attachment of epidermal stem cells to culture substrates was due to expression of a specific integrin.

As well as differences in the level of β-1 integrin expression, it has been proposed that skin basal epidermal cells also differ in their intercellular adhesiveness (reviewed in Watt, 1998), with the stem cells adhering more strongly to each other. This is a feature that these cells have in common with the GE cells, which are almost impossible to separate into a single cell suspension, and which adhere very tightly to the DP *in vivo*. However, given the differences in the number of hemidesmosomes and the level of integrin expression between

skin basal cells and GE cells, some other factor must be responsible for the "adhesiveness" of the GE cells. DIF-14b is a candidate molecule for promoting adhesion between the GE cells; however, as figure 3.11 shows (chapter 3), its expression appears not to be confined to the GE cells alone, so DIF-14b cannot be proposed as a specific marker of GE cells in the follicle.

Although none of the clones described in this study appear to be GE cell specific, the novel genes reported here provide a useful starting point for characterisation of the fundamental signalling mechanisms operating within the germinative epidermis.

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TECHNICAL APPENDIX A

MEDIA AND BUFFERS

LB medium (per litre)

10.0g Bacto-tryptone

10.0g NaCl

5.0g Yeast extract

ddH_2O to 1l

Adjust to pH 7.5

2XL medium (per litre)

20.0g Bacto-tryptone

10.0g yeast extract

1g NaCl

900ml ddH2O

Autoclave, cool and add

100ml sterile filtered 20%

glucose solution (2% final

conc).

NZY Agar

15.0g agar

1l NZY broth

NZY Broth (per litre)

5.0g NaCl

2.0g MgSO4.7H2O

5.0g Yeast extract

10.0g NZ amine

ddH2O to 1l

Adjust to pH 7.5

Top Agar

0.7g agarose

100ml NZY broth

2X YT Broth (per litre)

10.0g NaCl

10.0g Yeast extract

16.0g Bacto-tryptone

Superbroth (per litre)

5.0g NaCl

20.0g Yeast extract

35.0g Bacto tryptone

1ml 5M NaOH

ddH2O to 1l

50x Denhardts

5.0g Ficoll

5.0g polyvinylpyrrolidone

5.0g BSA

ddH_2O to 500ml

Sterile filter, aliquot and

store at -20°C

SM Buffer (per litre)

5.8g NaCl

2.0g MgSO4

50ml 1M Tris pH 7.5

5ml 2% gelatin

ddH2O up to 1l

ETHIDIUM BROMIDE PLATE QUANTITATION OF NUCLEIC ACID CONCENTRATION

Quantitation of small volumes of nucleic acid can be obtained by UV visualisation of samples mixed with ethidium bromide solution and "spotted" onto a 90mm petri dish.

EtBr Stock Solution - 1ml ddH2O

- 1µl 10mgml^{-1} EtBr solution

DNA Standards - 1ngµl^{-1}, 5ngµl^{-1}, 10ngµl^{-1}, 25ngµl^{-1}, 50ngµl^{-1}, 75ngµl^{-1} and 100ngµl^{-1} DNA samples made from a DNA stock of known concentration diluted in ddH_2O.

Label a 90mm petri dish to indicate where the sample to be quantified and the standards are to be spotted. Spot 1µl EtBr stock solution for each DNA sample. Add 1µl of the relevant DNA sample to each EtBr spot and incubate at room temperature for 10 minutes (all DNA samples must be spotted within 10 minutes of each other). Photograph the plate on a UV transilluminator and compare the intensity of the sample and standard spots to quantify the unknown sample.

PREPARATION OF RAT FOLLICLE ACETONE POWDER FOR USE IN NON-RADIOACTIVE *IN SITU* HYBRIDISATION PROTOCOL

- Freeze freshly isolated rat vibrissa follicles in liquid nitrogen and grind to a fine powder.
- Add 500µl ice cold acetone, mix well and incubate on ice for 30 minutes with occasional vortexing.
- Centrifuge at 10000g for 10 minutes at 4°C, remove the supernatant and resuspend the pellet in a further 500µl ice cold acetone.
- Spin again, remove the supernatant and transfer the pellet to a sheet of filter paper.

- Grind to a fine powder and allow to dry. Store at 4°C.

ALKALINE AGAROSE GEL ELECTROPHORESIS

Alkaline agarose gel electrophoresis is used to demonstrate the size range of first and second strand cDNA during production of cDNA libraries (chapter 2).

10x alkaline buffer
- 3ml 5M NaOH
- 2ml 0.5M EDTA
- 45ml H_20

2x loading buffer
- 200µl glycerol
- 750µl H_20
- 46µl saturated bromophenol blue
- 5µl 5M NaOH

- Suspend a minigel comb over a 5x7.5cm glass plate using plasticene, so that the teeth of the comb do not touch the surface of the plate.
- Prepare 1% alkaline agarose by melting 0.3g agarose in 27ml water. Allow to cool to 55°C and add 3ml 10x alkaline buffer.
- Pour 10ml molten alkaline agarose into the middle of the glass plate. Surface tension will prevent overflow, thus producing a small, thin gel which can be autoradiographed without drying.
- Load the radiolabelled cDNA samples in an equal volume of 2x loading buffer, and run in 1x alkaline buffer at 100mA or less, making sure that the temperature does not rise above 37°C.
- After running, seal the gel in a plastic bag, taking care not to get air into the bag, and expose to X-ray film.

APPENDIX B

PRIMER SEQUENCES

Primer identification	Sequence	Clone	Comments
587	5' ATC GAA TTC CCC CCC CCC CCC CCC 3'		Used for amplification of tailed cDNA in preparation of PCR-generated cDNA libraries.
588	5' ATC CTC GAG CCT TTT TTT TTT TTT TTT 3'		Primer for reverse transcription of polyA RNA.
332	5' CAG GAA ACA GCT ATG AC 3'	Vector specific	pBluescript forward primer.
1214	5' CCCCTCGAGGTGGACCCACAGACAAATCTA 3'	DIF-R6b	DIF-R6b 5'RACE antisense primer.
1215	5' CCCCTCGAGCACAAATCCAGTACCGTCCAC 3'	DIF-14b	DIF-14b antisense primer 1.
15b	5' TTTGCTGAGAGAAGTGAAAGCC 3'	DIF-15	DIF-15 5'RACE antisense primer
19b	5' ACTGCTGAGCCATTTCACTGGC 3'	DIF-19b	DIF-19b 5'RACE antisense primer.
14b5'	5' AAAACTAACGCCACGGGGGA 3'	DIF-14b	DIF-14b antisense primer 2.
14bRACE	5' GGTCCTTGCACCAAGCACCTG 3'	DIF-14b	DIF-14b antisense primer 3.
14bISH	5' AAGAAGCATATTCTATTAAGG 3'	DIF-14b	DIF-14b antisense primer 4.
BC1	5' CTCGAGCTCTACCACTGAGCTAAATCC 3'	All clones	Antisense primer specific to 5' end of BC1 repeat.
MIF5'	5'CGGGGAACATTGGTGTTCACG 3'	MIF-B2	Antisense primer to obtain 5'end of MIF cDNA.
MIFRACE	5' ATCTTGCCGATGCTGTGCAGG 3'	masCL-32	Antisense primer to obtain 5'end of MIF cDNA.