An Investigation of Hair Follicle Cell Immortalisation and Hair Keratin Gene Regulation

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Thesis Summary

This thesis presents results from an investigation into the regulation of hair-specific gene expression, including attempts to produce an immortalised hair follicle cortical cell line for this purpose and the use of mouse transgenesis and *in vitro* gel mobility shift assays.

A major aim of this project was to produce an immortalised hair follicle cell line derived from differentiating cortical cells. To achieve this, SV-40 T antigen (TAg) expression was targeted to the hair follicles of transgenic mice using the promoter of a sheep type II intermediate filament wool keratin gene, *K2.10*. Expression of TAg in the follicle cortex altered the normal protein profile and disrupted normal fibre ultrastructure, producing a marked phenotypic effect. *K2.10-TAg* transgenic mice developed various other abnormalities including vertebral abnormalities and bladder, liver and intestinal tumours which resulted in reduced life expectancy. However, no follicle tumours or neoplasia were apparent and immortalisation of cortical cells could not be established in culture. *In situ* hybridisation studies in the hair follicle using histone H3 as a cell proliferation marker suggested that cell proliferation had ceased prior to commencement of *K2.10-TAg* expression and was not re-established in the differentiating cortical cells. Hence, TAg was unable to induce cell immortalisation at this stage of cortical cell differentiation. Given this unsuccessful attempt to produce an immortalised cortical cell line, the use of more appropriate gene promoters which would target TAg expression earlier in follicle cell differentiation is briefly discussed.

The second part of this thesis investigates the regulation of a hair-specific keratin intermediate filament gene, *K2.10*. Initially, mouse transgenesis was used to analyse a small series of promoter deletion constructs utilising the *lacZ* reporter gene to detect expression in the hair follicle. It was shown that 400 bp of 5' proximal *K2.10* promoter was sufficient to confer correct cortical-specific gene expression. Mutation of a highly conserved putative regulatory element (designated HK-1) within this region failed to abolish cortical-specific expression, although expression levels appeared reduced. No expression was observed when the promoter was shortened to 150 bp of 5' proximal *K2.10* sequence.

Further analysis of the HK-1 sequence in gel shift assays showed that a protein (or proteins) present in hair follicle nuclear extracts binds specifically to this sequence. Finally, a series of overlapping 35-mer oligonucleotides spanning the 400 bp *K2.10* promoter region was tested in gel

shift assays and a number of specific complexes were shown to be formed, suggesting that the regulation of this promoter may be quite complex.

Declaration

This thesis has been submitted to the Faculty of Science at the University of Adelaide for examination for the degree of Doctor of Philosophy.

This thesis contains no material which has been accepted for the award of any other degree or diploma in any University. To the best of my knowledge, this thesis contains no material that has been previously published or written by another person, except where due reference is made in the text.

I consent to this thesis being made available for photocopying and loan as the University deems fitting, should the thesis be accepted for the award of the Degree.

Rebecca Keough

April, 1995

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Abbreviations

BCIP	5-bromo-4-chloro-3-indolyl-phosphate
bp	base pair
cDNA	complementary DNA
Chaps	3-[(3-chloramidopropyl)dimethylammonio]-propanesulfonate
CHO	Chinese hampster ovary
cRNA	complementary RNA (synthesised *in vitro*)
DNA	deoxyribonucleic acid
IF	intermediate filament
IRS	inner root sheath
KAP	keratin associated protein
kb	kilobase
kD	kilodalton
KRT, K	keratin
mAb	monoclonal antibody
mRNA	messenger RNA
NBT	4-nitro blue tetrazolium chloride
nt	nucleotide
oligo	oligonucleotide
ORS	outer root sheath
poly(A)	polyadenylic acid
RNA	ribonucleic acid
TAg	large tumour (T) antigen
Tricine	N-tris(hydoxymethyl)methylglycine
UTR	untranslated region
UV	ultraviolet

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Chapter One

Introduction

1.1 General Introduction

Wool production is of major economic significance in Australia and, as a consequence, research into the basic biology of the wool (hair) follicle has been encouraged in order to discover ways of improving fibre quality, characteristics and growth.

The hair follicle is a specialised structure which derives from the mammalian epidermis and provides an interesting model for the study of development, proliferation and differentiation. The work in our laboratory is directed at understanding the molecular mechanisms of follicle growth and development and the expression of the proteins which constitute the hair fibre.

Several of the genes for keratin intermediate filament (IF) proteins and keratin-associated proteins (KAPs) expressed in the hair follicle have been characterised (Huh *et al.*, 1994; Winter *et al.*, 1994 and for review see Powell and Rogers, 1994) however, little is known about the regulatory mechanisms controlling the expression of these genes during follicle differentiation. Studies of hair keratin gene regulation have been hampered to some extent by the lack of follicle keratinocyte cell lines. The use of cell lines has been a major tool in studying eukaryotic gene expression, providing a low cost, rapid and efficient system to complement mouse transgenesis approaches. In recent times, targeted oncogene expression in transgenic mice has been used very successfully to produce cell lines from a wide variety of tissues (for reviews see Hanahan, 1988; Adams and Cory, 1991). The use of SV-40 T antigen (TAg), in particular, has yielded cell lines which continue to express differentiation-specific markers (Paul *et al.*, 1988; Efrat *et al.*, 1988a, 1988b; Nakamura *et al.*, 1989; Bryce *et al.*, 1993).

The work presented in this thesis describes the attempt to produce an immortalised hair keratinocyte cell line using targeted expression of SV-40 TAg, and the preliminary analysis of the promoter region of a hair keratin IF gene, *K2.10*, using *in vitro* DNA-protein binding assays (gel mobility shift assays) and mouse transgenesis to define putative control elements involved in *K2.10* gene expression.

This chapter will give the background required for the evaluation of the experimental work presented in this thesis. It will describe the development and structure of the hair follicle and present a review of current work related to the control of keratin gene expression, the production of immortalised cell lines exhibiting differentiation-specific characteristics and the culture of intact hair follicles and cells derived from the follicle.

1.2 The hair follicle

1.2.1 Hair follicle morphogenesis

The hair follicle is a specialised appendage of the mammalian epidermis which initially forms during embryogenesis via a series of ectodermal-mesenchymal interactions. The ectoderm is an epithelial layer which gives rise to the epidermis and the hair follicle, whilst the dermis is derived from underlying mesoderm and contributes a specialised subpopulation of cells to the follicle which form the dermal papilla.

Hair follicle morphogenesis (see figure 1.1; for reviews see Serri and Cerimele, 1990; Holbrook, 1991; Hardy, 1992; Messenger, 1993) begins with a localised condensation of mesenchymal cells which interact with the adjacent ectoderm causing epithelial cells to proliferate and grow down into the dermis to form a hair plug. As this follicle plug grows it is thought to signal the aggregated mesenchymal cells to form a dermal papilla, a compact population of specialised dermal cells surrounded by the developing hair peg. The dermal papilla then stimulates the adjacent epithelial cells of the hair plug, termed hair follicle bulb cells, to divide. The cells of the hair bulb are one of the most actively proliferating populations in the body, and as they divide they form a cone of cells which moves up through the hair peg toward the skin surface. These cells subsequently differentiate into the various cell types which compose the mature follicle.

Upon formation, the hair follicle is in an active growth phase (anagen) during which time the hair follicle bulb cells continue to proliferate rapidly and the hair fibre lengthens. After a period of growth, follicles enter catagen, a phase in which cell division and hair growth ceases, the lower follicle regresses and a club is formed at the base of the fibre. The fibre remains anchored in the follicle during the subsequent resting phase, termed telogen. Eventually, the follicle will re-enter anagen and a new hair fibre will be produced. This cycle of induction, morphogenesis, differentiation and regression (i.e. the hair cycle; Dry, 1926) continues throughout the life of the animal (see figure 1.2).

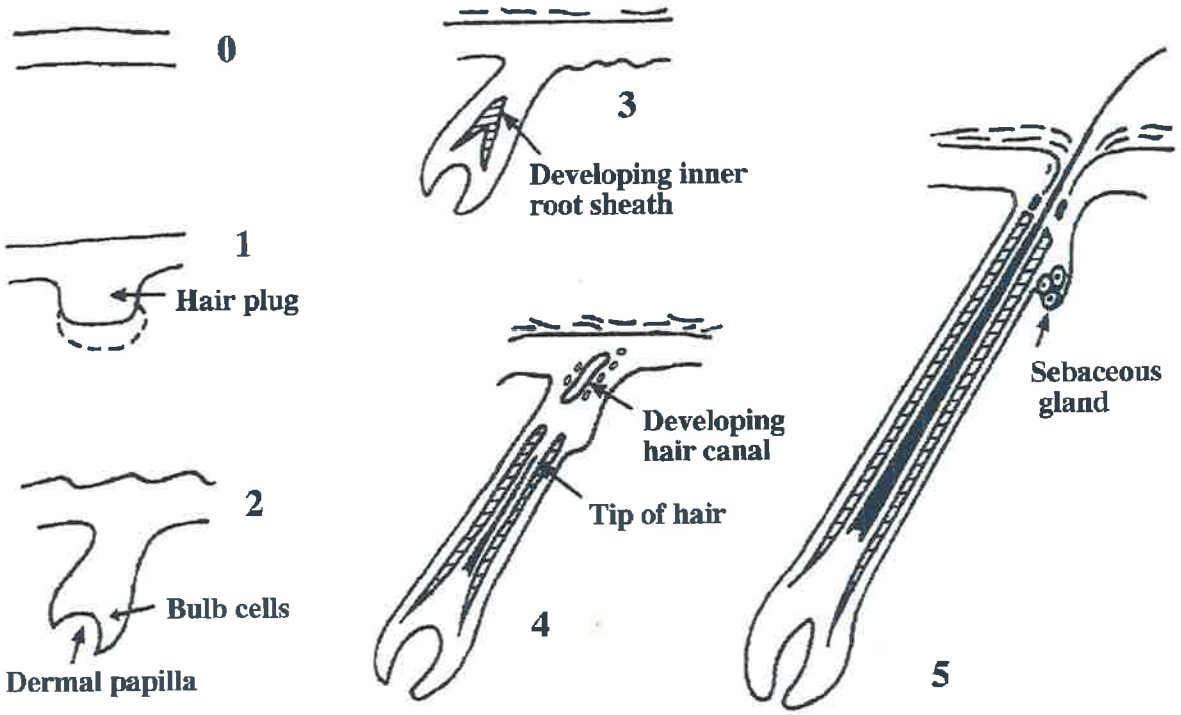


Figure 1.1 Hair follicle morphogenesis.

Beneath the developing epidermis (stage 0), a dermal condensation forms (represented as a dashed line) and there is a downgrowth of epithelial cells (stage 1). The epithelial hair peg surrounds the dermal papilla (stage 2) and the hair follicle bulb cells proliferate and differentiate (stage 3). The hair and hair canal develop (stage 4). The sebaceous gland is formed and the fully keratinised hair fibre emerges (stage 5). Modified from Hardy (1992).

The length of each phase of the hair cycle differs between mammals and between different hair types, such as pelage and vibrissa hairs. In mice, the anagen period for pelage follicles is typically 14-17 days (Dry, 1926) followed by catagen for 1-2 days. Follicles then enter telogen which continues for approximately 10 days. This contrasts with vibrissa follicles which undergo little follicle regression during catagen and experience a very short telogen phase (Young and Oliver, 1976). The hair cycle in mice is also synchronised such that waves of new hair growth occur across the body of the mouse (Dry, 1926; Chase and Eaton, 1959), unlike the random pattern of active hair follicles interspersed with telogen follicles characteristic of human hair growth and sheep wool growth.

Induction of a new follicle after telogen requires the re-establishment of the lower part of the follicle, including a new population of follicle bulb cells. It has been proposed that stem cells, which are thought to reside in the follicle bulge of the outer root sheath (ORS) (Cotsarelis *et al.*, 1990) , are stimulated to proliferate at the start of anagen (Wilson *et al.*, 1994) and form a down-growth of epithelial cells, surrounding the dermal papilla and forming the new follicle bulb. The stem cells remaining in the bulge region then return to their normal slow-cycling state until the next hair cycle. The cells of the follicle bulb form the "transiently amplifying" cell population and proliferate and then terminally differentiate once their proliferative potential has been exhausted (Cotsarelis *et al.*, 1990). A group of cells, termed germinative epithelial cells (Reynolds and Jahoda, 1991), located in the follicle bulb at the base of the dermal papilla (see 1.6.2) are also thought to exhibit classical stem cell properties. They appear to be undifferentiated and are proposed to give rise to the "transiently amplifying" cells of the follicle bulb (Reynolds and Jahoda, 1991). However, isolated germinative epithelial cells are stimulated to proliferate when grown over dermal papilla fibroblasts in culture (Reynolds and Jahoda, 1991), contradicting the definition of stem cells as "slow cycling" cells, although the culture system may not reflect the *in vivo* situation or may correspond to the induction of stem cell division at the start of anagen.

As yet, neither the ORS cells of the bulge, nor the germinative epithelial cells, have been conclusively shown to be the stem cells for follicle regeneration. It is possible that the cells of the bulge move down and form the germinative epithelial cell population at the beginning of each anagen and the germinative epithelial cells then contribute to the "transiently amplifying" bulb cell population. However, retraction of the dermal papilla with follicle regression during catagen is also thought to be critical for the "bulge cell activation hypothesis" of Cotsarelis *et al.* (1990) in which the dermal papilla cells are brought in to close proximity to the bulge region and activate the stem cells to

proliferate and move down to form the new follicle. In vibrissa follicles slow cycling cells have been identified at a site analogous to the bulge in pelage follicles (Lavker *et al.*, 1991) but there is virtually no movement of the dermal papilla during the hair cycle (Young and Oliver, 1976). This apparent anomaly in the "bulge activation hypothesis" between mouse pelage and vibrissal follicles is yet to be resolved.

Dermal-epidermal interactions are not only important for follicle formation during embryogenesis, but continue to play a vital role throughout the hair cycle. The production of hair follicles by normally hairless epithelia into which dermal papillae have been transplanted (Reynolds and Jahoda, 1992) demonstrates the inductive capabilities of the papilla. It has also been shown that hair growth only occurs in the presence of a dermal papilla (for a review see Jahoda and Oliver, 1990) and that the dermal papilla is responsible for determining the type and size of the follicle produced (Kollar, 1966; Oliver, 1970; Ibrahim and Wright, 1982).

Whilst little is known about the mechanisms which regulate hair growth and the hair cycle, an increasing number of growth factors have been implicated. Recent gene knockout of fibroblast growth factor 5 *(Fgf5)* produced mice in which the progression of the follicle from anagen to catagen is delayed, producing abnormally long hairs (Hebert *et al.*, 1994). This mutation corresponds to the naturally occurring angora *(go)* mouse mutation. FGF5 is expressed in the ORS of the lower third of the follicle during the final stage of anagen and, thus, appears to function as an inhibitor of hair elongation prior to the onset of catagen (Hebert *et al.*, 1994). The vitamin D_3 steroid hormone nuclear receptor (VDR) also appears to undergo changes in expression during the hair cycle. Antibody staining for VDR detected expression in the ORS and dermal papilla which increased during mid to late anagen and catagen and decreased in telogen and early anagen (Reichrath *et al.*, 1994). Given the modulatory effects of vitamin D_3 on keratinocyte proliferation and differentiation (Tomic *et al.*, 1992; Bikle and Pillai, 1993) it is possible that it plays a role in regulating the hair cycle.

Transforming growth factor-α (TGFα) is another molecule which appears to play a role in hair follicle formation, influencing the proper positioning of follicles (Luetteke *et al.*, 1993; Mann *et al.*, 1993). TGFα knockout mice have irregularly distributed follicles which emerge at various angles from the skin. Knockout of the *TGFα* gene corresponds to the naturally occurring waved-1 (*wa-1*) mutation in mice, whilst mutations in the epidermal growth factor/TGFα receptor produce a similar phenotype, apparently equivalent to the spontaneous mutation termed waved-2 (*wa-2*) (Luetteke *et al.*, 1994). There is also a large number of other spontaneous mouse mutations which affect hair

growth and the hair cycle (for a review see Sundberg, 1994). For example, the fuzzy (*fz*) mutation results in hair shaft abnormalities associated with abnormalities of dermal papilla in anagen follicles; the hairless (*hr*) mutation prevents upward movement of the dermal papilla upon follicle regression during catagen of the first hair cycle and results in no subsequent hair cycles; and the soft coat (*soc*) mutation produces abnormally short, squat and rounded follicle bulbs. Molecular characterisation of these and numerous other mutations should provide further insight into the control of the hair cycle and follicle growth.

1.2.2 Structure of the hair follicle

1.2.2.1 Follicle cell development

The hair follicle is a complex and dynamic structure composed of several different cell types which form the various layers of ORS and inner root sheath (IRS) and the medullary, cortical and surrounding cuticle layers of the hair shaft (see figure 1.3). As the cells of the follicle bulb divide they move up the follicle and rapidly differentiate into inner root sheath, cuticle, cortical and medullary keratinocytes. The follicle has been divided into a number of zones of differentiation (Orwin, 1976, 1979) which are summarised here and depicted in figure 1.3.

The zone of proliferation depicts the rapidly dividing, relatively undifferentiated follicle bulb cells and covers the area from the base of the follicle to the top of the dermal papilla. Above the dermal papilla, cells begin to differentiate into the different cell types of the fibre which is characterised by changes in cell shape and the initiation of keratin synthesis in cortical cells. In the keratogenous zone cells are fully differentiated and undergo a period of major protein synthesis. At this stage the innermost layer of the inner root sheath hardens. Once the cells become packed with protein (in the zone of keratinisation), the proteins are cross-linked, organelles and nuclei are degraded and the cells dehydrate. Beyond this region the hardened inner root sheath is sloughed off and the fully keratinised fibre emerges.

Very little is known about the control of follicle cell differentiation or the determination of cell fate. However, with the localisation of expression in the follicle of various genes known to be involved in other developmental and differentiation pathways, such as *Notch* (Kopan and Weintraub, 1993), *E2A* (Roberts *et al.*, 1993), *BMP-2* (Lyons *et al.*, 1990), *Fgf5* (Hebert *et al.*, 1994) and *BMP-4* (Jones *et al.*, 1991), these mechanisms may soon be elucidated.

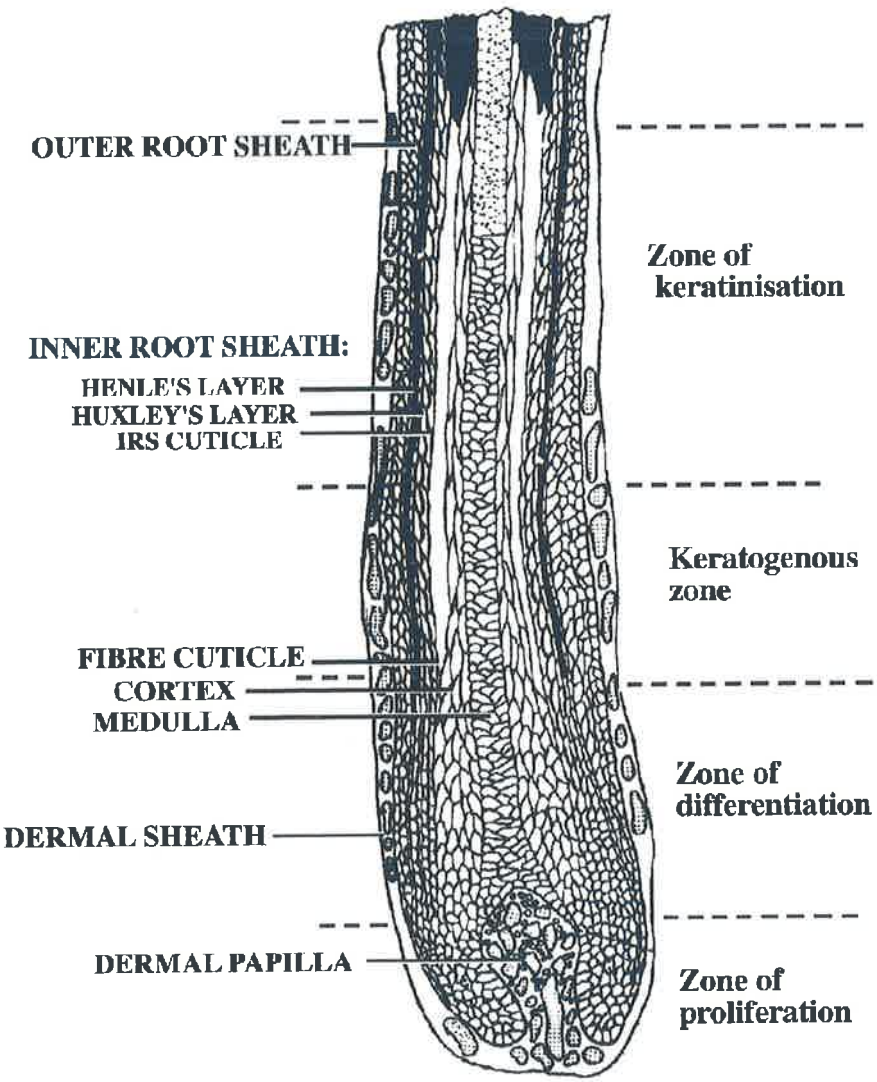


Figure 1.3 Schematic longitudinal section through a medullated follicle.
The cell layers of the follicle are labelled and the zones of differentiation are indicated by dashed lines. (See text for explanation). Modified from Orwin (1979).

1.2.2.2 Follicle cell layers

With the exception of the ORS, all of the cell layers of the follicle are derived from follicle bulb cells. The ORS layer is continuous with, but distinct from, the basal layer of the epidermis. It is composed of several distinguishable cell layers with the innermost layer, known as the companion layer (Orwin, 1971), more closely associated with the IRS than the ORS, such that it moves up the follicle with the IRS as the fibre grows. The cells of the companion layer degrade and slough off at the neck of the follicle in a similar manner to those of the hardened IRS.

The IRS consists of three distinct cell layers: Henle's layer, the outermost layer which is closely associated with the ORS, Huxley's layer and the IRS cuticle, the latter being associated with the fibre cuticle. The earliest marker of follicle bulb cell differentiation is seen in developing IRS cells with the expression of trichohyalin (Rothnagel and Rogers, 1986; Fietz *et al.*, 1990) which initially forms amorphous granules in the cells. As the cells differentiate further, filaments form, the granules disappear and the cellular proteins become highly cross-linked, resulting in a hardening of the IRS (Birbeck and Mercer, 1957; Rogers, 1964). The precise role that the IRS plays in follicle development is poorly understood, however, it is known that disruption of the IRS affects fibre growth (Konyukhov and Kupriyanov, 1990; Crish *et al.*, 1993; Murthy *et al.*, 1993).

The hair itself is composed of a protective cuticular layer, the hair cortex and, in many fibres, a central medulla (see figure 1.3). The cortex is the major constituent of the hair fibre and is responsible for many of the physical properties of the hair such as strength, elasticity and waviness. Cortical cells are produced from the bulb cells that laterally surround the dermal papilla and which elongate to form a characteristic spindle shape as they differentiate. Hardened cortical cells are packed with keratin IF which form filaments of 8-10 nm in diameter and are aligned parallel with the direction of hair growth. The filaments become embedded in a proteinaceous matrix of keratin IF-associated proteins (KAP) which is strengthened by disulphide bond crosslinkages (Chapman and Gemmell, 1971). The surrounding cells of the hair cuticle, however, contain few filaments. Rather, aggregates of protein are distributed along the outer edge of the cells and coalesce in the final stages of differentiation to form the hardened exocuticle. The inner region of the cell is termed the endocuticle and consists of cytoplasmic debris (for reviews see Orwin, 1979; Powell and Rogers, 1986).

The medulla is composed of arrays of regularly packed cells and air spaces and forms the central core of the fibre. It develops from cells positioned over the tip of the dermal papilla and the

formation of large trichohyalin-containing granules accompanies cell differentiation. However, unlike the situation in the IRS, the granules in the medulla persist and eventually harden.

1.3 Proteins of the hair cortex

X-ray and electron microscopic studies (Rogers, 1959a; Fraser *et al.*, 1972) of the hair fibre have shown that cortical cells contain an ordered array of closely packed filaments associated with interfilamentous protein. The filaments are assembled during cell differentiation from 2 families of keratin IF proteins and are crosslinked with an interfilamentous matrix of keratin-associated proteins (KAP) from at least 7 different protein families (Powell *et al.*, 1991). It is estimated that some 50 or more different keratin and KAP proteins are present in a fully differentiated cortical cell (Powell *et al.*, 1992; Powell and Rogers, 1994).

1.3.1 Keratin IF proteins

Keratin IF proteins form a large family of proteins within the diverse IF superfamily (for review see Steinert and Roop, 1988). All epithelial cells contain keratin as a component of their cytoskeleton to provide structural integrity to the epithelia, and specialised structures such as hair, nail and hoof contain an abundance of crosslinked keratin which forms the hardened tissue.

Keratin IFs exhibit the characteristic IF structure consisting of a central conserved α-helical rod domain which is flanked by non-helical amino- and carboxy- terminal domains (Steinert and Roop, 1988). Approximately 30 different keratin IF proteins have been identified and these are divided into 2 families, type I and type II, based on size and charge (Moll *et al.*, 1982). Type I keratins are acidic and range in size from 40-56 kD whilst type II keratins are larger, ranging from 50-70 kD, and have a basic or neutral charge.

Keratin filaments are comprised of heterodimers consisting of one type I and one type II protein. Each member of the type I family has a particular type II partner with which it is normally co-expressed and with which it dimerises (Sun *et al.*, 1984). The expression pattern of these keratin pairs can be correlated with specific programmes of differentiation (Sun *et al.*, 1984).

Five pairs of hair keratin IF have been isolated from the follicle (Crewther *et al.*, 1980; Heid *et al.*, 1986). Four of these pairs are expressed in the hair follicle cortex (Powell *et al.*, 1992; B. Powell, personal communication), whilst the minor component may be expressed in the hair cuticle

(Winter *et al.*, 1994). These and other hair-like keratins (Heid *et al.*, 1988b; Tobiasch *et al.*, 1992; Kitahara and Ogawa, 1993) comprise a special subset of keratin IF, often known as "hard" keratins, based on distinct amino acid sequence similarities and a higher cysteine content compared with "soft" keratin IF (Conway and Parry, 1988). Expression studies (Powell *et al.*, 1992) of hair keratin type II genes indicate that these pairs may be expressed at different stages of cortical cell differentiation (see 1.4).

1.3.2 Keratin-associated proteins

The small keratin-associated proteins which form the interfilamentous matrix surrounding the IF in cortical cells comprise at least 7 different families, most with multiple members. These proteins can be broadly classified into two types; the cysteine-rich KAP families (KAP 1-4 and KAP 9) and the glycine/tyrosine-rich KAP families (KAP 6-8) (for review see Powell and Rogers, 1994). The cysteine-rich keratins range in size from 11 kD to approximately 23 kD and contain 16-37 mol% cysteine. Within families, proteins are highly related and often contain characteristic repeated sequences (Swart *et al.*, 1976; MacKinnon *et al.*, 1990; Rogers *et al.*, 1991). The smaller glycine/tyrosine-rich proteins (6-9 kD) contain between 43-65 mol% of glycine and tyrosine. The KAP 6 family contains multiple members, possibly up to 20 different proteins in the mouse (Fratini *et al.*, 1994), whilst the KAP7 and KAP 8 proteins appear to be unique (Powell and Rogers, 1994).

It is known that differential expression of various KAP proteins results in differences in the ultrastructure of cortical cells (Chapman and Gemmell, 1971; Chapman, 1976), and that changes in the normal keratin protein profile of a cortical cell disrupt the integrity of the hair (Powell and Rogers, 1990; Keough *et al.*, 1995). However, the precise protein interactions between KAP and IF and between the KAPs themselves are unknown, although a high degree of disulphide crosslinking is predicted. It has also been suggested that the glycine/tyrosine-rich proteins may interact with IF via glycine loops (Fratini *et al.*, 1993). The function of different, highly similar members of the KAP families expressed within the same cell is also unknown.

1.4 Keratin gene expression in the hair cortex

It is generally recognised that the expression of hair keratin IF is the earliest marker of a differentiating cortical cell. Immunolocalisation studies using antibodies that detect hair keratin IF (French and Hewish, 1986; Lynch *et al.*, 1986; Heid *et al.*, 1988a) show staining in the

keratinocytes around the apex of the dermal papilla and in the cells above (although weak staining is also observed in the follicle bulb). *In situ* hybridisation studies using riboprobes to localise hair IF mRNAs (Kopan and Fuchs, 1989; Powell *et al.*, 1992; Tobiasch *et al.*, 1992) also indicate that hair-specific IF expression is first detectable in the upper region of the follicle bulb around the tip of the dermal papilla and continues into the keratinised region of the follicle cortex.

Differentiation of cortical cells is accompanied by the activation of a large number (approximately 50 or so) of hair-specific keratin IF and KAP genes. The expression patterns of these gene families has been extensively mapped in the wool follicle (Powell *et al.*, 1992; Powell and Rogers, 1994) and has shown a complex, coordinated pattern of expression throughout cortical cell differentiation. A brief description of cortical keratin gene expression data will be summarised here and in figure 1.4 (see also Powell and Rogers, 1994).

As previously mentioned, 4 pairs of keratin IF genes are thought to be expressed in the follicle cortex. Mapping of the expression of the type II genes has shown that the genes are activated sequentially. *K2.12* is transcribed first in the cells around the tip of the dermal papilla (B. Powell, personal communication; see figure 4.4), followed by *K2.9* and *K2.10* expression in cells above the dermal papilla (Powell *et al.*, 1992; Keough *et al.*, 1995) and lastly, *K2.11* is activated at a slightly later stage (Powell *et al.*, 1992). It is predicted that type I IF genes are similarly differentially expressed (Powell *et al.*, 1992).

Expression of the KAP genes has been determined using general probes which detect multiple members within a gene family. In Merino wool follicles 2 types of cortical cells, ortho- and para-cortical cells, are distributed in a bi-lateral arrangement (Rogers, 1959b) and differential gene expression in each cortical cell type is clearly distinguishable (Fratini *et al.*, 1993, 1994). In other follicle types ortho- and para-cortical cells are distributed differently (Fraser and Rogers, 1955; Orwin *et al.*, 1984).

KAP genes are activated once IF gene expression has commenced. The genes encoding the glycine/tyrosine-rich KAPs (KAP 6-8) are first expressed in cells of one half of the cortex in Merino follicles (Fratini *et al.*, 1993). The cysteine-rich KAP genes are subsequently activated in cells of the other half of the cortex. As the cells continue to differentiate, both glycine/tyrosine-rich and cysteine-rich KAP genes are expressed in all cortical cells, with the exception of the *KAP4* genes which remain confined to one cell type (Fratini *et al.*, 1994).

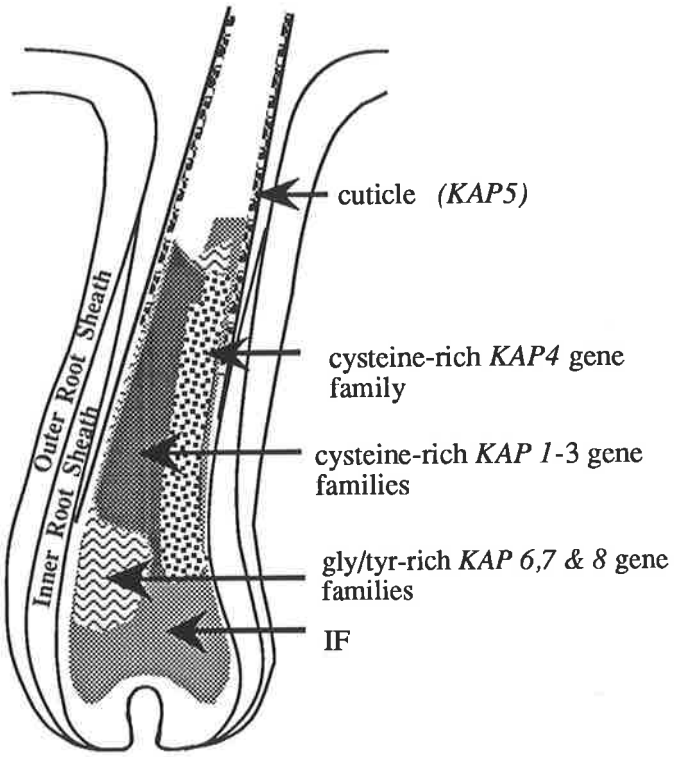


Figure 1.4 Sequential expression of keratin genes in wool follicle differentiation.

A schematic summary showing the overlapping pattern of keratin gene expression. Adapted from Powell *et al.* (1991).

The expression of hair keratin IF and KAP genes is restricted to the hair follicle in the epidermis. Expression of a hair keratin type I gene, *mHa2* , has recently been localised to the hair cuticle (Winter *et al.*, 1994) and *KAP5* genes are expressed in this cell layer as well (MacKinnon *et al.*, 1990; Jenkins and Powell, 1994). However, hair keratin IF (or hair-related keratins) are also expressed in other hardened epidermal appendages such as nail and hoof (Lynch *et al.*, 1986; Moll *et al.*, 1988a; Heid *et al.*, 1988b; Kitahara and Ogawa, 1993) and in subpopulations of cells in the tongue (Dhouailly *et al.*, 1989; Tobiasch *et al.*, 1992; Winter *et al.*, 1994).

1.5 Regulation of keratin gene expression

1.5.1 Regulation of keratin IF gene expression

Keratin IF are the predominant intermediate filaments in keratinocytes and other epithelial cells and at least 30 different epithelial keratin IF have been described. The distribution of these keratins is generally restricted to specific epithelial cell types as expression is linked to various differentiation programmes or disease states (for review see Oshima, 1992).

The regulation of keratin *IF* gene expression has been widely studied and factors such as calcium levels, retinoids and vitamin D_3 have been shown to regulate the expression of a number of these genes (Yuspa *et al.*, 1989; Stellmach *et al.*, 1991; Tomic *et al.*, 1992; Tomic-Canic *et al.*, 1992). Specific elements which mediate these responses have recently been identified in the human *K1* gene (Lu *et al.*, 1994). The calcium responsive element was shown to be composed of an AP-1 binding site and a steroid hormone receptor binding element through which retinoic acid and vitamin D_3 can modulate the effect of AP-1 binding. An AP-1 binding site has also been found in the 3' enhancer of human *K19* (Hu and Gudas, 1994) and in the intron enhancer of *K18* (Oshima *et al.*, 1990).

Another factor which has been shown to be important for epidermal keratin gene expression (and possibly the expression of other epidermal genes (Behrens *et al.*, 1991; Tamai *et al.*, 1994) is AP-2 (Leask *et al.*, 1991; Snape *et al.*, 1991; Byrne and Fuchs, 1993). AP-2 alone, however, is not sufficient to confer keratinocyte specificity (Leask *et al.*, 1991). Whilst both AP-1 and AP-2 are considered to be general transcription factors which act on a wide range of genes in a variety of tissues (Rittling *et al.*, 1989; Ney *et al.*, 1990; Jarman *et al.*, 1991; Mitchell *et al.*, 1991; Park *et al.*, 1992), they appear to play an important role in epidermal gene expression and may be regulated by other factors (for example, steroid hormone receptors) which confer specificity of expression.

Two other transcriptional regulators have been identified which are selectively expressed in terminally differentiating suprabasal epidermal cells and hair follicle cortical cells, Skin-1a (Skn-1a) and Skin-1i (Skn-1i) (Andersen *et al.*, 1993). These factors belong to the POU domain family of transcriptional regulators, and are most highly related to Oct-2, a transcription factor believed to be important in the terminal differentiation of B lymphocytes (Clerc *et al.*, 1988). Skn-1a and Skn-1i appear to be encoded by the same gene, differing only in the amino terminal domain as a consequence of alternative splicing. Skn-1i mRNA is most abundant in the post-mitotic cells of the epidermis and appears to act by inhibiting the activity of Oct-1, a POU domain transcription factor believed to be important in cellular proliferation events (Andersen *et al.*, 1993). Unlike Skn-1i, Skn-1a is a DNA-binding protein and has been shown to activate transcription from the human *K10* promoter in HeLa cells (Andersen *et al.*, 1993). Thus, the tissue-restricted expression of the *Skn-1a/i* gene and the effect of these factors on epidermal gene expression suggest that they play regulatory roles with respect to epidermal and follicle cortical cell differentiation.

Perhaps the most well characterised keratin promoters are those of the human *K14* gene and its complementary type II partner, *K5*. *K14* and *K5* are expressed in basal cells of all stratified epithelia, and notably in basal epidermal keratinocytes and keratinocytes of the hair follicle ORS. Several regulatory elements and their transcription factors have been identified in the *K14* promoter; these include a functional AP-2 site (Leask *et al.*, 1990, 1991) and a cluster of retinoic acid receptor and thyroid hormone receptor binding half-sites (Tomic-Canic *et al.*, 1992) in the proximal promoter. Neither of these elements confers tissue-specificity of expression on the promoter, however, evidence suggests that a distal element (located between -1700 and -2100 from the transcription start site) acts together with AP-2 to confer tissue-specific expression (Leask *et al.*, 1990). A binding site for the transcription factor Sp1 has also been identified in the proximal promoter by *in vitro* gel mobility shift analysis (Byrne and Fuchs, 1993). Whilst Sp1 is a ubiquitous factor, there is increasing evidence that Sp1 expression levels vary during development and differentiation in a tissue-specific manner (Saffer *et al.*, 1991; Minie *et al.*, 1992; Robidoux *et al.*, 1992). An Sp1 homologue, SPR-1, also exhibits elevated expression levels in a tissue-specific pattern (Hagen *et al.*, 1992).

Analysis of the human *K5* promoter has shown that a 90 bp region of the proximal promoter was sufficient to direct gene expression to stratified epithelia including epidermis, hair follicles and tongue, although the specificity of expression was altered from basal cells to suprabasal cells and from ORS to IRS in the hair follicle (Byrne and Fuchs, 1993). *In vitro* gel mobility shift analysis of

the *K5* proximal promoter has identified binding sites for Sp1 and AP-2, as well as the binding site GTTCCTGGGTAAC which is similar to the consensus sequence for several POU-homeodomain transcription factors (Byrne and Fuchs, 1993). The *K5* promoter also contains a putative thyroid hormone receptor and retinoic acid receptor binding half-site cluster, analogous to that found in the *K14* gene (Tomic-Canic *et al.*, 1992) and an AP-1 site has been identified in an enhancer region between -762 and -1009 of the bovine *K5* gene which may have epithelial-specific activity (Casatorres *et al.*, 1994). Thus, it appears that the regulation of *K5* (and possibly *K14*) may be highly complex with a number of factors, both positive and negative regulators, being required to confer correct expression.

Elements determining the tissue-specificity of keratin expression have yet to be identified. However, a prime candidate for this role in epidermal keratinocytes may be the "CK8-mer" motif (AAPuCCAAA) which is present in various human, bovine and mouse keratin genes (Blessing *et al.*, 1987) , the human involucrin gene (Eckert and Green, 1986) and the bullous pemphigoid antigen gene (Tamai *et al.*, 1994).

1.5.2 Regulation of hair keratin gene expression

The complex pattern of hair keratin gene expression during cortical cell development suggests that a hierarchy of gene expression is set up in which the timing and location of transcriptional activation is precisely controlled. However, apart from keratin gene expression determined by the terminal differentiation programme, other factors, such as nutrition, can influence gene expression. It has been shown that the expression of the cysteine-rich *KAP4* genes in the wool follicle is markedly increased upon increasing dietary cysteine, and furthermore, this increase is rapid and is apparent at the level of mRNA specific for these genes (Fratini *et al.*, 1994). It is not known whether this effect is due to an increase in gene transcription or mRNA stabilisation, however, it adds to the complexity of keratin gene regulation in the follicle.

To date, little is known about the control of hair keratin gene expression. Several keratin gene fragments containing variable lengths of flanking sequence have mimicked endogenous gene expression when used to generate transgenic mice (McNab *et al.*, 1990; Powell and Rogers, 1990; Powell *et al.*, 1991; Keough *et al.*, 1995), indicating that the controlling elements lie within these gene fragments. The faithful expression of sheep keratin genes in mice also suggests that the regulatory mechanisms are highly conserved between mammals.

No regulatory elements or transcription factors have yet been identified which indicate how these genes are activated or what determines tissue-specific and differentiation-specific expression. However, sequence analysis of the promoter regions of a number of keratin genes has enabled the identification of putative binding sites for known transcription factors (Kuczek and Rogers, 1987; Wilson *et al.*, 1988; Kaytes *et al.*, 1991; Powell *et al.*, 1992; Fratini *et al.*, 1993) and comparison of hair keratin gene promoters has defined a number of conserved sequences which may be important in gene regulation (Powell *et al.*, 1991, 1992).

Consensus sequences for AP-1 and AP-2 transcription factor binding sites have been discovered in the promoter regions of several type II hair keratin genes (Powell *et al.*, 1992) and a *KAP6* gene (Fratini *et al.*, 1993). Both AP-1 and AP-2 sites are found in epidermal keratin IF genes (Oshima *et al.*, 1990; Snape *et al.*, 1990; Leask *et al.*, 1991; Magnaldo *et al.*, 1993; Hu and Gudas, 1994; Lu *et al.*, 1994) where they have been shown to function in gene expression. Another element present in epidermal keratin genes is the "CK8-mer" motif (Blessing *et al.*, 1987) which is also found in hair keratin *IF* genes (Powell *et al.*, 1992). Whether these sites are functional in hair keratin genes is yet to be determined, however, localisation of *c-fos* expression to the follicle (Fisher *et al.*, 1991) strengthens the possibility that AP-1 may be involved.

The SV-40 core enhancer sequence has been found in glycine/tyrosine-rich KAP gene promoters (Kuczek and Rogers, 1987; Fratini *et al.*, 1993) and an inverted core enhancer sequence is present in two type I hair keratin gene promoters and is conserved between mouse and sheep genes (Kaytes *et al.*, 1991). The SV-40 core enhancer, NTGTGG(a/t)(a/t)AG (Sassone-Corsi and Bourelli, 1986), binds the ubiquitously expressed TEF-1 transcription factor (Xiao *et al.*, 1991) which has also been implicated in tissue-specific gene expression in muscle upon interaction with a muscle-specific factor (Farrance *et al.*, 1992; Shimizu *et al.*, 1993).

Comparison of the promoter regions of the numerous hair keratin genes sequenced from each of the different gene families has identified a number of conserved sequences which may be considered as potential regulatory elements (Powell *et al.*, 1991 and see table 1.1). Eight previously unidentified motifs were found from a comparison of 15 hair keratin genes (Powell *et al.*, 1991). These motifs include a putative cuticle-specific element (CU-1), 2 perfectly conserved elements found in all glycine/tyrosine-rich genes sequenced to date (HGT-1, HGT-2) and 3 sequences which are found in many of the cysteine-rich KAP genes. Another conserved motif, termed HK-1, is found in the promoter region of all hair keratin IF genes sequenced to date and also in 3 different KAP genes (Kaytes *et al.*, 1991; Powell *et al.*, 1991). This palindromic element is located 180-240

Table 1.1 Possible regulatory motifs involved in hair keratin gene expression[a]

Gene	HK-1 (CTTTGAAG)	HGT-1 (TCAGTTT)	HGT-2 (TAATCAGA)	HS-1 (CCAAAGGCAAAG)	HS-2 (ACAAAAGCAGGA)	HS-3 (AAAAATGCT)	UHS-1 (ACAAGGAAA)	CU-1 (CAGGAGGAAGG)
sK1.2	✓							✓(10/11)
mHKA1[b]	✓							
sK2.9	✓							
sK2.10	2✓(7/8)			✓(10/12)				
sKAP7		✓	✓					
sKAP8		✓	✓				✓(7/9)	
rKAP6.1		✓	2✓				✓(8/9)	
sKAP1.1				✓	✓(11/12)	2✓(8/9)		
sKAP1.3				✓(10/12)	✓(11/12)	3✓(2 x 8/9)		
sKAP1.4	✓(7/8)			✓(11/12)	✓(11/12)	✓(8/9)	✓8/9)	
sKAP2.9								
sKAP3.4					✓(9/12)			
rKAP4L							✓	
mKAP9.1					✓(9/12)		✓	2✓(9/11)(10/11)
hKAP5.1							✓	✓
sKAP5.1					✓(9/12)			2✓(10/11)

[a]Where a motif is identified (✓) the preceeding number indicated two or more copies, and the numbers in parentheses show the match to the consensus if there are differences. Thus, (8/11) indicates that 8 nucleotides match the 11-nucleotide consensus. The arbitrary cut-off for inclusion in this table was a maximum mismatch of 3 bases on the larger consensus sequences. s, sheep; m, mouse; r, rabbit; h, human.

[b]Kaytes *et al.* (1991).

Adapted from Powell *et al.* (1991).

bp upstream of the transcription initiation site and is present in sheep and mouse genes (table 1.2). A hair-related sheep IF gene, *K2.13* (Powell *et al.*, 1993) which is located within the hair keratin type II gene cluster (Powell and Beltrame, 1994) and is not expressed in the hair follicle lacks the HK-1 element found in the hair-specific genes.

1.5.2.1 Regulation of the hair keratin type II *K2.10* gene

Sequence analysis of the 5' proximal promoter of the sheep *K2.10* gene has revealed a number of conserved sequences and putative regulatory elements (see figure 1.5; Powell *et al.*, 1991, 1992). These include two CK8-mer elements (Blessing *et al.*, 1987), two HK-1 elements and AP-1 and AP-2 binding sites. Most of these elements are also found in the co-expressed *K2.9* gene, frequently within longer conserved regions (figure 1.5). It is interesting to note that the AP-2 site at approximately -150 (relative to the transcription start site at +1) is more similar to the AP-2 binding site discovered in epidermal keratin genes (Snape *et al.*, 1990) than it is to the AP-2 consensus sequence, CCc/gCc/gGGC (Williams and Tjian, 1991).

The function of these putative control elements has not yet been established. This is due, in part, to the lack of efficient systems for the analysis of hair-specific gene expression such as suitable cell lines. The work presented in this thesis addresses these problems.

1.6 Follicle cell culture systems

1.6.1 Culture of hair follicles as intact organoids

The maintenance and growth of intact hair follicles in culture provides an important *in vitro* system for the study of the mechanisms involved in hair growth and the effect of various drugs and growth factors on the follicle.

Several different methods for the maintenance of hair follicles in culture have been described. These include the use of bovine eye lens capsule as a basement membrane-like substrate for follicles (Weterings *et al.*, 1981), the use of a collagen matrix to maintain follicle morphology (Rogers *et al.*, 1987) and the culture of small skin biopsies containing hair follicles (Holbrook and Minami, 1991). However, these techniques for culturing intact hair follicles have met with limited success. One reason for this appears to be the method of follicle isolation. Evidence suggests that the follicle is particularly sensitive to physical damage or trauma (Philpott *et al.*, 1989; Kondo *et al.*, 1990) which occurs by plucking (Ludwig, 1967), and enzymatic release of follicles from dermis using

Table 1.2 Comparison of HK-1 motifs in hair keratin genes

sheep	*K2.10*	-180	GTAAACGGCTTTGAAGATGAAACAGGCCTGAGGCC
sheep	*K2.10*	-676	CCTAAGAACTCTGAAGAACAACCTTGGTTCTCTCC
sheep	*K2.9*	-210	GCAAGGCACTTTGAAGATGAAACATGCTGAGTCCC
sheep	*K1.2*	-230	GTCCAGCCCTTTGAAGCCAACCATGAAGCTCTTAT
mouse	*HKA1*	-230	CGTGACTTCTTTGAAGGGCCACCGCAGAACTCTGT
sheep	*K2.11*	-267	AGCTGGCCCTTTGAGGACAAAAATTGTGCAGTTTA
sheep	*KAP1.4*	-240	GGAGGGCTCTTTGAACATGGAAAACCACAAACCTA
sheep	*KAP7*	-200	GGCGCTGACTTTGAAGATACAACACCATTCCTTTG
sheep	*KAP8*	-230	ACCCAGGGCTTTGAAGACTCTGCAAAGCGGTATTT
consensus sequence			CTTTGAAG

Adapted from Powell *et al.* (1992)

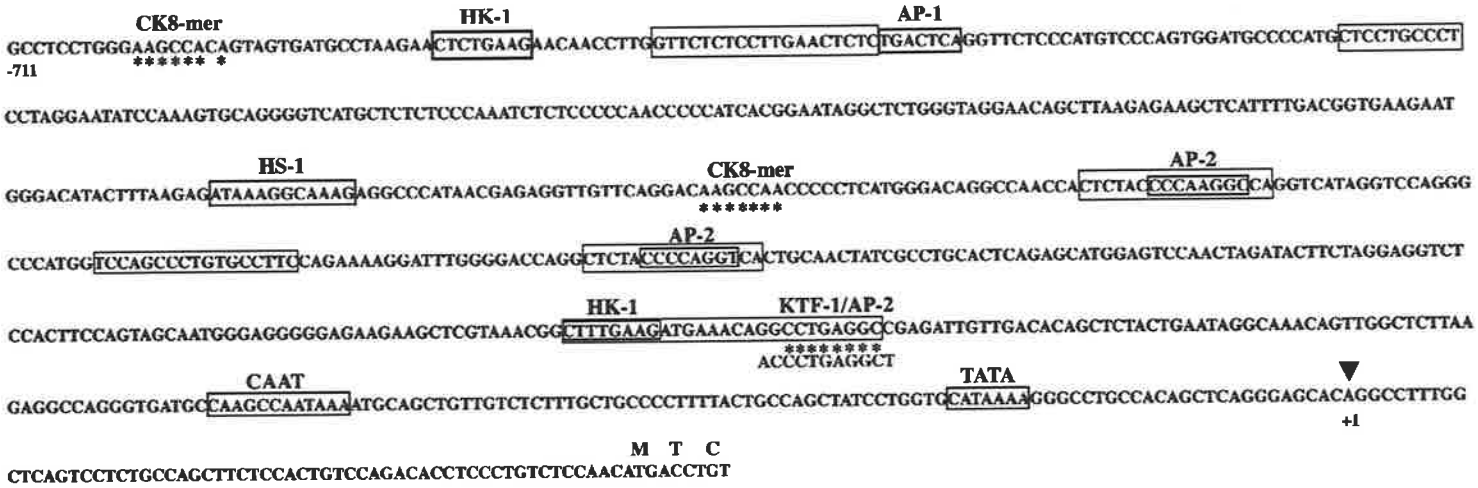


Figure 1.5 Putative regulatory motifs in the promoter of the sheep *K2.10* gene.

A number of conserved DNA sequence motifs have been identified within 700 bp of the transcriptional start site (+1) of the gene by comparison with other hair keratin genes and consensus binding sites of known transcription factors. The HK-1 and HS-1 motifs were identified in other hair keratin genes (see table 1.1) and the unlabelled boxes represent homologous sequences conserved between the sheep *K2.9* and *K2.10* genes. Asterisks (*) indicate nucleotides which match consensus sequences for the given motifs. The "CK-8-mer" consensus is AAPuCCAAA (Blessing *et al.*, 1987); the KTF-1/AP-2 sequence is ACCCTGAGGCT (Snape *et al.*, 1990). M, T, C represent methionine, threonine and cysteine, the first 3 amino acids of exon 1. Adapted from Powell *et al.*, (1991).

collagenase disrupts the extracellular matrix and basal lamina of the dermal papilla (Link *et al.*, 1990).

It is only recently that the successful maintenance of hair follicles and hair shaft elongation in culture has been achieved (Kondo *et al.*, 1990; Philpott *et al.*, 1990, 1992; Li *et al.*, 1992). The method described by (Philpott *et al.*, 1990, 1992) uses carefully dissected hair follicles which are maintained free-floating in serum-free medium. Follicle growth is observed for up to 10 days in this system and the use of defined culture medium without added growth factors or hormones suggests that the follicles can regulate their own growth (for review see Kealey and Philpott, 1994). Hair follicle growth *in vitro* has also been reported using gelatin sponge-supported culture of human scalp skin (Li *et al.*, 1992). In this system intact, full-thickness skin is used enabling the role of other cell types in follicle growth to be studied, such as macrophages and mast cells. Hair shaft elongation in anagen follicles is seen over a 10 day period, although the growth rate is not as great as that observed by Philpott *et al.* (1990).

1.6.2 Culture of hair follicle cells

Most hair follicle cell cultures have been derived from outer root sheath cells (for reviews see Schaart *et al.*, 1990; Kealey and Philpott, 1994). ORS keratinocytes grow well from plucked or enzyme-isolated follicles, and it appears that the only epithelial cell growth observed from these follicles is the ORS (Kealey and Philpott, 1994). Primary ORS keratinocyte cultures plated onto fibroblast feeder layers or eye lens capsules have been shown to express the normal complement of keratins seen *in vivo* (Weterings *et al.*, 1980; Stark *et al.*, 1987). If ORS keratinocytes are cultured at an air-liquid interface, then interfollicular epidermal keratinocyte markers are expressed (Lenoir *et al.*, 1988) indicating the phenotypic plasticity of ORS cells.

Culturing of other follicle cell types has proven more difficult. Attempts to culture microdissected IRS cells have been unsuccessful (Stark *et al.*, 1987) and there have been few reports of successful cortical cell and follicle matrix cell culture. Frater (1975) describes the *in vitro* differentiation of cortical and cuticle cells from a single-cell suspension of newborn mouse follicles over a 48 hour culture period, however, little cell proliferation was seen. The culture of follicle bulb cells dissected from plucked human hair follicles and plated onto a fibroblast feeder layer has also been reported (Jones *et al.*, 1988). These cells were capable of limited hair keratin protein synthesis, but cell proliferation was not conclusively demonstrated.

Successful isolation and culture of germinative epithelial cells from rat vibrissae and human hair follicles has recently been achieved (Reynolds and Jahoda, 1991; Reynolds *et al.*, 1993). These cells reside at the very base of the hair follicle bulb and are thought to be distinct from the other cells of the hair bulb and ORS since they appear to behave differently in culture and exhibit a different protein profile (Reynolds and Jahoda, 1991). Growth of germinative epithelial cells requires a feeder layer of dermal papilla fibroblasts, indicating the need for dermal papilla-epithelial interactions for cell division to occur. Furthermore, when grown over dermal papilla cells, germinative epithelial cells formed multilayered colonies with an intact basement membrane structure between the germinative cells and the papilla cells (Reynolds and Jahoda, 1991; Reynolds *et al.*, 1993).

Despite the ability to culture germinative cells from the hair follicle bulb, these cells remain completely undifferentiated and have not yet been shown to be capable of differentiation in culture. The isolation and culture of pluripotential hair follicle cells (whether germinative epithelial cells or stem cells) should enable the investigation of factors and conditions which will allow the cells to differentiate into the different follicle cell types, facilitating research into growth control mechanisms.

1.6.3 Hair follicle-derived cell lines

The ability to culture follicles as intact organoids is a major breakthrough for the study of follicle growth and factors which can influence growth, and the culture and manipulation of germinative epithelial cells should provide further opportunities to examine dermal-epidermal interactions in hair growth and hair cell differentiation. However, cell lines derived from the various cell types of the follicle would not only complement these studies but also enable the rapid analysis of hair-specific gene expression in different cell types which the present culture systems do not allow.

Ideally, useful hair-derived cell lines would express differentiation-specific markers appropriate to their cell type. To date, no such cell lines exist for the hair shaft, however, a cell line from a human trichilemmoma originating from the ORS has been established (reported in Kealey and Philpott, 1994).

1.7 Production of immortalised cell lines by targeted oncogene expression

The targeting of oncogene expression in transgenic mice to produce specific, immortalised cell lines which exhibit differentiated phenotypes has been a considerable breakthrough in many different systems, often producing useful cell lines where no naturally occurring cell lines previously existed (Efrat *et al.*, 1988b; Nakamura *et al.*, 1989; Bryce *et al.*, 1993; Lew *et al.*, 1993). Targeting is achieved by placing the oncogene under the transcriptional regulation of a tissue-specific gene promoter in transgenic mice. Cells from resultant tissue-specifc tumours can then be isolated and cultured to produce cell lines. The major advantages of this technique are the avoidance of viral transfection of primary cells for the production of cell lines, and the ability to immortalise cells for which primary cultures cannot be established or which de-differentiate upon culturing.

A large number of different viral and cellular oncogenes have been used to generate cell lines in this manner (for reviews see Hanahan, 1988; Adams and Cory, 1991), and it is clear that particular oncogenes are more oncogenic in certain cell types. In contrast, Simian Virus 40 large tumor antigen (SV-40 TAg) has been shown to transform a wide variety of different cell types, including cells expressing markers of terminal differentiation (Bryce *et al.*, 1993). Highly differentiated β-pancreatic cell lines (Efrat *et al.*, 1988a), eye lens cell lines (Yamada *et al.*, 1990; Bryce *et al.*, 1993), neuronal cell lines (Efrat *et al.*, 1988b; Mellon *et al.*, 1990; Suri *et al.*, 1993) and hepatocyte cell lines (Paul *et al.*, 1988) have been produced by this method.

An alternative approach to targeted oncogenesis is the use of more widely or ubiquitously expressed promoters [for example, metallothionein gene promoters (Morahan *et al.*, 1989; Shanahan *et al.*, 1989; Ohta *et al.*, 1990) and the major histocompatibility complex $H\text{-}2K^b$ class I promoter (Jat *et al.*, 1991)] to direct the expression of a conditional oncogene in transgenic mice. A widely used example of such an oncogene is the SV-40 TAg temperature-sensitive mutant, *tsA58*, which is capable of immortalisation only at permissive temperatures (33°C) and has restricted growth potential at higher temperatures (39°C) (Jat and Sharp, 1989; Radna *et al.*, 1989; Jat *et al.*, 1991). Cells of interest can be isolated from transgenic mice and cultured at the permissive temperature in order to induce cell immortalisation. A number of cell lines have been produced by this method, including hepatocyte cell lines (Yanai *et al.*, 1991), colon and intestinal cell lines (Whitehead *et al.*, 1993) and hematopoietic cell lines (Ravid *et al.*, 1993; Cairns *et al.*, 1994). An attractive feature of this approach is the ability to produce transgenic mice which express *tsA58* in a broad range of tissues such that cell lines can be generated from a variety of different cell types (Jat *et al.*, 1991;

Whitehead *et al.*, 1993). Conditional immortalisation of cells in this manner has also provided the opportunity to study normal cell differentiation upon inactivation of the oncoprotein at the restrictive temperature (Ravid *et al.*, 1993).

It should be mentioned that, whilst immortalised cell lines can display differentiation characteristics, many such cell lines do not exhibit the normal complement of gene expression seen in the cells from which they are derived. In epithelial cells the pattern of keratin expression may be altered, with immortalised cells often expressing a different keratin profile. Examples are seen in cell lines derived from naturally occurring tumours, such as mammary epithelial cell lines (Trask *et al.*, 1990), as well as in cell lines produced by viral transformation such as human epidermal keratinocytes transformed with SV-40 (Banks-Schlegel and Howley, 1983; Bernard *et al.*, 1985) where simple epithelial-type keratins such as K7, K8, K18 and K19 predominate. However, cell lines which appear to express normal differentiation-specific keratins do exist, including the HaCaT human epidermal keratinocyte cell line (Boukamp *et al.*, 1988) and an SV40 TAg-immortalised human thyroid epithelial cell line (Wyllie *et al.*, 1992).

1.8 Aims of the project

The hair follicle is fascinating model for studying development, terminal differentiation and regeneration yet many aspects of follicle cell differentiation remain elusive. What determines cell fate in the follicle bulb, what triggers and controls the terminal differentiation of bulb cells, and how are the numerous keratin genes co-ordinately regulated throughout terminal differentiation? One approach that may begin to shed light on the molecular mechanisms of differentiation is to study the control of the expression of the products of terminal differentiation, the keratin genes.

The aims of this project were two-fold:

1. To produce an immortalised hair follicle cell line that would facilitate the study of hair keratin gene regulation by targeted expression of SV-40 TAg in the hair follicle cortex of transgenic mice.

2. To define control elements important for hair keratin gene expression. In particular, to examine the sheep *K2.10* gene promoter using mouse transgenesis and *in vitro* techniques such as gel mobility shift assays.

Chapter Two

Materials and Methods

2.1 Materials

2.1.1 Tissue

Mouse tissues were collected from transgenic and non-transgenic animals which were housed in special containment facilities in the University of Adelaide.

2.1.2 Bacterial strains

The following *E.coli* K12 strains were used in the experiments described in this thesis:

BB4: *supF58, supE44, hsdR514, galK2, galT22, trpR55, metB1, tonA, ΔlacU169F'[proAB*$^+$*, lacI, Δ(lacZ)M15, Tn10(tet*^{r}*)]* (Bullock, 1987).

XL1Blue: *recA1, endA1, gyrA96, thi-1, hsdR17, supE44, relA1, lac,* [F' *proAB, lacI*^{q}*, ZΔM15* Tn*10*(tet^r)] (Bullock, 1987).

2.1.3 Phagemid Strains

pGEM-3Zf(+), pGEM-5Zf(+), pGEM-7Zf(+) and pGEM-11Zf(+) were obtained from Promega Corporation.

pBluescript II SK(+) and pBluescript II KS(+) were obtained from Stratagene.

2.1.4 Enzymes

Restriction endonucleases were purchased from either Boehringer Mannheim, Pharmacia or New England Biolabs.

Deoxyribonuclease I (DNase I) was purchased from Boehringer Mannheim, Worthington and Promega.

Collagenase (Class I), Lysozyme, Ribonuclease T1 (RNase T1), and Ribonuclease A (RNase A) were purchased from Sigma Chemical Company.

E.coli DNA polymerase I (Klenow fragment), SP6 RNA polymerase, Taq polymerase and RNase inhibitor (RNasin) were purchased from Bresatec Ltd.

T7 RNA polymerase and T4 DNA ligase were purchased from Promega Corporation.

T4 DNA polymerase, T3 RNA polymerase, Calf intestinal phosphatase (CIP) and Proteinase K were supplied by Boehringer Mannheim.

2.1.5 Antibodies

Sheep anti-mouse IgG-FITC conjugate was purchased from Silenus Laboratories, Australia.

The following antibodies, described by Harlow *et al.* (1981), were used to detect SV-40 TAg:

Mouse monoclonal antibody PAb 419 was kindly supplied as ascites fluid by Dr T. Adams (University of Melbourne, Victoria).

Mouse monoclonal antibody PAb 416 was purchased from Oncogene Science Inc.

2.1.6 Radiochemicals

[α-^{32}P]dATP (specific activity, 3000 Ci/mmole), [α-^{32}P]dCTP (specific activity, 3000 Ci/mmole), [α-^{32}P]UTP (specific activity, 3000 Ci/mmole), [γ-^{32}P]ATP (specific activity, 3000 Ci/mmole), [α–^{35}S]dATP (specific activity, 1000-1500 Ci/mmole), and [α-^{35}S]UTP (specific activity, 1000-1500 Ci/mmole) were purchased from Bresatec Ltd.

2.1.7 Molecular Biology Kits

Oligo-labelling kits (GIGAprime kit), nick translation kits (NTK-B), terminal kinasing kits (TKK-1) and riboprobe kits (MMK-1) were purchased from Bresatec Ltd.

Geneclean kits were purchased from Bio-101.

Sequenase II kits were purchased from USB.

2.1.8 General Chemicals and Reagents.

The following chemicals were purchased from the Sigma Chemical Co.: acrylamide and bis-acrylamide, agarose, ampicillin, rATP, chloramphenicol, EDTA (ethylenediaminetetraacetic acid), IPTG (isopropylthiogalactoside), 2-mercaptoethanol, mineral oil, salmon sperm DNA, SDS (sodium dodecyl sulphate), TEMED (N,N,N',N'-tetramethylethylenediamine), TESPA (3-aminopropyltriethoxysilane) and tetracycline.

BCIG (5-bromo-4-chloro-3-indolyl-3-D-β-galactoside) glycogen and polydIdC (poly-deoxy-inosinic-deoxy-cytidylic acid) were purchased from Boehringer Mannheim.

CsCl (technical grade) was purchased from Metallgesellschaft.

Dextran sulphate and Ficoll (type 400) were purchased from Pharmacia.

Gene-Screen membrane was purchased from NEN-Dupont.

Low melting point agarose (type I) was obtained from BRL.

Magnesium chloride, Nonidet P40 (NP40), polyethylene glycol (6000), potassium ferricyanide, and potassium ferrocyanide were purchased from BDH Laboratories.

Nitrocellulose was obtained from Schleicher and Schuell.

OCT (optimal cutting temperature) embedding compound was purchased from Miles Laboratories, Inc.

Tetramethylammoniumchloride (TMACl) was purchased from Aldrich.

Urea (ultra pure) was obtained from Merck.

Zetaprobe GT was obtained from Bio-rad.

General chemicals not listed above were obtained from one of the following suppliers: Ajax Chemicals Pty. Ltd., BDH Chemicals Pty. Ltd., May and Baker Pty Ltd., Merck, Pharmacia or Sigma Chemical Co. Chemicals were of the highest purity available.

2.1.9 Cell culture reagents

Listed below are the sources of reagents and equipment used in the cell culture work. All heat-labile solutions and media were filter sterilized (Sartorius Minisart NML, 0.2 μm) before use.

Dulbecco's modified eagle medium (DMEM), Ham's F12 Medium and Hank's balanced saline solution (HBSS) were purchased from Gibco.

Foetal calf serum (FCS) : Commonwealth Serum Laboratories (CSL).

Trypsin (Difco) was made as a 1% solution in 1 x Versene (EDTA; CSL).

Plasticware (sterile) used was obtained from Lux (Nunc. Inc.) (60 and 100 mm dishes), Corning (25, 75 and 150 cm^2 flasks) and Disposable Products (10 and 20 ml polypropylene tubes).

"Pipet-aid" pipetting devices (Drummond Scientific Co.) were used in work carried out in the Biohazard and Laminar flow hoods.

2.1.10 Media and Buffers

All buffers and media were prepared with distilled and deionized water and sterilized by autoclaving, except heat labile reagents, which were filter sterilized.

(i) Growth Media

The following media were made as described in Sambrook *et al.* (1989):

LB Medium - used for the growth of *E.coli* XL1Blue and BB4 containing plasmids and phagemids.

2 x YT Medium - used for the growth of XL1Blue and BB4.

SOC Medium - used in the transformation of phagemids and plasmids into *E.coli.*

TYP Medium - 1.6% (w/v) bacto-tryptone, 1.6% (w/v) bacto-yeast extract, 85 mM NaCl, 14 mM K_2HPO_4 was used for the growth of XL1Blue during the preparation of single-stranded phagemid.

Agar plates were made using LB medium as described by Sambrook *et al.* (1989). Antibiotics were added when required as described by Sambrook *et al.* (1989).

(ii) Buffers

Denhardt's - 0.02% (w/v) polyvinyl pyrollidone, 0.02% (w/v) BSA, 0.02% (w/v) Ficoll.

PBS: - 136 mM NaCl, 2.6 mM KCl, 1.5 mM K_3PO_4, 8.0 mM Na_3PO_4, pH 7.3.

SSC - 150 mM NaCl, 15 mM Na citrate, 1 mM EDTA pH 7.0

SSPE - 150 mM NaCl, 20 mM sodium dihydrogen phosphate, 1 mM EDTA pH 7.4

TAE - 40 mM Tris-acetate pH 8.2, 20 mM Na acetate, 1 mM EDTA

TBE - 130 mM Tris, 50 mM Boric acid, 2.5 mM EDTA pH 8.3

TE - 10 mM Tris-HCl pH 8.0, 0.1 mM EDTA

Tris-glycine - 40 mM Tris-HCl, 600 mM glycine, pH 8.5

2.1.11 Oligonucleotides

Oligonucleotides for primers, gel mobility shift assays, and site-directed mutagenesis were synthesised by Bresatec Ltd. The oligo sequences are listed below.

K2.10 mutagenesis oligo to introduce a Sal 1 restriction site at +53 in the 5'UTR of the *K2.10* gene:

5'-AGG TCA TGT TGT CGA CAG GGA GGT-3'

Mut-HK-1 mutagenesis oligo to mutate the HK-1 element in the 400 bp *K2.10* promoter of the transgenesis construct, *K2.10(400ΔHK-1)-lacZ.* The introduced nucleotide changes are underlined.

5'-TCA GGC CTG TTT CAT CCT AGA CGC CGT TTA CGA GCT TC-3'

Overlapping 35-mer oligos spanning the *K2.10* promoter from -350 to +5:

1A. 5'-CCC ATG GTC CAG CCC TGT GCC TTC CAG AAA AGG AT-3'

1B. 5'-AT CCT TTT CTG GAA GGC ACA GGG CTG GAC CAT GGG-3'

2A. 5'-CTT CCA GAA AAG GAT TTG GGG ACC AGG CTC TAC CC-3'

2B. 5'-GG GTA GAG CCT GGT CCC CAA ATC CTT TTC TGG AAG-3'

3A. 5'-GAC CAG GCT CTA CCC CAG GTC ACT GCA ACT ATC GC-3'

3B. 5'-GC GAT AGT TGC AGT GAC CTG GGG TAG AGC CTG GTC-3'

4A. 5'-CAC TGC AAC TAT CGC CTG CAC TCA GAG CAT GGA GT-3'

4B. 5'-AC TCC ATG CTC TGA GTG CAG GCG ATA GTT GCA GTG-3'

5A. 5'-CTC AGA GCA TGG AGT CCA ACT AGA TAC TTC TAG GA-3'

5B. 5'-TC CTA GAA GTA TCT AGT TGG ACT CCA TGC TCT GAG-3'

6A. 5'-TAG ATA CTT CTA GGA GGT CTC CAC TTC CAG TAG CA-3'

6B. 5'-TG CTA CTG GAA GTG GAG ACC TCC TAG AAG TAT CTA-3'

7A. 5'-CCA CTT CCA GTA GCA ATG GGA GGG GGA GAA GAA GC-3'

7B. 5'-GC TTC TTC TCC CCC TCC CAT TGC TAC TGG AAG TGG-3'

8A. 5'-AGG GGG AGA AGA AGC TCG TAA ACG GCT TTG AAG AT-3'

8B. 5'-AT CTT CAA AGC CGT TTA CGA GCT TCT TCT CCC CCT-3'

9A. 5'-AAC GGC TTT GAA GAT GAA ACA GGC CTG AGG CCG AG-3'

9B. 5'-CT CGG CCT CAG CCG TGT TTC ATC TTC AAA GCC GTT-3'

10A. 5'-AGG CCT GAG GCC GAG ATT GTT GAC ACA GCT CTA CT-3'

10B. 5'-AG TAG AGC TGT GTC AAC AAT CTC GGC CTC AGG CCT-3'

11A. 5'-TGA CAC AGC TCT ACT GAA TAG GCA AAC AGT TGG CT-3'

11B. 5'-AG CCA ACT GTT TGC CTA TTC AGT AGA GCT GTG TCA-3'

12A. 5'-GGC AAA CAG TTG GCT CTT AAG AGG CCA GGG TGA TG-3'

12B. 5'-CA TCA CCC TGG CCT CTT AAG AGC CAA CTG TTT GCC-3'

13A. 5'-GAG GCC AGG GTG ATG CCA AGC CAA TAA AAT GCA GC-3'

13B. 5'-GC TGC ATT TTA TTG GCT TGG CAT CAC CCT GGC CTC-3'

14A. 5'-CCA ATA AAA TGC AGC TGT TGT CTC TTT GCT GCC CC-3'

14B. 5'-GG GGC AGC AAA GAG ACA ACA GCT GCA TTT TAT TGG-3'

15A. 5'-TCT CTT TGC TGC CCC TTT TAC TGC CAG CTA TCC TG-3'

15B. 5'-CA GGA TAG CTG GCA GTA AAA GGG GCA GCA AAG AGA-3'

16A. 5'-CTG CCA GCT ATC CTG GTG CAT AAA AGG GCC TGC CA-3'

16B. 5'-TG GCA GGC CCT TTT ATG CAC CAG GAT AGC TGG CAG-3'

17A. 5'-TAA AAG GGC CTG CCA CAG CTC AGG GAG CAC AGG CC-3'

17B. 5'-GG CCT GTG CTC CCT GAG CTG TGG CAG GCC CTT TTA-3'

K2.10 HK-1 Oligos:

The 8 bp HK-1 core sequence is underlined and mutated bases are highlighted.

HK-1A 5'-AAC GGC TTT GAA GAT GAA AC-3'

HK-1B 5'-GTT TCA TCT TCA AAG CCG TT-3'

ΔHK-1A 5'-AAC.GGA CGA TCT GAT GAA AC-3'

ΔHK-1B 5'-GTT TCA TCA GAT CGT CCG TT-3'

HK-1 Variant Series:

The HK-1 core sequence is underlined and nucleotides which vary from the *K2.10* sequence are highlighted.

HKv-1A 5'-CTG GCC CTT TGA GGA CAA AA-3'

HKv-1B 5'-TTT TGT CCT CAA AGG GCC AG-3'

HKv-2A. 5'-AAC GGC TTT GAG GAT GAA AC-3'

HKv-2B. 5'-GTT TCA TCC TCA AAG CCG TT-3'

HKv-3A. 5'-AAC GGC TCT GAA GAT GAA AC-3'

HKv-3B. 5'-GTT TCA TCT TCA GAG CCG TT-3'

HKv-4A. 5'-AAC GGA TTT GAA GAT GAA AC-3'

HKv-4B. 5'-GTT TCA TCT TCA AAT CCG TT-3'

HKv-5A. 5'-AAC GGC GTT GAA GAT GAA AC-3'

HKv-5B. 5'-GTT TCA TCT TCA ACG CCG TT-3'

HKv-6A. 5'-AAC GGC TTA CAA GAT GAA AC-3'

HKv-6B. 5'-GTT TCA TCT TGT AAG CCG TT-3'

HKv-7A. 5'-AAC GGC TTT GCA GAT GAA AC-3'

HKv-7B. 5'-GTT TCA TCT GCA AAG CCG TT-3'

HKv-8A. 5'-GAC TTC TTT GAA GGG CCA CC-3'

HKv-8B. 5'-GGT GGC CCT TCA AAG AAG TC-3'

K2.10 KTF-1 oligo:

KTF-1A 5'-AAA CAG GCC TGA GGC CGA GAT TG-3'

KTF-1B 5'-CAA TCT CGG CCT CAG GCC TGT TT-3'

Non-specific competitor oligos:

NS-1A 5'-TGG CTC AGG AGG AAG ATC CGC-3'

NS-1B 5'-GCG GAT CTT CCT CCT GAG CCA-3'

NS-2A 5'-GCC TGT TAA GTC TCC TCA AAT AGC G-3'

NS-2B 5'-CGC TAT TTG AGG AGA CTT AAC AGG C-3'

AP-1 binding site (TRE) oligo (Risse *et al.*, 1989):

AP-1A 5'-AAG CAT GAG TCA GAC AC-3'

AP-1B 5'-GTG TCT GAC TCA TGC TT-3'

2.1.12 Miscellaneous

Cloned DNA sequences

pG2TAg : Dra I/Bam HI fragment (194 bp) spanning nucleotides 2533-2727 of the SV-40 genome cloned into the Sma I site of pGEM-2. Linearisation with Eco RI and transcription with T7 RNA polymerase produces a 253 nt transcript. pG2TAg was constructed by T. Occhiodoro.

Sheep *K2.10* gene fragment (14.5 kb) was obtained from Dr B. Powell.

SV-40 *tsA58* DNA was a gift from Dr P. Jat.

Conserved hair keratin type II probes

C-terminal probe : 325 bp Alu I fragment from the sheep *K2.9* gene spanning nt. 5847-6171 (Powell *et al.*, 1992).

N-terminal probe : 220 bp exon 1 fragment of *K2.9* spanning nt. 638-858 (Powell *et al.*, 1992).

exon 4 probe : 376 bp Eco RI/Pst I fragment of *K2.9* from nt. 3951-4327 (Powell *et al.*, 1992).

exon 5 and 6 probe : 785 bp Pst I fragment of *K2.9* from nt. 4324-5109 (Powell *et al.*, 1992).

Note that exon 4, 5 and 6 probes span a continuous region from nt. 3951-5109 of *K2.9*. These probes were obtained from Dr B. Powell.

DNA Markers

DMW-S1 : *Eco*RI digested SPP1 phage (360 bp-8.51 kb) : Bresatec

DMW-P1 : *Hpa*I digested pUC19 (26-501 bp) : Bresatec

DMW-L1 : *Hin*dIII digested λ DNA (564 bp-23.13 kb) : Bresatec

Sheep follicle and *K2.10* transgenic mouse whisker follicle sections were gifts from Dr B. Powell.

Sheep genomic cosmid library was purchased from Clontech.

Rat tissue nuclear extracts and several cell line nuclear extracts were obtained from Dr G. Braidotti. AP-2 protein extracts and HeLa cell nuclear extracts were purchased from Promega.

X-ray film was obtained from Konica Corporation or Fuji Photo Film Corporation. Hyperfilm was purchased from Amersham.

Ektachrome film and D19 developer (used for *in situ* hybridizations) were purchased from Kodak Ltd.

L4 emulsion (used for *in situ* hybridizations), Hypam Rapid Fixer and Pan F film were purchased from Ilford Ltd.

Qiagen Columns were obtained from Diagen Inc.

TLC plates (Kieselgel 60 F_{254}) were purchased from Merck.

2.2 Methods

2.2.1 DNA Methods

2.2.1.1 Ethanol precipitation

Ethanol precipitation was conducted by the method of Sambrook *et al.* (1989). In situations of low concentrations of DNA, 1-2 µl of glycogen carrier was added to facilitate precipitation of the DNA.

2.2.1.2 Phenol extraction

Before use, phenol (Wako) was equilibrated with buffer, one time with 1 M Tris-HCl pH 8.0 followed by three further equilibrations with 10 mM Tris-HCl pH 8.0, 0.1 mM EDTA. The extraction was carried out by mixing the aqueous sample with an equal volume of phenol/chloroform (1:1). After centrifugation the upper aqueous phase was transferred to a new tube.

2.2.1.3 Isolation of plasmid DNA

(i) Preparative scale procedure

Recombinant plasmid DNA was prepared by a modified procedure of Birnboim and Doly (1980) described by Sambrook *et al.* (1989) using caesium chloride density gradient equilibration centrifugation (Radloff *et al.*, 1967) for final purification of the plasmid DNA.

(ii) Preparation through columns

Plasmid DNA was prepared through Qiagen columns following the manufacturers protocol.

(iii) Analytical scale procedure - "minipreps"

Small scale preparation of plasmid DNA was conducted essentially by the method of Ish-Horowicz and Burke (1981).

2.2.1.4 Restriction enzyme digestion and analysis of DNA

Restriction endonuclease digestions were performed using the conditions recommended by the supplier for each enzyme. Usually 2-5 units of enzyme were used per µg of DNA and reactions were performed for approximately 2 hours.

2.2.1.5 Agarose gel electrophoresis of DNA

DNA was size fractionated by agarose gel electrophoresis, which was performed in a horizontal apparatus, essentially by the method described by Sambrook *et al.* (1989). Analytical electrophoresis was performed on gels of 75 mm x 55 mm whilst larger gels were used for DNA preparation and Southern analysis.

All gels were run in TAE buffer and samples were loaded in 2.5% Ficoll, 0.1% lauryl sarkosyl, 0.025% bromophenol blue, 0.025% xylene cyanol. Electrophoresis was performed at 60-100 mA. After electrophoresis, DNA was detected by staining with 0.1% ethidium bromide for 5 minutes and viewing under short wave UV light. Gels were photographed using a Polaroid camera with 667 (ASA 3000) Polaroid film or using a Tracktel GDS-2 gel documentation system (Vision Systems).

2.2.1.6 Polyacrylamide gel electrophoresis

Electrophoresis of oligonucleotides and DNA fragments of less than 500 bp in length was carried out on a vertical 14 cm x 14 cm x 0.5 mm slab gel containing 5-20% (w/v) acrylamide/bisacrylamide (50:1). The acrylamide was polymerised in 1 x TBE buffer by the addition of 0.1% (w/v) APS and 0.1% (v/v) TEMED and the gel was pre-electrophoresed at 15-20 mA for 20 minutes before loading. Gels were electrophoresed at 15-20 mA until the loading dyes had migrated the desired distance. DNA was visualised under UV light after ethidium bromide staining, radiolabelled DNA was detected by autoradiography (2.2.3) and oligonucleotides were visualised by UV shadowing using a fluorescent TLC plate (Merck).

2.2.1.7 Isolation of DNA from gels

(i) Restriction fragments of size greater than 8 kb were isolated from low melting point Agarose (type I) gels by phenol extraction of the melted agarose followed by ethanol precipitation.

(ii) Restriction fragments ranging in size from 500 bp to 8 kb were extracted from agarose using a "Geneclean" kit obtained from BIO 101 according to the manufacturers instructions. This method is based on the procedure described by Vogelstein and Gillespie (1979).

(iii) Restriction fragments ranging in size from 80 bp to 1 kb were extracted from either agarose or acrylamide gels by elution from the gel slice in 0.5 M ammonium acetate, 10 mM magnesium acetate, 1 mM EDTA, 0.1% SDS at 37°C for 16 hours. The DNA was then ethanol precipitated (2.2.1.1).

2.2.1.8 DNA subcloning

(i) Vector preparation

Phagemid cloning vectors were prepared by linearization with the appropriate restriction endonuclease(s) followed by removal of the 5' terminal phosphate groups as described by Sambrook *et al.* (1989). The vectors were then subjected to agarose gel electrophoresis, the linear vector band excised and the DNA prepared using the "Geneclean" kit (2.2.1.7).

(ii) Endfilling of DNA fragments

DNA fragments with 5' protruding termini were endfilled with Klenow fragment. Either Klenow fragment or T4 DNA polymerase was used to "blunt" 3' overhangs.

(iii) Ligation

Approximately 40 ng of vector DNA was combined with the desired DNA fragment in a 1:3 molar ratio. The ligations were performed in 50 mM Tris-HCl pH 7.4, 10 mM magnesium chloride, 10 mM dithiothreitol, 1 mM rATP, 100 µg/ml BSA. Approximately 1 unit of T4 DNA ligase was added and ligation was allowed to proceed at 16°C overnight.

(iv) Plasmid and phagemid transformation

E.coli XL1Blue cells and BB4 cells were made competent and transformed by a modification of the calcium chloride method of Sambrook *et al.* (1989) in which the cells were resuspended once in 0.1 M calcium chloride, 0.02 M magnesium chloride.

One half of the ligation mix was normally used for each transformation.

2.2.1.9 Preparation of labelled DNA

(i) Oligo-labelling

DNA restriction fragments were oligo-labelled using a kit obtained from Bresatec Ltd according to the method provided by the manufacturers which is based on the procedure of Feinberg and Vogelstein (1983).

The labelled DNA was purified by Sepharose CL-6B chromatography (Sambrook *et al.*, 1989).

(ii) 5' End-labelling of oligonucleotides

Synthetic DNA oligonucleotides were radiolabelled using [γ-^{32}P]ATP and T4 polynucleotide kinase using a kit purchased from Bresatec. The oligos were subsequently purified on a 20% non-denaturing polyacrylamide gel (2.2.1.6) The labelled oligos were excised from the gel and eluted at 37^{o}C in 0.4 ml TE.

2.2.1.10 Preparation of single-stranded phagemid DNA

Single-stranded phagemid DNA was produced essentially as described by Sambrook *et al.* (1989), and the template DNA was then prepared in an identical fashion to single stranded M13 template DNA described by Winter and Fields (1980).

2.2.1.11 Site-directed oligonucleotide mutagenesis

Kinased universal sequencing primer (10 pmol) and kinased mutagenesis primer (10 pmol) were annealed with single-stranded phagemid DNA (0.5 pmol) in 50 mM Tris-HCl pH 7.4, 10 mM $MgCl_2$, 660 mM NaCl at 70^{o}C for 5 min and slowly cooled to room temperature. The following components were then added to the annealed template: 50 µM dNTPs, 1 mM rATP, 1 mM DTT, 2 units Klenow fragment, 5 units T4 DNA ligase and the reaction was incubated overnight at room temperature. One-tenth and one-fiftieth of the mutagenesis reaction was transformed into XL1Blue cells (see 2.2.1.8). Colonies were selected by either Grunstein's colony screening (see 2.2.1.16) or by restriction enzyme digestion, where mutagenesis resulted in creation of a new restriction enzyme recognition site. All mutations were confirmed by DNA sequencing.

2.2.1.12 DNA sequencing

Double-stranded sequencing was performed using supercoiled phagemid DNA purified by caesium chloride gradient centrifugation or Qiagen columns. Templates were RNAse A treated (1 µg/µl at 37^{o}C, 15 minutes) and then alkali denatured (0.2 M NaOH, 0.2 mM EDTA at 37^{o}C, 15 minutes).

Both double-stranded and single-stranded phagemid template DNA was sequenced using the dideoxy chain termination method (Sanger *et al.*, 1977). Sequencing was performed using Sequenase (modified T7 DNA polymerase) and the Sequenase II sequenase kit according to the manufacturer's instructions. Products of the sequencing reactions were separated by electrophoresis on 6% (w/v) polyacrylamide/ 8M urea gels containing TBE buffer. Gels were pre-electrophoresed at 800 V for 30 minutes before use and were run in TBE buffer at 1300 V. After transfer to Whatman 3MM chromatography paper and drying on a vacuum gel dryer, gels were autoradiographed overnight at room temperature.

2.2.1.13 Preparation of genomic DNA from mouse tail

Tail samples were minced with a scalpel blade and treated with Proteinase K (200 µg/ml) in 1% SDS, 50 mM Tris-HCl, 10 mM EDTA, 150 mM NaCl, pH 7.5 for 16 hours at 37°C. High molecular weight DNA was purified by phenol/chloroform extraction as described by Sambrook *et al.* (1989).

2.2.1.14 Southern transfer

Plasmid and genomic DNA, which had been cleaved by restriction endonuclease(s) and size fractionated in agarose gels, were transferred to Bio-Rad Zetaprobe membrane by the modified method of Southern (1977) reported by Reed and Mann (1985) using 0.4 M sodium hydroxide.

The filters were hybridised according to the instructions for Zetaprobe GT membranes. The stringency of the individual washing conditions is reported for each of the hybridised filters.

The filters were autoradiographed at -80°C in the presence of an intensifying screen.

Filters which were to be re-used were washed in boiling 0.1 x SSC, 0.1% SDS and shaken for 15 minutes. This wash was then repeated and the filter autoradiographed prior to re-use.

2.2.1.15 Dot blot analysis

Transfer of undigested, heat-denatured genomic DNA (5 µg) by vacuum blotting in 0.1 M NaOH to Genescreen membrane was performed using a "Minifold" vacublotter (Schleicher and Schuell) according to the manufacturer's instructions. Copy number control series were prepared by serial dilution of plasmid DNA together with negative control genomic DNA (5 µg). Blotted samples were neutralised with 0.5 M Tris pH 7.5 and the DNA was fixed to the membrane by UV crosslinking.

2.2.1.16 Grunstein's colony screening

Replica filters were made of colonies which had been grown on nitrocellulose filters layered on nutrient agar plates containing the appropriate antibiotic. The resultant filters were screened following an adaptation of the method of Grunstein and Hogness as described in Sambrook *et al.* (1989).

2.2.1.17 Gel mobility shift assays

(i) Probe preparation

Gel mobility shift assays were performed using synthetic oligonucleotide probes. One strand of each complementary oligonucleotide was kinased with γ-^{32}P-ATP (2.2.1.9ii) and gel purified. Approximately 1.5 ng of ^{32}P-labelled oligonucleotide was combined with 300 ng of unlabelled complementary oligonucleotide in a 15 µl annealing reaction containing 200 mM NaCl. The mixture was heated to 100^{o}C for 45 seconds and then slowly cooled to RT in the heating block.

(ii) DNA binding reactions

DNA binding activity was assayed essentially as described by Schneider *et al.* (1986). Protein extract (typically, 5-6 µg of crude nuclear extract) was combined with competitor DNA and 0.2 ng of oligonucleotide probe in a 10-20 µl reaction volume containing 25 mM Hepes-NaOH pH 7.5, 1 mM EDTA, 5 mM DTT, 100 mM NaCl, 100 ng polydIdC and 20% (v/v) glycerol. Most binding reactions also contained 2.5% Chaps to enhance complex formation (Hassanain *et al.*, 1993). After incubation at RT for 20 minutes, Tris-glycine loading buffer was added to the reaction and the samples were electrophoresed on a 10% non-denaturing Tris-glycine polyacrylamide gel. The gels were of the dimensions 14 x 14 x 0.05 cm^3 and were run in Tris-glycine buffer (2.1.10ii) at 300-350 V at 4^{o}C after pre-electrophoresis for 30-40 minutes. After electrophoresis, the gel was dried onto Whatman 3MM paper and autoradiographed or exposed to a PhosphorImage screen (2.2.3) for 16-48 hours.

2.2.2 RNA Methods

2.2.2.1 Preparation of total RNA

Total RNA was isolated from mouse skin, isolated mouse follicles and various cell lines using the acid guanidinium thiocyanate/phenol/chloroform extraction procedure described by Chomczynski and Sacchi (1987).

2.2.2.2 *In vitro* transcription

Inserts in pGEM or pBluescript phagemid vectors were transcribed with either SP6, T7 or T3 RNA polymerase according to the method of (Kreig and Melton, 1987) using a kit purchased from Bresatec Ltd. The RNA was labelled to high specific activity by the incorporation of [α-^{35}S]UTP or [α-^{32}P]UTP. Riboprobes of length greater than 500 nucleotides were reduced in length by treatment with 40 mM $NaHCO_3$/60 mM Na_2CO_3, pH 10.2 at 60°C following the method of Cox *et al.* (1984) when used for *in situ* hybridisation.

Transcripts were either phenol extracted and ethanol precipitated or purified through Sephadex G25 fine "Quick spin" columns (from Amersham) prior to further use.

2.2.2.3 RNA protection assay

RNA protection assays were performed on total RNA as described by Kreig and Melton (1987) using 100 000-180 000 cpm riboprobe hybridised with 5-50 µg total RNA. Mouse β-actin control and yeast tRNA were supplied from the Ambion RNA protection kit.

2.2.2.4 Tissue *in situ* hybridisation

Tissue sections were fixed in 4% paraformaldehyde/0.25% glutaraldehyde for 4 hours at room temperature, rinsed for 1 hour in 50% ethanol and then stored at 4°C in 70% ethanol until processed through paraffin wax for histological analysis or *in situ* hybridization. Sections (7 µm) were cut and transferred to TESPA subbed slides (Rentrop *et al.*, 1986).

The *in situ* hybridization procedure was based on the method of Cox *et al.* (1984) with the modifications of Powell and Rogers (1990). For histological examination sections were stained with the tripartite SACPIC stain of Auber (1950) which distinguishes the histological components of the hair follicle and their relative states of differentiation.

2.2.3 Autoradiography

Radiolabelled DNA and RNA was visualised by autoradiography either using storage phosphor technology and PhosphorImager (Molecular Dynamics) or using X-ray film in an Ilford autoradiography cassette. X-ray film exposures were performed at room temperature or at -80°C using a tungsten intensifying screen. After exposure, X-ray film was processed using a Curix60 automatic processor (Agfa).

2.2.4 Protein Methods

2.2.4.1 Extraction and S-carboxymethylation of hair protein

Protein extraction and S-carboxymethylation of hair samples was performed essentially as described in Raphael *et al.* (1984). Washed hair samples (10 mg) were solubilised in 1 ml 8 M urea, 50m M Tris, 0.05 M DTT (pH 10) for 18 hours at room temperature. Samples were sonicated briefly to ensure complete cell disruption and centrifuged to pellet cell debris. To 500 µl of extract, 25 µl of 1 M DTT was added and incubated at room temperature for 10 minutes. 250 µl 1 M iodoacetamide, 2 M Tris-HCl pH 10 was then added and, after incubation at room temperature for 30 minutes, 10 µl of 14.2 M 2-mercaptoethanol was added and the samples stored at -20°C.

^{14}C-Labelled proteins were S-carboxymethylated with iodo-[2-^{14}C]-acetic acid as described by (Raphael *et al.*, 1984).

2.2.4.2 Protein gel electrophoresis

(i) Two-dimensional gel electrophoresis

Electrophoresis in the first dimension was at pH 8.9 in 8 M urea and in the second dimension by SDS slab gel (Marshall and Gillespie, 1982). Following electrophoresis the proteins were detected by fluorography (Bonner and Laskey, 1974).

(ii) Tricine gel electrophoresis

A tricine SDS polyacrylamide gel system was used to resolve low molecular weight proteins. Gels were prepared and run essentially as described by Schagger and von Jagow (1987).

2.2.4.3 Western blotting

After SDS-polyacrylamide gel electrophoresis of samples, proteins were transferred to nitrocellulose using a semi-dry transfer system (Pharmacia NovaBlot). The filter was treated with blocking solution (1X PBS, 0.05% Tween) and then probed with 1° antibody diluted appropriately in PBS-Tween at RT for 2-18 hours with agitation. The filter was then washed in PBS-Tween followed by two washes in 150 mM NaCl, 50 mM Tris pH 7.5.

The 2° antibody, anti-mouse IgG-alkaline phosphatase conjugate, was diluted 1:1000 in 150 mM NaCl, 50 mM Tris pH 7.5, 5% blotto and applied to the filter. After incubation at RT for 1 hour, the filter was washed several times in 150 mM NaCl, 50 mM Tris pH 7.5 and rinsed briefly in alkaline phosphatase buffer (100 mM NaCl, 5 mM $MgCl_2$, 100 mM Tris pH 9.5) prior to the detection reaction.

For detection of alkaline phosphatase activity the filter was incubated in alkaline phosphatase buffer containing 25 µg/ml NBT and 22 µg/ml BCIP until the desired signal was produced. The reaction was stopped by washing the filter in PBS containing 0.1 M EDTA pH 8.

2.2.4.4 Preparation of nuclear extracts

(i) From cell lines

Cells in monolayer cultures (6x 150 cm^2 flasks) were harvested by trypsinisation, washed twice with PBS and resuspended in 0.5 ml Buffer A (10 mM Hepes pH 7.9, 1.5 mM $MgCl_2$, 10 mM KCl, 0.5 mM PMSF, 1 mM DTT). Cells were incubated on ice 15 minutes and lysed by passing through a 25 gauge needle 10 times. Nuclei were pelleted by centrifugation in an Eppendorf centrifuge at RT for 20 seconds and then resuspended in 0.4 ml Buffer C (20 mM Hepes pH 7.9, 420 mM NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 25% glycerol, 0.5 mM PMSF, 1 mM DTT). Nuclei were incubated on ice for 30 minutes with intermittent mixing. Debris was pelleted by centrifuging at 4°C for 5 minutes. Nuclear extract was aliquoted into tubes, snap frozen and stored at -80°C.

(ii) From mouse hair follicles

Hair follicles were isolated from 10-12 Swiss or BalbC mice (3-4 days old) (see 2.2.6.3). Follicles were washed three times in PBS, resuspended in 1.0 ml cold lysis buffer A (10 mM Hepes pH 7.9, 10 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA, 1 mM DTT, 0.5 mM PMSF, 0.6% NP40) and transferred to a Dounce homogeniser on ice. Cells were lysed by 25-30 strokes of the homogeniser. The extent of lysis was checked by staining an aliquot with trypan blue and viewing under a light microscope. Nuclei were pelleted by centrifugation in an Eppendorf centrifuge for 20 seconds at room temperature and washed in 1.0 ml cold lysis buffer A without NP40. Pelleted nuclei were resuspended in 350-400 µl extraction buffer (10 mM Hepes pH 7.9, 0.1 mM EDTA, 0.1 mM EGTA, 1.5 mM $MgCl_2$, 400 mM KCl, 25% glycerol, 0.5 mM DTT, 0.5 mM PMSF) and incubated on ice for 20 minutes. Debris was pelleted by centrifuging at 4°C for 5 minutes. Nuclear extract was aliquoted, snap frozen in liquid nitrogen and stored at -80°C.

The protein concentration of all nuclear extracts was estimated using the Bradford assay (Bradford, 1976).

2.2.5 Histochemical techniques

2.2.5.1 Immunohistochemistry

Unfixed mouse dorsal skin containing hair follicles in anagen phase was embedded in OCT compound. Cryostat sections (14 μm thick) were air-dried and stained for SV-40 TAg using mouse monoclonal antibodies Pab416 or Pab419 essentially as described by Hanahan (1985). Antigen-antibody complexes were visualised by fluorescein-conjugated secondary antibody.

2.2.5.2 Assay for β-galactosidase activity

Skin sections were prepared as for immunochemistry (2.2.5.1) and air-dried sections were fixed in 0.2% glutaraldehyde/PBS for 5 minutes at RT and washed twice in PBS prior to staining in 0.04% BCIG, 0.45 mM potassium-ferrocyanide, 0.45 mM potassium-ferricyanide, 1 mM $MgCl_2$ in PBS at 37°C for 16-20 hours as described in Sanes *et al.* (1986). After staining, sections were rinsed in PBS, counterstained in eosin and mounted in Gurr's Depex mounting medium.

2.2.5.3 Electron microscopy

Hairs were prepared for transmission electron microscopy as described by Filshie and Rogers (1961) and ultrathin sections were cut with an LKB Ultratome. The sections were post-stained with 2% uranyl acetate followed by 20% lead citrate solution. Micrographs were taken using a Philips 300 electron microscope.

2.2.6 Cell culture techniques

2.2.6.1 Maintenance of cell lines

Where sterile conditions and technique were required to maintain viability, cell culture handling in the methods described below was routinely performed in a biohazard safety hood (BH series, Class II, Gelman Sciences Australia Pty Ltd) and according to the C1 containment handling guidelines specified by the Biohazards Committee (University of Adelaide).

2.2.6.2 Cell counting

Estimates of viable cells from harvested cultures (homogeneous cell suspensions) were performed using a haemocytometer (Neubauer, Improved) and a preparation of 0.5% trypan blue in PBS. Cell were diluted 1 in 10 in this solution prior to cell counts.

2.2.6.3 Culture of mouse hair follicles

Hair follicles were isolated from the skin of 2-4 day old mice by trypsin and collagenase digestion as described by Rogers *et al.* (1987). Follicles were purified through a 9% Ficoll gradient as detailed in Weinberg *et al.* (1990) and washed 3-5 times in DMEM before plating.

2.2.7 Production and analysis of transgenic mice

The following methods were performed by personnel in the Department of Obstetrics and Gynaecology, University of Adelaide, South Australia, and by Bresatec Ltd essentially according to the methods described by Steinert (1988) and Hogan *et al.* (1986).

(a) maintenance of the C57BL/6J mouse strain

(b) preparation of pseudopregnant female recipients

(c) preparation of donor females / vasectomised males

(d) collection and fertilisation of ova

(e) microinjection and transfer of embryos.

2.2.7.1 Preparation of the DNA fragments for microinjection

Transgene DNA fragments were excised from vector DNA and prepared for microinjection by preparative agarose gel electrophoresis and purification via elution from glass powder (Geneclean kit).

2.2.7.2 Identification of transgenic mice

DNA dot blots and Southern transfer analyses of mouse tail DNA were used to identify transgenic mice.

2.2.7.3 Analysis of transgene expression

Immunofluorescent staining was used to identify expression of TAg in the skin of *K2.10-TAg* transgenic mice (2.2.5.1). Expression in other tissues and tumours was analysed by RNA protection assay (2.2.2.3).

Transgenic mice carrying the *lacZ* reporter gene were analysed for expression in the skin by staining for β-galactosidase activity (2.2.5.2).

2.2.7.4 Maintenance of mice

Mice were housed and cared for by Animal Services, University of Adelaide.

2.2.8 Computer programmes

DNA sequence data was compiled and analysed on a VAX 11-785 computer using the programmes ANALYSEQ (Briggs *et al.*, 1986), SPCOMP (a programme written locally by Dr. A.V.Sivaprasad and Dr Ian Dodd), Signal Scan (Prestridge, 1991) and a number of the programs from the Sequence Analysis Software Package of the University of Wisconsin Genetics Computer Group (UWGCG) (Busch and Sassone-Corsi, 1990).

2.2.9 Containment facilities

All work involving recombinant DNA was carried out under C1 containment conditions required for work involving viable organisms or C0 conditions required for work not involving viable organisms, as defined and approved by the Australian Academy of Science Committee on Recombinant DNA and by the University Council of the University of Adelaide.

2.2.10 Ethics approval

All transgenic mouse work was carried out under the ethics numbers M54/89 and S6/93. Non-transgenic mice were obtained for matings, tissue samples and follicle preparations under the ethics number S16/92. All animal work was carried out according to the guidelines set by the Ethics Committee of the University of Adelaide and by the Genetic Manipulations Advisory Council.

Chapter Three

Targeted expression of SV-40 TAg in the hair follicle of transgenic mice

3.1 Introduction

The study of hair keratin gene regulation and other aspects of hair keratinocyte differentiation have been hampered by the lack of good *in vitro* systems, in particular, a follicle keratinocyte cell line. The use of cell lines is a major tool in studying the molecular and cellular biology of eukaryotic systems and the targeting of oncogene expression in transgenic mice to produce specific, immortalised cell lines has been a considerable breakthrough in many systems where no naturally occurring cell lines previously existed (Nakamura *et al.*, 1989; Bryce *et al.*, 1993; Lew *et al.*, 1993).

Directed expression of SV-40 TAg in transgenic mice has been shown to induce tissue-specific tumorigenesis and many useful cell lines exhibiting differentiated characteristics have been established from resultant tumour cells (for examples see Hanahan, 1985; Paul *et al.*, 1988; Nakamura *et al.*, 1989). In an attempt to produce such a cell line derived from hair follicle cortical cells, SV-40 TAg expression was targeted to the follicle cortex in transgenic mice using the sheep wool *KRT2.10 (K2.10)* gene promoter.

The sheep *K2.10* gene had previously been expressed in transgenic mice (Powell and Rogers, 1990) and expression was localised to the follicle cortex, mimicking its expression in the wool follicle. Since the cell line was to be used primarily for the study of hair keratin gene regulation, it was thought desirable that the cell line should exhibit some of the differentiated characteristics of a cortical cell such that expression of keratin gene constructs would be possible. Of the genes available to us at the time, the *K2.10* gene was expressed earliest in cortical cell differentiation and therefore appeared to be a useful promoter for use in this work.

This chapter describes the production and analysis of *K2.10-TAg* transgenic mice and the effect of transgene expression on these mice.

3.2 Results

3.2.1 Generation and identification of *K2.10-TAg* transgenic mice

In order to generate mice carrying the *K2.10-TAg* transgene, a hybrid construct containing the sheep *K2.10* 5' promoter sequences fused to the SV-40 early region, encoding SV-40 large (T) and small (t) tumour antigens (figure 3.1), was microinjected into fertilised mouse oocytes (2.2.7).

Twenty founder (Fo) transgenic mice were identified from 160 mice by dot blot of tail DNA and 14 of these were confirmed by Southern blot analysis shown in figure 3.2. Of these transgenic mice, only 10 mice were shown to express TAg; Fo-40, Fo-49, Fo-50, Fo-75, Fo-94, Fo-119, Fo-127, Fo-131, Fo-138 and Fo-144. A line of transgenic mice was established from only one of the ten Fo mice, Fo-40, as the other mice failed to breed. The copy number of the transgene in Fo-40 was approximately 10-25 copies (figure 3.3A) and was stably inherited over at least 4 generations (figure 3.3B).

K2.10-TAg transgene expression was analysed by immunofluorescent staining of mouse pelage follicles using monoclonal antibodies directed against SV-40 TAg. Expression was directed to the cortex of transgenic hair follicles (figure 3.4), following the same pattern as observed with the entire sheep *K2.10* gene in transgenic mice (Powell and Rogers, 1990). Cortical expression was observed in all cases, however, patchy staining was seen in follicles from Fo-131 and Fo-138 and not all follicles in these skin sections were positive for TAg.

3.2.2 Aberrant hair phenotype of *K2.10-TAg* mice

Mice expressing the transgene were initially identifiable by wavy vibrissae, noticable 2-3 days after birth. Hairs of the pelage were also affected, producing a ruffled coat in the milder phenotype ranging to a stubble covering the back of the mouse in the more severe phenotypes (figure 3.5). Scaling of the epidermis was evident in severely affected newborn mice Fo-50, Fo-75 and Fo-94 and the skin of these newborn mice was wrinkled (figure 3.5C). Premature hair loss resulting in a thinner pelage or regions of temporary nudity were observed depending on the severity of the phenotype. This was thought to be due to weakening of the fibre at the base as it correlated with regions subjected to abrasion, such as the underside of suckling mothers and areas around the head, neck and face. When viewed by light microscopy, plucked hairs from *K2.10-TAg* mice show deformities which are absent in normal mice (figure 3.6). This varies from a slight crookedness

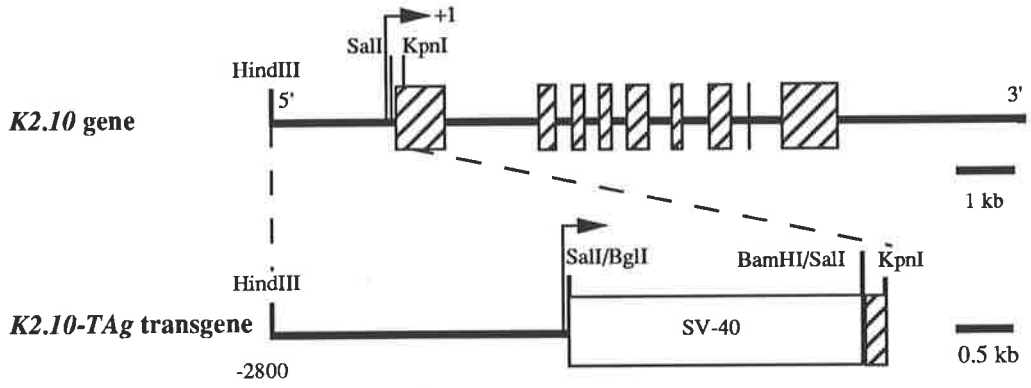


Figure 3.1 Construction of *K2.10-TAg* transgene.

The *K2.10* promoter fragment containing 5' flanking/5' untranslated region (UTR) sequence from a Hind III site (-2800) to a Kpn I site (+271) in the first exon was blunt-end cloned into the Sma I site of pGEM7Zf(+). A 2.7 kb BamH I/Bgl I fragment spanning nucleotides 2533-5235 of the SV-40 genome was ligated into an engineered Sal I site created by site-directed mutagenesis at +53 of the *K2.10* promoter. The SV-40 fragment encodes both small (t) and large (T) tumour antigens and contains the polyadenylation signals. The resulting 5.7 kb fusion construct, *K2.10-TAg*, was excised from the vector by digestion with Cla I (in pGEM7Zf(+) polylinker) and Kpn I. The arrow depicts the transcription start (+1) site; the hatched boxes depict *K2.10* exon sequences.

Figure 3.2 Southern blot analysis of *K2.10-TAg* Fo mice.

(A) Phosphorimage of a Southern blot of Hind III digested genomic DNA (~10 μg) from transgenic mice (Fo-24, Fo-32, Fo-35, Fo-40, Fo-49, Fo-50, Fo-75, Fo-84, Fo-85, Fo-87, Fo-94, Fo-109, Fo-119, Fo-127, Fo-131, Fo-133, Fo-138, Fo-144 and Fo-145; Fo-126 is a non-transgenic control) separated on a 1% agarose gel and then transferred to Zeta-probe GT membrane. The filter was hybridised with the entire 2.7 kb *TAg* probe and washed in 2X SSPE, 0.1% SDS at 65°C.

Despite being identified as transgenic by dot blot analysis (data not shown), no signal could be detected by Southern blot in Fo-32, Fo-35, Fo-109, Fo-133 and Fo-145. This may be due to the lack of sensitivity of this Southern blot to detect 1-2 copies of the transgene. (DNA from Fo-57, a non-expressing *K2.10-TAg* transgenic mouse, was omitted from this blot).

(B) A diagrammatic representation of the *K2.10-TAg* transgene fragment and the possible arrangements of multiple copy insertions in the mouse genome.

1. A line diagram of the *K2.10-TAg* Cla I/Kpn I transgene fragment (5.7 kb). Hind III restriction sites and fragment sizes are marked. The filled region indicates the *TAg* encoded sequences and represents the probe fragment used to identify *K2.10-TAg* transgenics.

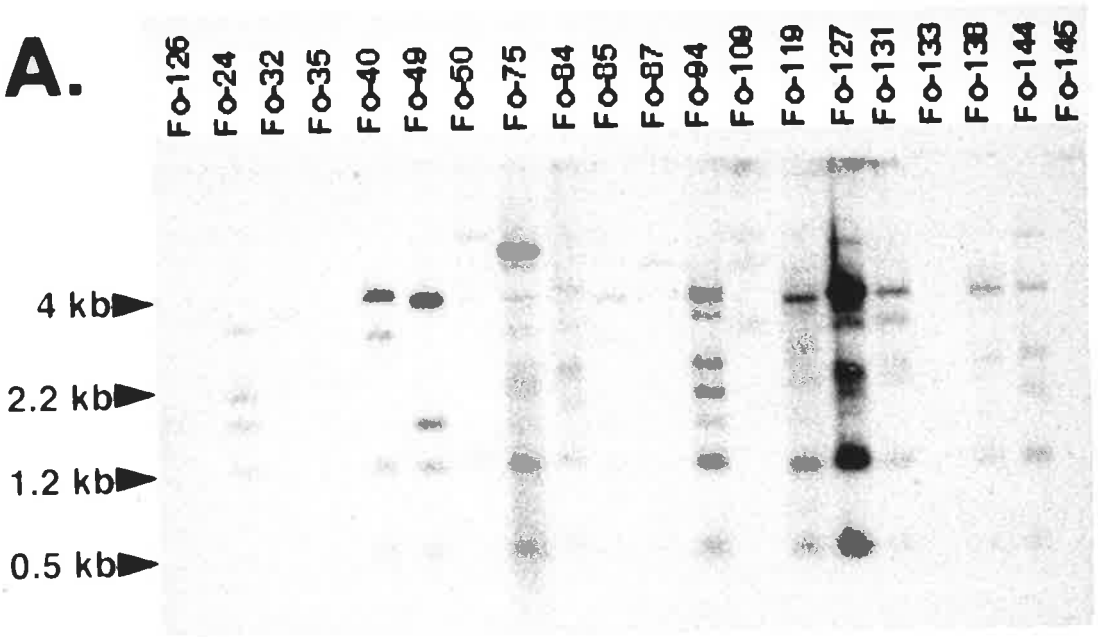
2. A head-to-tail tandem array occurs when multiple copies of the fragment insert into a single locus such that transcription of all transgenes is in the same direction. In this situation Hind III bands of 4.0 kb, 1.2 kb and 0.5 kb will be detected together with a possible minor band >1.1 kb whose size depends upon the position of an adjacent Hind III site in the mouse genome. This pattern occurred in samples Fo-40, Fo-49, Fo-85, Fo-119 and Fo-127.

3. A tail-to-tail tandem array occurs when multiple copies of the fragment insert into a single locus such that adjacent fragments are in opposite directions. In this situation bands of 2.2 kb, 1.2 kb, 0.5 kb and minor bands of variable sized "end fragments" will be seen.

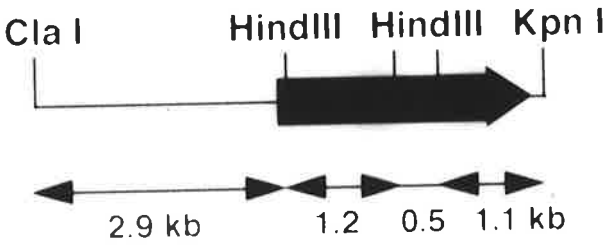
A head-to-tail-to-tail-to-head array (i.e. a combination of the above possibilities in a single locus) will result in all of the above major bands being detected as well as variable minor "end fragments". A similar pattern of hybridising fragments will also arise if there has been multi-copy integration into several loci. Transgenics Fo-75, Fo-84, Fo-94, Fo-131, Fo-138 and Fo-144 show this pattern of integration.

The appearance of major bands of sizes greater than 4 kb indicate either incomplete digestion with Hind III or chromosomal rearrangements. This may explain the pattern seen in samples Fo-50, Fo-75 and Fo-87. The absence of a major band at 4 kb or 2.2 kb, as occurs in Fo-24, suggests DNA rearrangement or mutation.

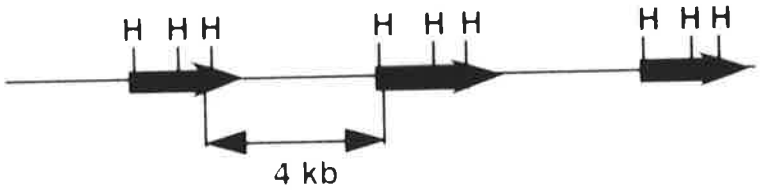
A.



B. 1. *K2.10-TAg* transgene fragment



2. Head-to-tail array



3. Tail-to-tail array

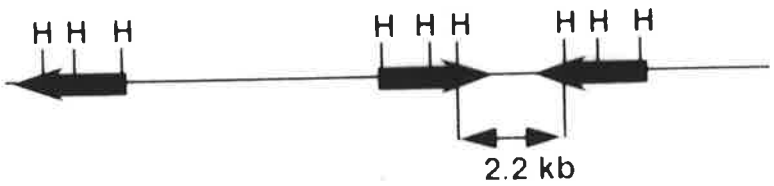
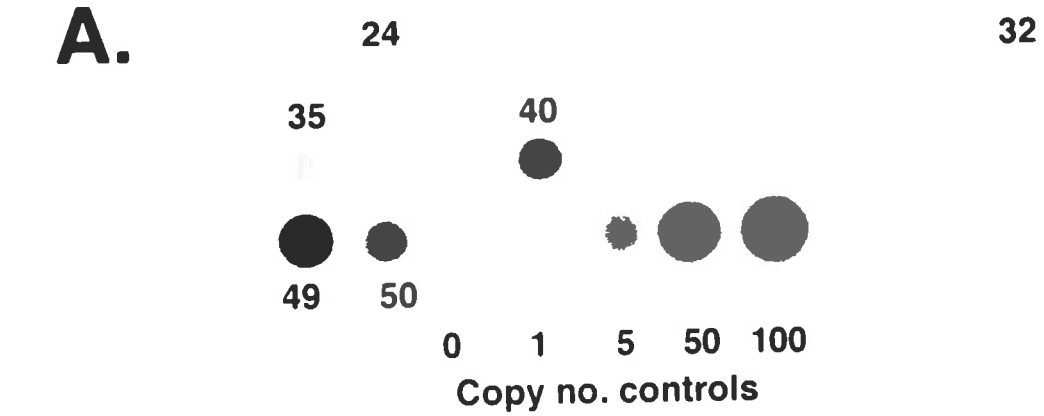


Figure 3.3 Analysis of *K2.10-TAg* transgenic line #40 by dot blot and Southern.

(A) Dot blot of *K2.10-TAg* Fo mouse genomic DNA showing copy number controls equivalent to 1, 5, 50 and 100 transgene copies in 5 μg of mouse genomic DNA. DNA was blotted onto Genescreen membrane and hybridised with *TAg* probe. The filter was washed in 2X SSC, 0.1% SDS at 65°C and autoradiographed. Fo-24, Fo-32, Fo-35, Fo-40, Fo-49 and Fo-50 are identified as transgenic. The copy number of Fo-40 is estimated to be 10-25 copies. DNA loading variations were normalised by re-probing the filter with a mouse *K14* gene probe (data not shown).

(B) Southern blot analysis of line #40 transgenic mice from generations F_0 to F_4. Approximately 10 μg of Hind III digested genomic was separated on a 1% agarose gel and transferred to Zetaprobe GT membrane. The filter was hybridised with TAg probe and washed in 2X SSC, 0.1% SDS at 65°C. The blot shows that the transgene has integrated in a head-to-tail array and is stably inherited over 4 generations. Variation in band intensities is due to loading irregularities. M1, λ digested with Hind III; M2, SPP1 digested with Eco RI.


A.
24
32
35
40
49
50
0
1
5
50
100
Copy no. controls

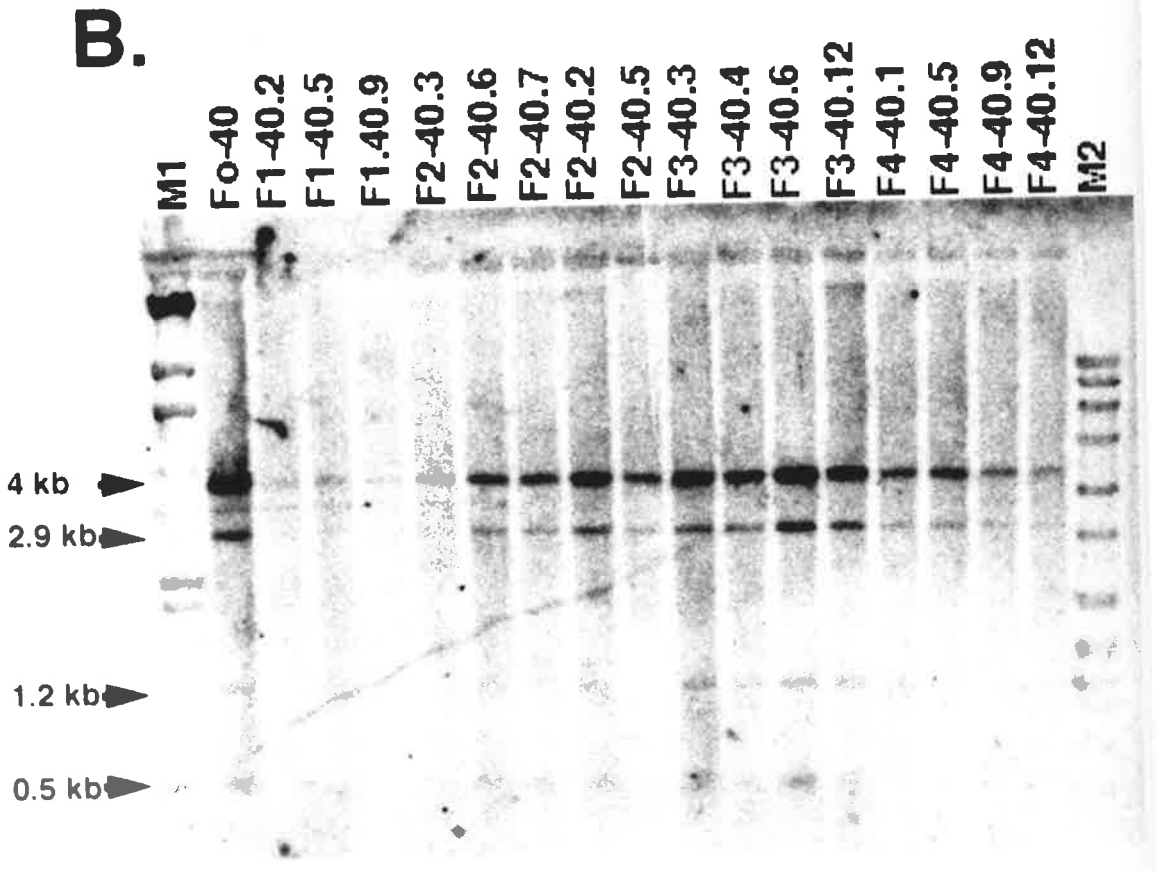

B.
M1
Fo-40
F1-40.2
F1-40.5
F1.40.9
F2-40.3
F2-40.6
F2-40.7
F2-40.2
F2-40.5
F3-40.3
F3-40.4
F3-40.6
F3-40.12
F4-40.1
F4-40.5
F4-40.9
F4-40.12
M2
4 kb
2.9 kb
1.2 kb
0.5 kb

Figure 3.4 Expression of the *K2.10-TAg* gene in the skin of transgenic mice as determined by immunofluorescent staining.

(A) Specific staining with anti-SV40 TAg mAb is localised to the hair follicle cortex (bright image; arrowheads);

(B) Non-specific background staining to outer root sheath and muscle is evident when anti-SV40 TAg mAb is omitted. Note the absence of staining in the follicle bulb and medulla. Bar, (A) 75 μm; (B) 150 μm.

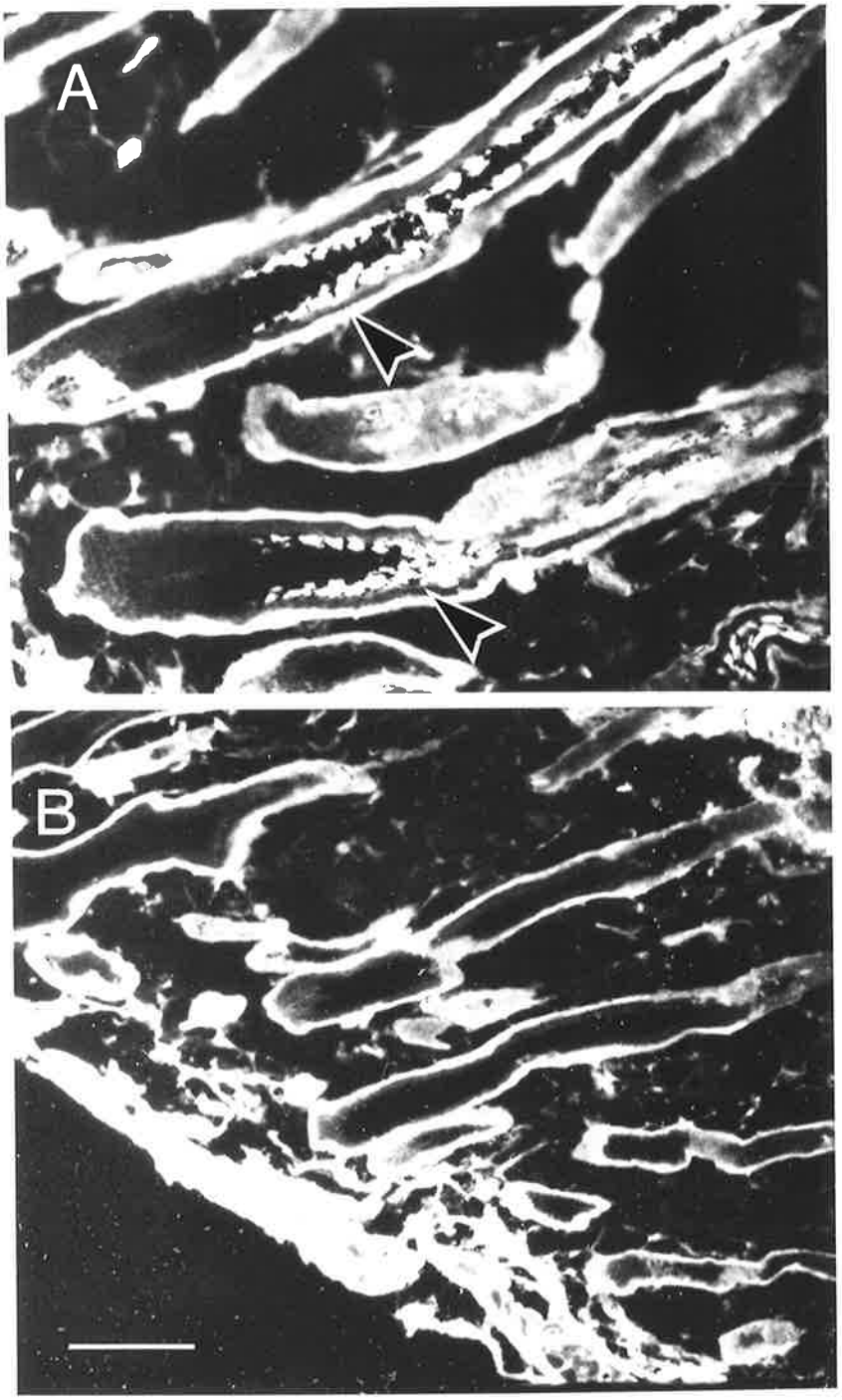

A
B

Figure 3.5 Aberrant hair phenotype of *K2.10-TAg* transgenic mice.

(A) F2-40.7 at 4 months old exhibits the moderate transgenic phenotype with a ruffled coat and some hairloss typical of transgenic mice of the #40 line;

(B) mouse Fo-119 at 1 month of age showing the stubble typical of a strong transgenic phenotype;

(C) mouse Fo-94 (right) along with non-transgenic littermate at 11 days old.

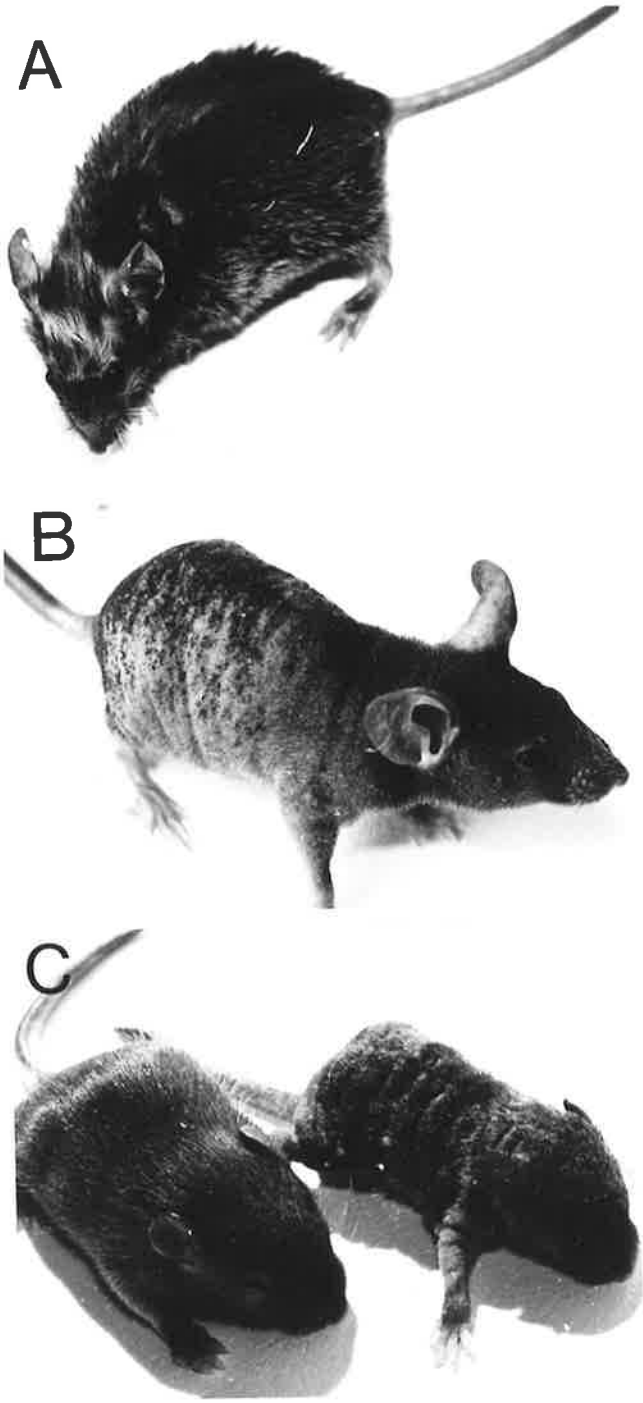
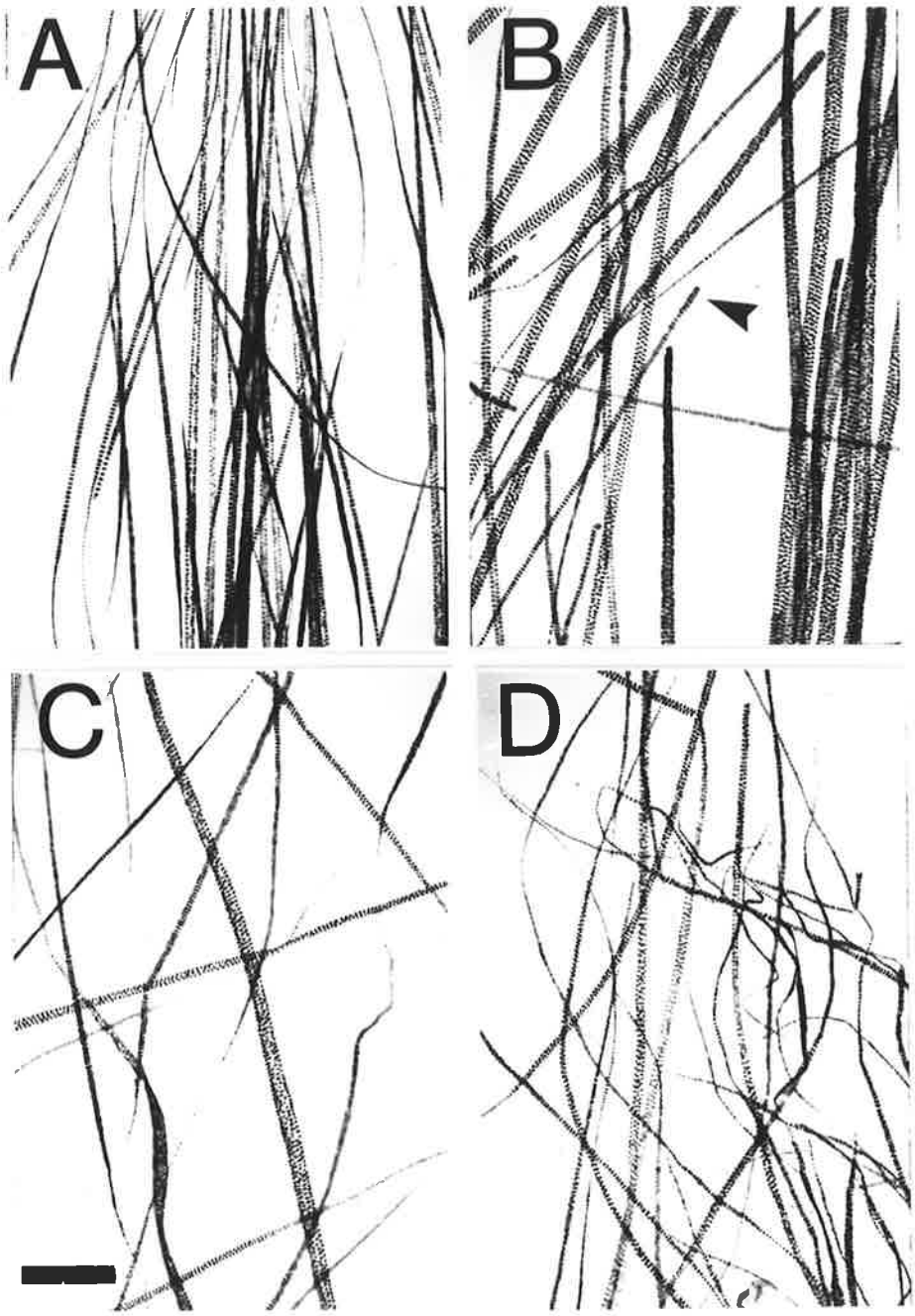

A
B
C

Figure 3.6 Hair fibre phenotypes observed by light microscopy.

Comparison of hairs from a normal mouse (A) with hairs from the moderate phenotype Fo-40 mouse (B) and the severe phenotype mice Fo-49 (C) and Fo-50 (D). Arrowhead in (B) shows broken fibres typical of plucked hairs from these transgenic mice. The ends of plucked hairs from normal mice are usually encased in a capsule. Bar, 2 mm.


A
B
C
D

along the hair shaft in some of the fibres of mice displaying the mild phenotype (#40 line) to a noticable kinkiness and fibre deformation in the more severe phenotypes (e.g. Fo-50).

Skin sections from mice which exhibited severe phenotypes, such as Fo-49, Fo-50 and Fo-75, show malformation of the follicle shafts (figure 3.7). Follicles from less severe phenotypes appear normal. Skin sections from mice Fo-49 and Fo-50 show extensive follicle abnormalities and deformed, folded hairs, many of which appear encysted and unable to pierce the epidermis. An inflammatory response is also evident in the dermis of these mice.

Electron micrographs of the transgenic hairs from Fo-40, Fo-49, Fo-50 and F_1-40.9 show that the fibres are composed of a mixed population of normal cortical cells and abnormal cortical cells in which the cellular ultrastructure appears grossly altered (figure 3.8). The proportion of affected cells varied between fibres from approximately 30-75% of cortical cells. Normal cortical cells exhibit a characteristic "fingerprint pattern" due to the closely-packed ordered arrays of IFs and densely-staining KAPs. This pattern is less obvious or completely absent in the abnormal cells, appearing instead as a densely-stained structureless mass. Clearly, keratin assembly and packing is disrupted in these abnormal cortical cells.

3.2.3 Analysis of hair protein

To further investigate the abnormal ultrastructure of the cortical cells of the *K2.10-TAg* hairs, amino acid analyses were performed on transgenic hair samples. Table 3.1 shows the amino acid composition of hair from a normal mouse, a *K2.10* hair-loss mouse (Powell and Rogers, 1990), a severe phenotype *K2.10-TAg* mouse (Fo-127), and two mice of the #40 line - a *K2.10-TAg* heterozygote (F_3-40.3) and a homozygote (F_3-40.3.6). Amino acid compositions of small (t) and large (T) tumour antigens calculated from the protein sequences are also included. There are significant differences in the composition of many amino acids between the transgenic and normal mouse hair samples, the most dramatic differences being in hairs from F_3-40.3.6. Large, notable changes are an increase in glutamic acid/glutamine and a decrease in glycine and cysteine levels. These changes in the amino acid compositions are not consistent merely with an overexpression of the tumour antigen proteins and this is highlighted by the increase in glutamic acid/glutamine levels in transgenic hairs.

In order to examine the effect of TAg expression on the protein profile of the hair, one- and two-dimensional gel analyses of S-carboxymethylated proteins extracted from normal and transgenic

Figure 3.7 Histology of the skin of *K2.10-TAg* transgenics.

(A-C) Low power magnification of skin from a normal 27 day old mouse (A), Fo-40 at 4 months (B) and Fo-50 at 24 days old (C). Note the decreased thickness of the epithelium in the severely affected phenotype, Fo-50 (C), compared with the moderate phenotype, Fo-40, and normal mouse skin of approximately the same age (A). (D-F) Higher power magnification of skin from Fo-40 (D), Fo-49 (E) and Fo-50 (F). Note the presence of abnormal follicles and fibre deformation in all transgenic mice. Bars, (A-C) 100 μm; (D-F) 50 μm.

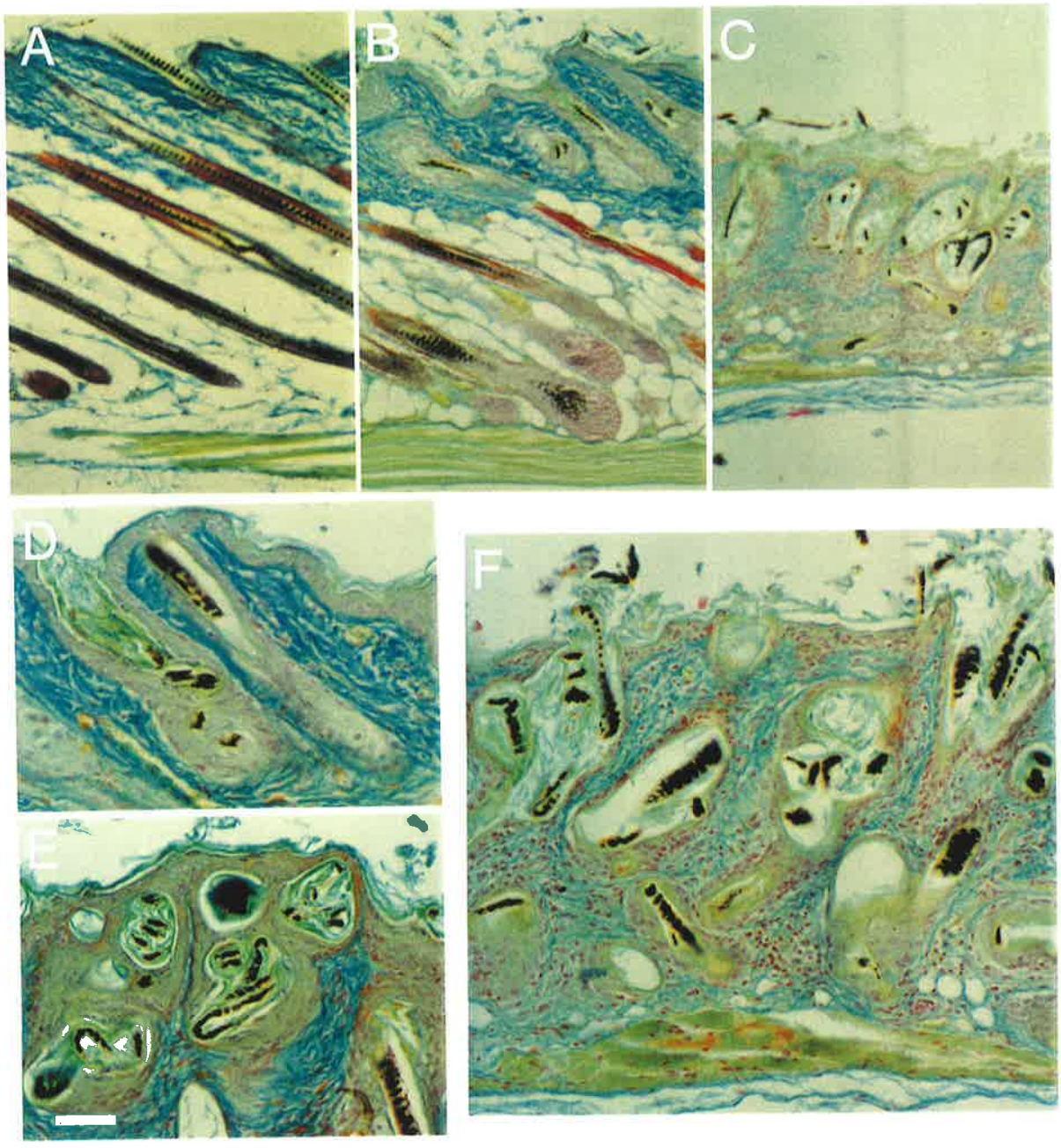

A
B
C
D
E
F

Figure 3.8 Electron micrograph of follicle cortical cells from Fo-49 transgenic mouse.

(A) Transgenic fibres contain a mixture of both normal (n) and abnormal (ab) cells.

(B) Higher magnification of cortical cells showing the distinct ultrastructure of normal cortical cells (n) and the abnormal cells (ab) which appear devoid of structure. Bars, (A) 300 nm; (B) 100 nm.

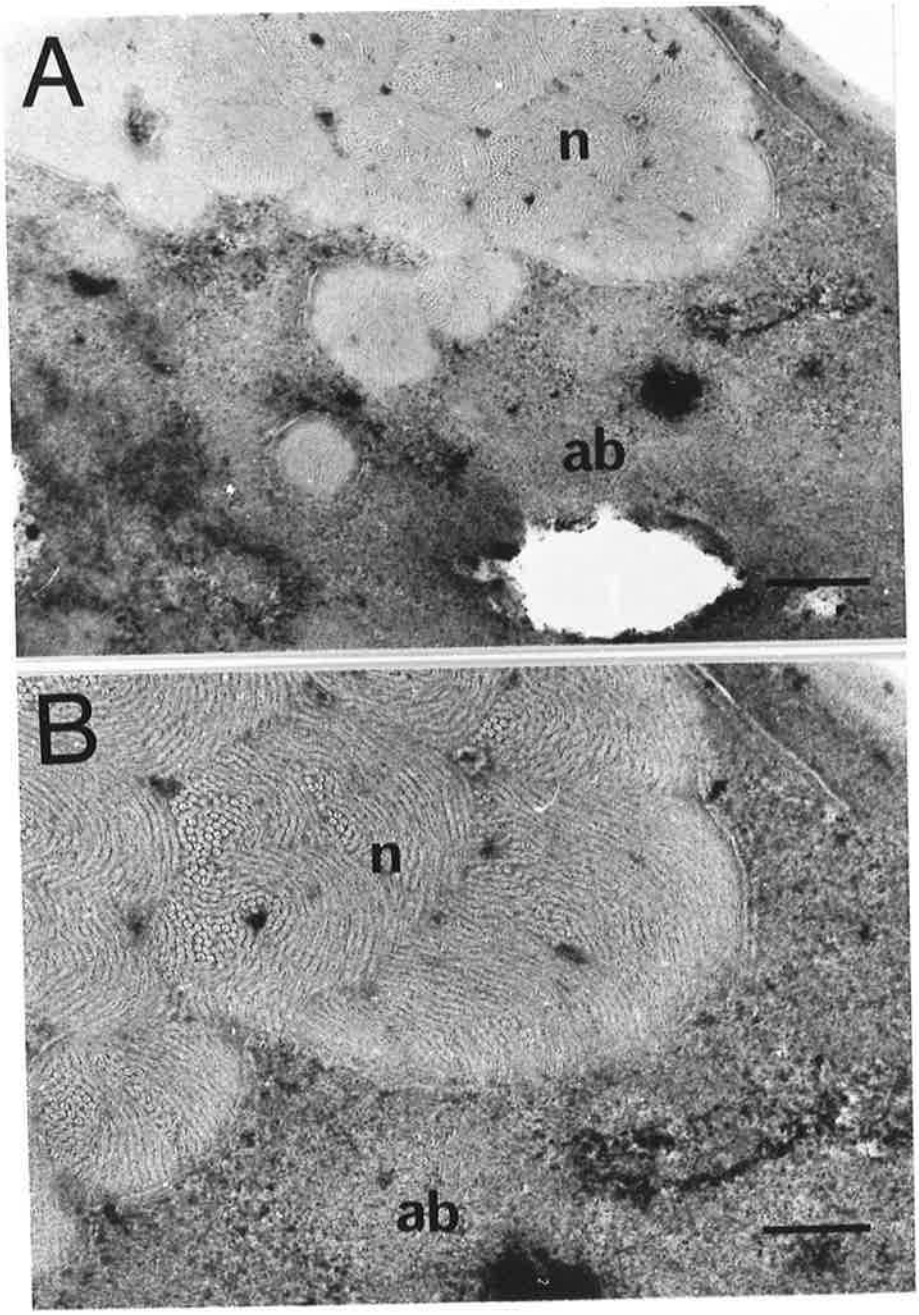

A
n
ab
B
n
ab

Table 3.1 Amino acid compositions of hair from *K2.10-TAg* transgenic mice and normal mice

	normal*	K2.10‡	F_3-40.3§	F_3-40.36‖	Fo-127¶	T antigen#	t antigen#
Asp	5.2	7.3	6.2	6.7	6.1	11.6	11.4
Thr	4.7	4.5	4.6	4.5	4.6	4.3	2.9
Ser	9.6	8.1	8.7	8.0	8.6	6.6	2.9
Glu	13.0	16.1	15.0	16.2	14.5	12.2	8.6
Pro	6.8	4.6	6.0	5.9	6.0	4.5	3.4
Gly	10.4	8.7	9.0	8.3	9.3	5.1	6.3
Ala	4.2	6.3	5.0	5.2	4.8	5.4	4.6
Cys	13.6	7.9	10.2	9.2	10.9	2.1	6.3
Val	4.2	5.2	4.6	4.7	4.4	4.8	3.4
Met	0.8	1.1	1.0	1.1	1.0	3.2	5.7
Ile	2.3	3.2	2.7	2.9	2.7	4.2	2.3
Leu	6.2	8.2	7.1	7.7	7.0	10.0	13.2
Tyr	5.0	3.2	4.1	3.8	4.5	3.7	4.0
Phe	2.7	2.9	2.9	3.0	2.9	5.1	4.0
His	1.1	1.1	1.3	1.4	1.2	2.7	1.7
Lys	3.0	4.9	3.8	4.3	3.7	8.9	10.9
Arg	7.0	6.5	7.7	6.9	7.7	3.9	4.6

*Normal littermate of F_3-40.36

‡*K2.10* hairloss transgenic mouse from [Powell, 1990 #46]

§F_3 mouse from #40 line (heterozygous for *K2.10-TAg*)

‖F_3 mouse from #40 line (homozygous for *K2.10-TAg*)

¶F_o-127, severe phenotype

#Calculated values from SV-40 large (T) and small (t) tumour antigen protein sequence obtained from Swiss-Protein Database

hairs were performed (figure 3.9). Two-dimensional gel analysis of ^{14}C-S-carboxymethylated proteins extracted from normal mouse hair and hair from Fo-40 suggests that, whilst the overall protein profile is similar, there is a decrease in the cysteine-rich and glycine/tyrosine containing KAP proteins in Fo-40 hair. Closer examination of the lower molecular weight KAP proteins from two normal mouse hair samples and three #40-line offspring hair samples analysed by tricine gel separation showed marked differences in the protein profiles of transgenic and non-transgenic hairs. A reduction in the intensity of many protein bands is evident in the transgenic samples whilst the IF proteins remain relatively unchanged. The affected proteins correspond to the expected molecular weights of proteins from many of the KAP families (Crewther, 1976); for example, the glycine/tyrosine containing KAP proteins (6-9 kD), the KAP3 proteins (11 kD), the KAP2 proteins (16-17 kD) and the KAP1 proteins (19-21 kD).

3.2.4 Other aspects of the *K2.10-TAg* phenotype

The *K2.10-TAg* transgenic phenotype was not restricted to the hairs of these mice. Transgenic mice were generally smaller than non-transgenic littermates and some showed severely retarded growth rates (figure 3.5). The vertebrae in many of these mice were also affected. Four founder mice, including Fo-40, had kinks in the tail, as did many but not all of the transgenic progeny of line #40. X-ray analysis of mouse Fo-40 showed malformed vertebral discs in the tail and curvature of the spine (figure 3.10). Mice with spinal abnormalities often developed partial paralysis of the hind legs. All transgenic mice had a reduced life expectancy ranging from a few days to 9 months (table 3.2) which tended to correlate with the severity of the phenotype.

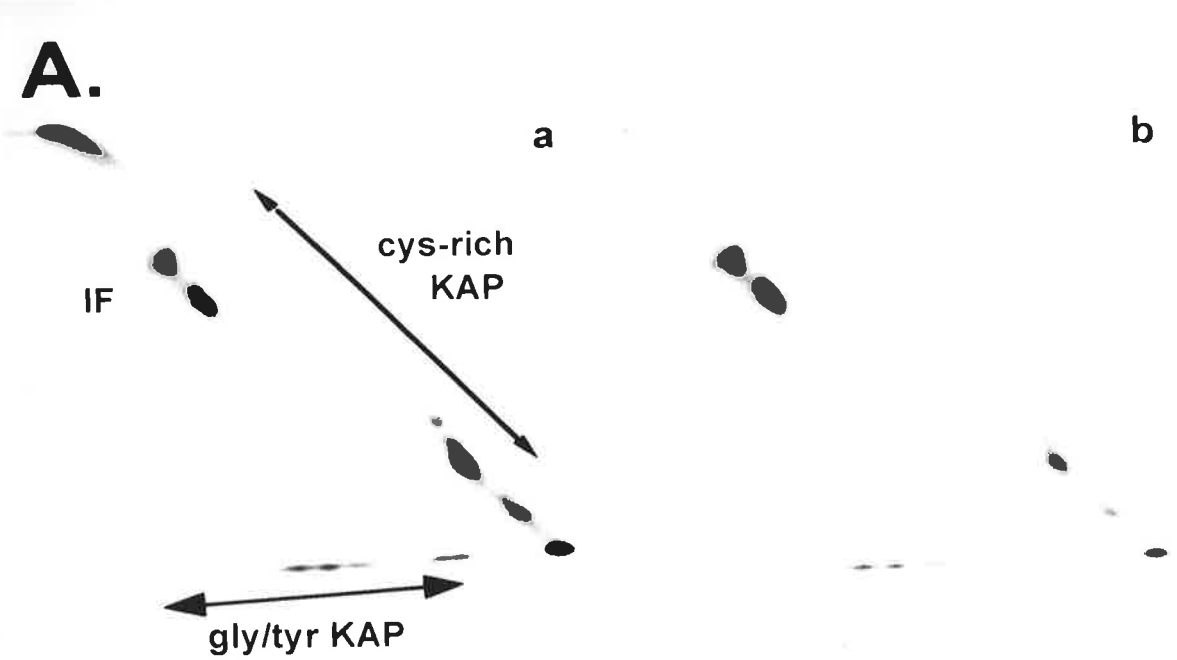
Many mice developed obvious ectopic tumours such as growths in the bladder (figure 3.11), stomach or intestine with secondary tumours in the liver. RNA protection analysis of several tumorous tissues showed the expression of TAg in these tumours (figure 3.12). However, no expression was detectable in equivalent normal, non-tumorous tissue in *K2.10-TAg* transgenic mice. The cause of distress or death was not obvious for all mice and extensive autopsies were not performed.

Despite the occurrence of ectopic tumours and an obvious effect of TAg expression in the hair follicle, none of the mice developed tumours in the skin or hair follicles and microscopic inspection of hair follicle sections failed to show any neoplastic lesions in the skin or hair follicles (see chapter 4).

Figure 3.9 Protein analyses of transgenic hair samples.

(A) Two-dimensional gel analysis of ^{14}C-S-carboxymethylated protein from normal mouse hair (a) and from Fo-40 hair (b). Proteins corresponding to IF, cysteine-rich KAP and glycine/tyrosine rich KAP families are indicated. Both protein samples (50 μg) were prepared and electrophoresed under identical conditions to enable comparison between the samples (see 2.2.4.2i).

(B) Tricine gel analysis of S-carboxymethylated protein from normal mouse hair (lanes 1 and 2) and protein from #40-line transgenic mice (lanes 3, 4 and 5). Samples (70 μg) were electrophoresed on a 14% polyacrylamide gel and stained with Coomassie Blue. Protein bands showing obvious changes in transgenic samples are indicated (arrowheads). Densitometric analysis of the gel indicates that there are significant decreases (30-50%) in these proteins in the transgenic samples compared with the normal samples. Loading variations were normalised to the IF proteins of the normal samples. M, 5 μg low molecular weight protein markers (Gibco BRL); sizes are as shown.


A.
a
b
IF
cys-rich
KAP
gly/tyr KAP

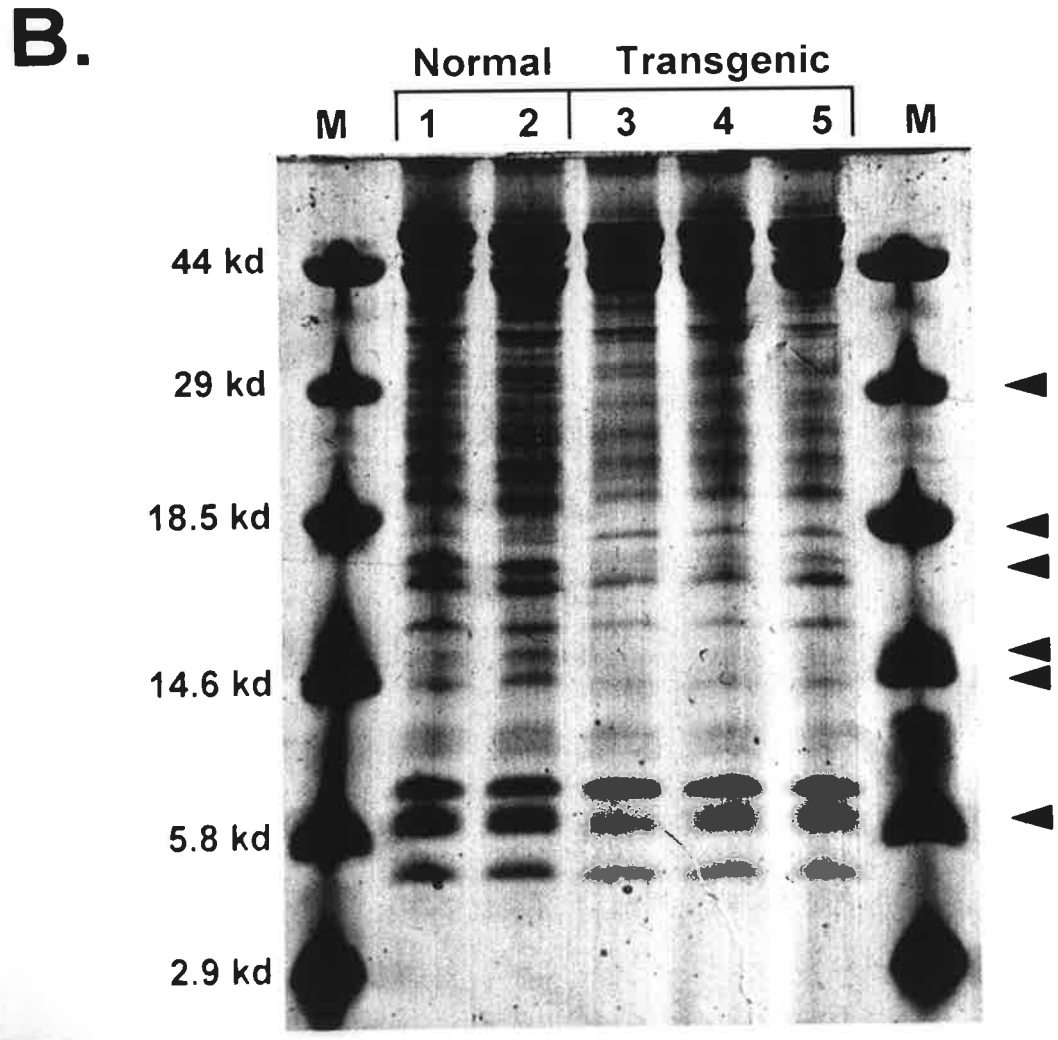

B.
Normal
Transgenic
M
1
2
3
4
5
M
44 kd
29 kd
18.5 kd
14.6 kd
5.8 kd
2.9 kd

Figure 3.10 Dorsal X-ray of Fo-40 at 5.5 months old.

Fo-40 displayed a moderate hair phenotype. At 5 months old Fo-40 displayed weight loss and partial paralysis of the hind legs and marked curvature of the spine. X-ray analysis showed malformed disks in the tail (arrowheads) and signs of wear on some spinal disks. Autopsy showed no obvious signs of tumours or neoplasia. Loss of function in the hind legs was thought to be due to pinching of the spinal cord near the shoulders.

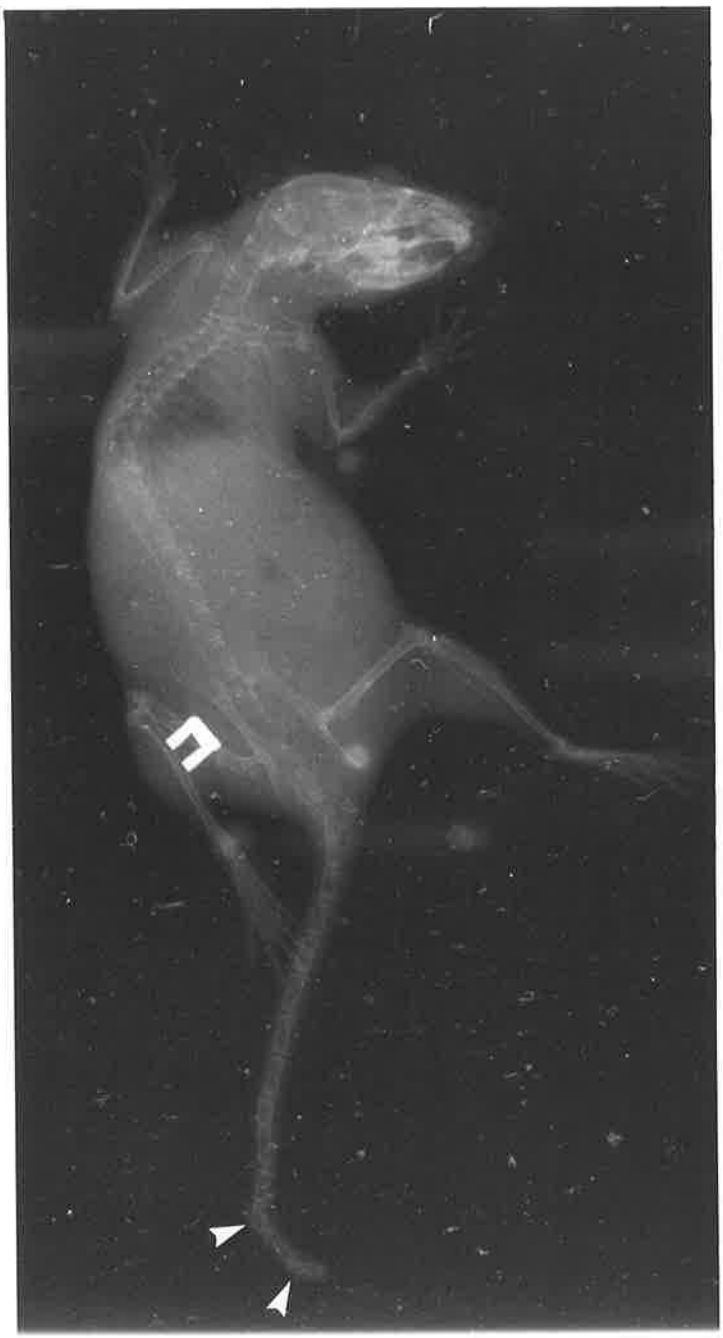


Table 3.2. Mortality of *K2.10-TAg* mice.

mouse	copy number*	lifespan‡	overall phenotype
F_o-49	25-50	3 months	severe; paralysis of hind legs
F_o-50	10-25	24 days	very severe
F_o-75	5-10	17 days	very severe
F_o-94	25-50	11 days	very severe
F_o-119	10-25	5 months	severe; intestinal tumour
F_o-127	50	2.5 months	severe
F_o-131	10-25	4 months	moderate
F_o-138	1-5	6 months	moderate
F_o-144	10-25	3 months	severe
F_o-40	25-50	5.5 months	moderate; paralysis of hind legs
F_1-40.1	25-50	8 months	moderate; stomach tumor; liver secondaries
F_1-40.2	25-50	5 months	moderate
F1-40.3	25-50	6 months	moderate
F_1-40.9	25-50	9 months	moderate; intestinal tumor; liver secondaries
F_2-40.5	25-50	5 months	moderate; bladder tumor
F_3-40.6	25-50	3.5 months	moderate; bladder tumor

* Approximate *K2.10-TAg* gene copy number.

‡ Approximate lifespan; mice were killed when they became sick or severely stressed.

Figure 3.11 ***K2.10-TAg*** **mouse F_3-40.6 showing bladder tumour.**

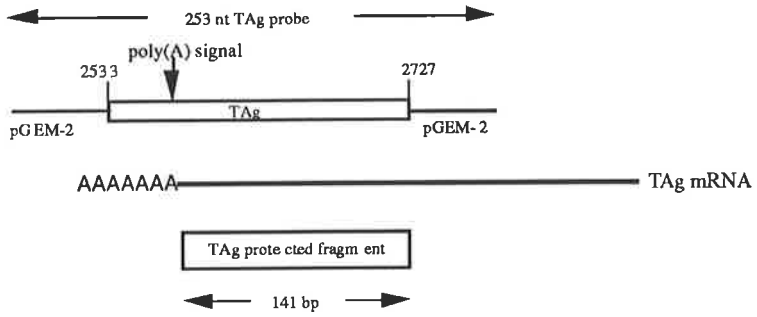
Autopsy at 3.5 months old revealed a tumorous growth blocking the base of the bladder such that the bladder was full and distended.

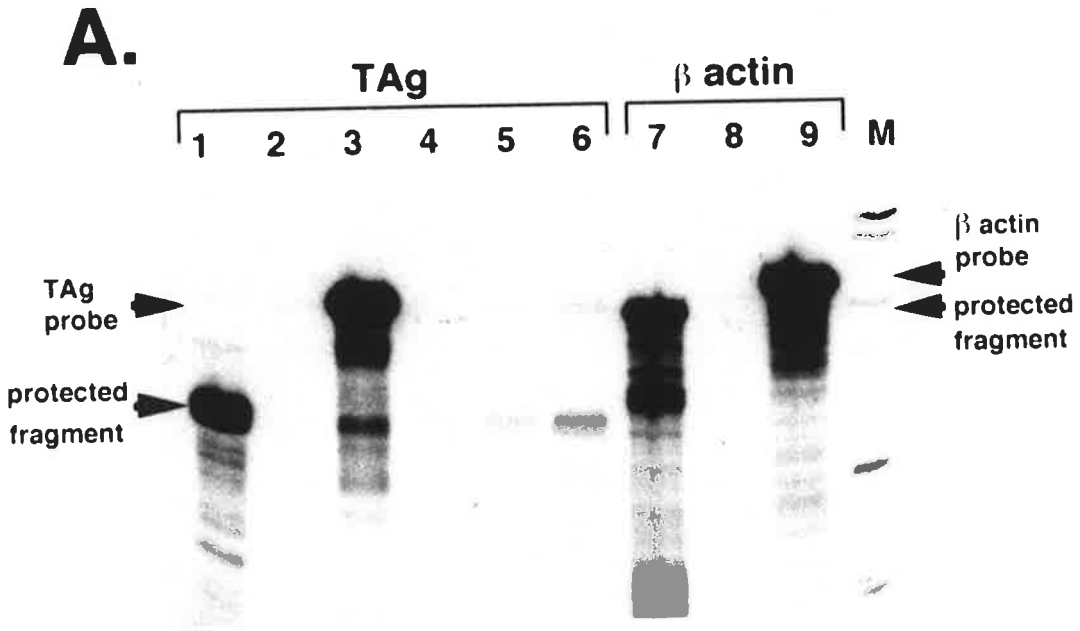
Figure 3.12 RNA protection analysis of TAg expression in tumours and non-tumorous tissues from *K2.10-TAg* transgenic mice.

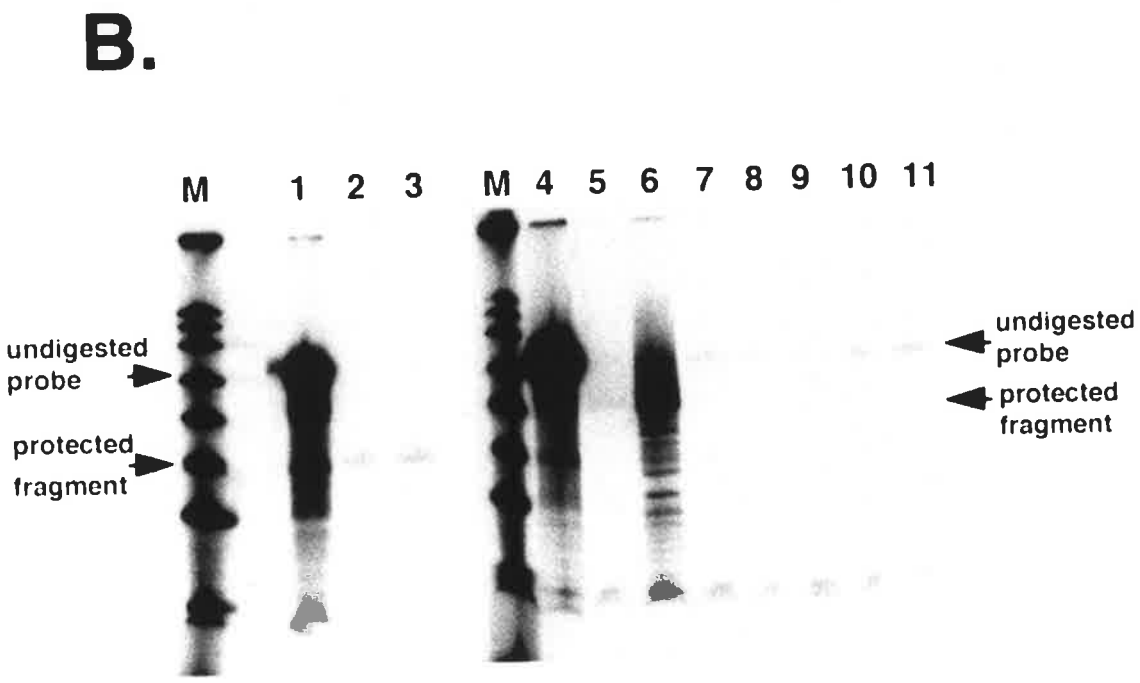
(A) RNA protection analysis of Fo-119 intestinal tumour RNA. Tumour RNA (10, 20 and 50 μg) was analysed for TAg expression (lanes 4, 5 and 6) together with COS cell RNA (10 μg) as a positive control (lane 1) and yeast RNA (10μg) probe controls (lanes 2 and 3; + RNase treatment and - RNase treatment respectively). RNA samples were hybridised with a TAg antisense riboprobe (253 nt) transcribed from pG2TAg (see 2.1.12) to produce a protected TAg fragment of 141 bp as shown. Tumour RNA was also hybridised with a 300 nt mouse β actin antisense probe (lane 7) and yielded a 250 bp protected fragment as shown. β actin probe controls with yeast RNA (10 μg), -RNase and +RNase treatment, were included (lanes 8 and 9 respectively). Samples were separated on a 6% polyacrylamide, 7M urea gel and autoradiographed. Note the low level of TAg expression compared with the relatively abundant β actin mRNA. M, labelled markers of pUC19 DNA digested with Hpa II.

(B) RNA protection analysis of liver tumour and non-tumorous tissues from transgenic mice. RNA was isolated from the skin (including hair follicles; lanes 2 and 6, 10 μg and 20 μg respectively), liver, spleen, intestine, stomach and heart (lanes 7-11 respectively; 50 μg each) from a 10 day old transgenic mouse and from a tumour-ridden adult mouse liver (lanes 3 and 5, 50 μg and 20 μg respectively). Lanes 1 and 4 are undigested probe controls. RNA was hybridised with TAg antisense riboprobe (253 nt) to yield a protected fragment of 141 bp. Samples were separated on a 6% polyacrylamide, 7M urea gel and autoradiographed. TAg expression was detected in transgenic skin (lanes 2 and 6) and the liver tumour (lanes 3 and 5) but not in non-tumorous transgenic tissues. M, labelled markers of pUC19 digested with Hpa II.

The line diagram below represents the 253 nt cRNA probe containing SV-40 sequences from nt 2533-2727 of the SV-40 genome. This genomic probe fragment spans the polyadenylation site in the 3' UTR of the TAg mRNA and hybridises with the TAg mRNA to yield a 141 bp protected fragment (spanning nt 2586-2727 of the SV-40 genome).




A.
TAg
β actin
1 2 3 4 5 6 7 8 9 M
TAg probe
protected fragment
β actin probe
protected fragment


B.
M 1 2 3 M 4 5 6 7 8 9 10 11
undigested probe
protected fragment
undigested probe
protected fragment

3.3 Discussion

3.3.1 Expression of SV-40 T antigen

The expression of the sheep *K2.10* gene, used to target SV-40 TAg expression to the follicle cortex in this study, had previously been analysed in transgenic mice (Powell and Rogers, 1990). The gene encodes a keratin IF type II protein and high level expression in mice produces a hair-loss phenotype. The 2.8 kb *K2.10* gene promoter fragment encompassing the immediate 5' flanking/5' UTR sequence is sufficient to direct the expression of SV-40 TAg to the hair follicle cortex (see figure 3.4), maintaining the localisation and timing of expression of the endogenous *K2.10* gene in the sheep follicle and the expression of the larger transgene seen in the *K2.10* hair-loss transgenic mice.

TAg expression in the hair follicle was demonstrated by immunolocalisation however, despite several attempts, TAg mRNA was not able to be detected by cRNA *in situ* hybridization to mouse skin from several *K2.10-TAg* transgenics (i.e. Fo-40, Fo-94, Fo-127, Fo-138, Fo-144). This suggests either very low level expression from the 2.8 kb *K2.10* promoter fragment or instability of the TAg mRNA. TAg mRNA was detectable in transgenic mouse skin (see figure 3.12) and in isolated hair follicles by RNA protection assay (data not shown).

Growth retardation (see figure 3.5), tumour formation in the bladder (see figure 3.11), stomach, intestine and other tissues and vertebral abnormalities (see figure 3.10) in *K2.10-TAg* mice appear to be a consequence of TAg expression, as different Fo mice show the same phenotype and *TAg* mRNA has been demonstrated in tumours from these mice (see figure 3.12). However, *TAg* expression could not be detected in non-tumorous tissue (see figure 3.12), and *in situ* hybridization experiments on day 16 *K2.10-TAg* mouse embryos did not detect any *TAg* expression (data not shown). However, *in situ* hybridization experiments on a day 14-15 *K2.10* hair-loss mouse embryo showed some *K2.10* expression in the jejunum of the intestine (B. Powell, personal communication). It is not known whether TAg expression in the tumours is promiscuous or whether low level expression of hair keratins normally occurs in these tissues or in subsets of cells within them. It is known that keratins such as K19 are expressed in small subpopulations of cells for short periods during embryonic development and under certain pathological conditions (Van Eyken *et al.*, 1988a, 1988b, 1988c). Control of expression of keratins K18 and K8 is also thought to be leaky as they are occassionally expressed in some non-epithelial cells and tumours (Knapp and

Franke, 1989). Non-follicular transgene expression may also be due to the lack of tight controlling sequences, gene silencers for example, which lie outside of the 2.8 kb *K2.10* gene promoter fragment used to drive TAg expression.

Interestingly, there are reports of novel expression patterns of several chimeric genes in transgenic mice where expression occurs in tissues in which the targeting promoter is normally inactive (Behringer *et al.*, 1988; Lew *et al.*, 1993). In growth hormone factor-1/TAg mice (Lew *et al.*, 1993), expression of TAg occurred in resultant intestinal tumours but not in adjacent non-tumorous intestinal tissue suggesting only a select population of cells was affected. In mouse protamine-1 (mP1)/SV40 mice (Behringer *et al.*, 1988) ectopic expression was thought to be due to novel regulatory elements generated by the juxtaposition of the *mP1* promoter and *TAg* sequences.

Hair-like keratins have been found in hoof, claw and nail in several species (Marshall and Gillespie, 1977; Lynch *et al.*, 1986; Heid *et al.*, 1988b), and in specific cell populstions of tongue and thymus (Heid *et al.*, 1988b; Tobiasch *et al.*, 1992), although no abnormalities in those tissues were obvious in *K2.10-TAg* mice (data not shown). The mechanisms by which TAg affected vertebral development and growth rates was not investigated in this work.

3.3.2 Effects of T antigen on hair and folicle morphology

Expression of TAg in the cortical cells of the hair follicle has obvious effects on the morphology of the hairs and whiskers of mice (see figures 3.5 and 3.6). In transgenic mice with a moderate phenotype the pelage fibres were crooked and the whiskers were distinctly curly, but follicle morphology appeared virtually normal. In the more severe phenotypes (such as Fo-49 and Fo-50) the follicles, fibres and skin were grossly affected. Many hairs appeared so weakened and malformed that they could not penetrate the epidermis, resulting in ingrown hairs. The follicles themselves were grossly altered in their morphology and an inflammatory response in the skin was evident.

The expression of several transgenes in mouse follicles has been shown to affect hair and follicle morphology (Powell and Rogers, 1990; Blessing *et al.*, 1993; Missero *et al.*, 1993) producing wavy fibres and hair-loss. Unlike the overexpression of sheep K2.10 in the mouse follicle, cyclic hair-loss is not seen in the *K2.10-TAg* mice. In the less severe phenotypes it appears that the limited hair-loss is due to abrasion, as hair-loss occurs predominately on the underside of the mice (especially on suckling mothers) and around the snout and head where scratching is more frequent.

This suggests an increased brittleness or susceptibility to breakage which is not as severe as in the *K2.10* hair-loss mice. Similar phenomena occur in follicles of mice carrying the *naked* (*N*) gene (Raphael *et al.*, 1982) and in wool follicles of wheat-fed sheep given methionine supplements (Chapman and Reis, 1978) where poor keratinisation of the cortical cells produces weakened fibres and limited hair-loss in moderate phenotypes, whilst severe phenotypes show gross follicle deformation similar to that seen in the skin of mice Fo-49 and Fo-50 (see figure 3.7).

SV-40 TAg expression altered the ultrastructure of the cortical cells, and affected cells lack the ordered arrays of IF, appearing as a structureless mass in electron micrographs (see figure 3.8). This suggests that macrofibril formation is incorrectly organised or retarded or indiscernible due to a lack of densely-staining KAP protein to contrast the IF structure. The amino acid analysis data show changes in the levels of many amino acids, most notably cysteine, glycine and glutamine/glutamic acid. These changes do not reflect the composition of TAg as the levels of glutamic acid/glutamine in transgenic hair are increased whilst the levels of arginine remain relatively normal which does not correlate with predicted changes due to TAg protein. Further, the magnitude of change of some of the other amino acids is inconsistent with predicted changes resulting from TAg overexpression; for example, the change in lysine content compared with the change in glycine. These data show that the tumour antigens do not form a significant component of transgenic hair protein and therefore, it is unlikely that the disruption of cellular ultrastructure is due to large deposits of TAg. However, both the amino acid analyses and protein gel data suggest that the expression of the transgene alters the normal protein composition of the cortical cells. The decrease in the content of cysteine, glycine and tyrosine in the transgenic hairs is consistent with a decrease in the cysteine-rich KAP families and the glycine/tyrosine-rich KAP families. A decrease in the abundance of several proteins corresponding to the molecular weights of these KAP protein families is also evident (see figure 3.9). It should also be noted that a decrease in cysteine-rich proteins was also seen in transgenic mice in which K2.10 was overexpressed (see table 3.1). This was attributed to a competition for common transcription factors and/or steric hindrance of the increased amounts of IF protein preventing translation of later KAP mRNAs (Powell and Rogers, 1990).

The expression of a foreign protein per se in the follicle (for example, β-galactosidase) will not necessarily produce morphological changes in the follicle or fibre (see 5.2). However, the hair-loss phenotype of mice over-expressing the sheep *K2.10* transgene is dramatic. A less severe phenotype is seen in sheep expressing the *K2.10* transgene, although changes in the expression levels of other hair keratins is evident at the mRNA level where there appears to be a reduction in expression of

both IF type I and other type II genes as well as many of the KAP genes (S. Bawden, personal communication).

It is not known how TAg is effecting the change in hair protein composition and it is possible that the hair phenotype of these transgenic mice is an indirect consequence of the systemic effect of TAg in various internal organs. TAg has been shown to affect the activity of a number of viral and cellular promoters, either through direct interaction with transcriptional activators and the transcriptional machinery (Mitchell *et al.*, 1987; Gruda *et al.*, 1993; Martin *et al.*, 1993), or indirectly by the induction of transcription factors such as Sp1 (Saffer *et al.*, 1990) and c-jun (Endo, 1992). In *K2.10-TAg* transgenic hairs TAg protein is present in the cortical cells, therefore the nuclear-localised TAg could be acting at the level of gene transcription, as it is known to bind transcription factors such as AP-2 (Mitchell and Tjian, 1989) which may be involved in the expression of hair keratin genes (Powell *et al.*, 1991).

The presence of fibres composed of normal and abnormal cortical cells in the *K2.10-TAg* mice is an interesting phenomenon. It is possible that several Fo mice may have been chimeras (no offspring could be obtained to determine this), and given the polyclonal nature of hair follicle origin (Schmidt *et al.*, 1987), it would then be possible to obtain fibres composed of mixed cortical cell populations. However, this does not explain the stable, heritable mosaic pattern of abnormal and normal cortical cells in the #40 line. A similar heritable mosaic pattern of reporter gene expression was seen in the intestinal villi of a pedigree of L-fatty acid binding protein-human growth hormone (*L-FABP-hGH*) transgenic mice (Roth *et al.*, 1991). In these mice, polyclonal villi comprised both hGH-positive and hGH-negative enterocyte populations. It was postulated that this expression pattern arose from "position-effect variegation" (Karpen and Spradling, 1991) associated with the mosaic expression of transgenes inserted adjacent to a junction between euchromatin and heterochromatin. We have observed hair follicle mosaicism in at least 2 Fo *K2.10-TAg* mice (e.g. Fo-40 and Fo-50), and in several other transgenic mice using a shorter *K2.10* promoter fragment (see 5.2) and a hair follicle KAP gene promoter (B. Powell, personal communication). Mosaic-like patterns of expression have also been reported in some *K19* transgenic mice (Bader and Franke, 1990). These genes may be predisposed to insertion at euchromatin/heterochromatin junctions or this phenomenon may be more frequent than is reported.

3.3.3 Lack of follicle cell immortalisation

Immortalisation of many different cell types has been achieved through the targeted expression of SV-40 TAg in transgenic mice (for example, Bryce *et al.*, 1993; Cartier *et al.*, 1993; Yamada *et al.*, 1990) with many of the resultant cell lines maintaining differentiated characteristics. It was reasoned that, in using the sheep *K2.10* promoter to drive TAg expression in the cortical cells of the follicle, the differentiation-dependent expression of TAg would result in useful cell lines with cortical cell characteristics. However, despite the expression of TAg in the follicle cortex and the occurrence of tumours in various tissues of the *K2.10-TAg* mice, no hyperplasia or neoplasia was detected in the hair follicles. This apparent lack of cortical cell immortalisation was investigated further in the following chapter.

Chapter Four

Investigation into the lack of *K2.10-TAg* follicle cell immortalisation

4.1 Introduction

SV-40 TAg is known to have a powerful oncogenic effect, producing tumours and cell immortalisation in a vast range of cell types (for reviews see Hanahan, 1988; Adams and Cory, 1991). However, targeted TAg expression in the hair follicle cortex failed to produce hair follicle tumours in the transgenic mice *in vivo*. Whilst initially surprising, this could have been due to several possibilities. One hypothesis is that any transformed cells would be rapidly pushed up the follicle by the continually dividing and differentiating bulb cells and be extruded with the hardened fibre, preventing tumour formation. Alternatively, TAg expression could have been targeted too late in follicle differentiation for cortical cell immortalisation to occur because the cells were no longer capable of cell division and had permanently exited from the cell cycle.

The work in this chapter addresses these two possibilities using follicle culture techniques to allow time for immortalisation to occur in culture, and *in situ* hybridisation analysis to examine the distribution of cycling and differentiating follicle cells. The use of alternative gene promoters to target TAg expression to the follicle is also investigated.

4.2 Results

4.2.1 Follicle cells in culture

Follicles from newborn 3-5 day old transgenic mice were isolated essentially as described by Rogers *et al.* (1987) and plated into dishes as intact follicles. Follicles were also dispersed into single cells by treatment with EDTA and trypsin (Weinberg *et al.*, 1991) and plated out, however this method appeared to reduce total cell viability by about 20-30% (data not shown). Isolated pelage follicles appear as elongated clumps under phase microscopy, with some follicles showing a well developed hair fibre (figure 4.1A). After maintaining follicles in culture overnight they adhered

Figure 4.1 Culturing of isolated mouse hair follicles.

(A) Freshly isolated intact hair follicles from 5 day old *K2.10-TAg* #40-line transgenic mice viewed under phase-contrast. Note that some follicles contain fully formed keratinised hair shafts (arrowhead).

(B) After 24 hours in culture, follicles are attached to the culture dish and outgrowths of epithelial cells (presumably outer root sheath keratinocytes) are apparent (arrowhead).

(C) Keratinocytes forming characteristic pavement-like colonies surround the attached follicles after 2 days in culture. Note that there is a progressive loss of follicular structure over time.

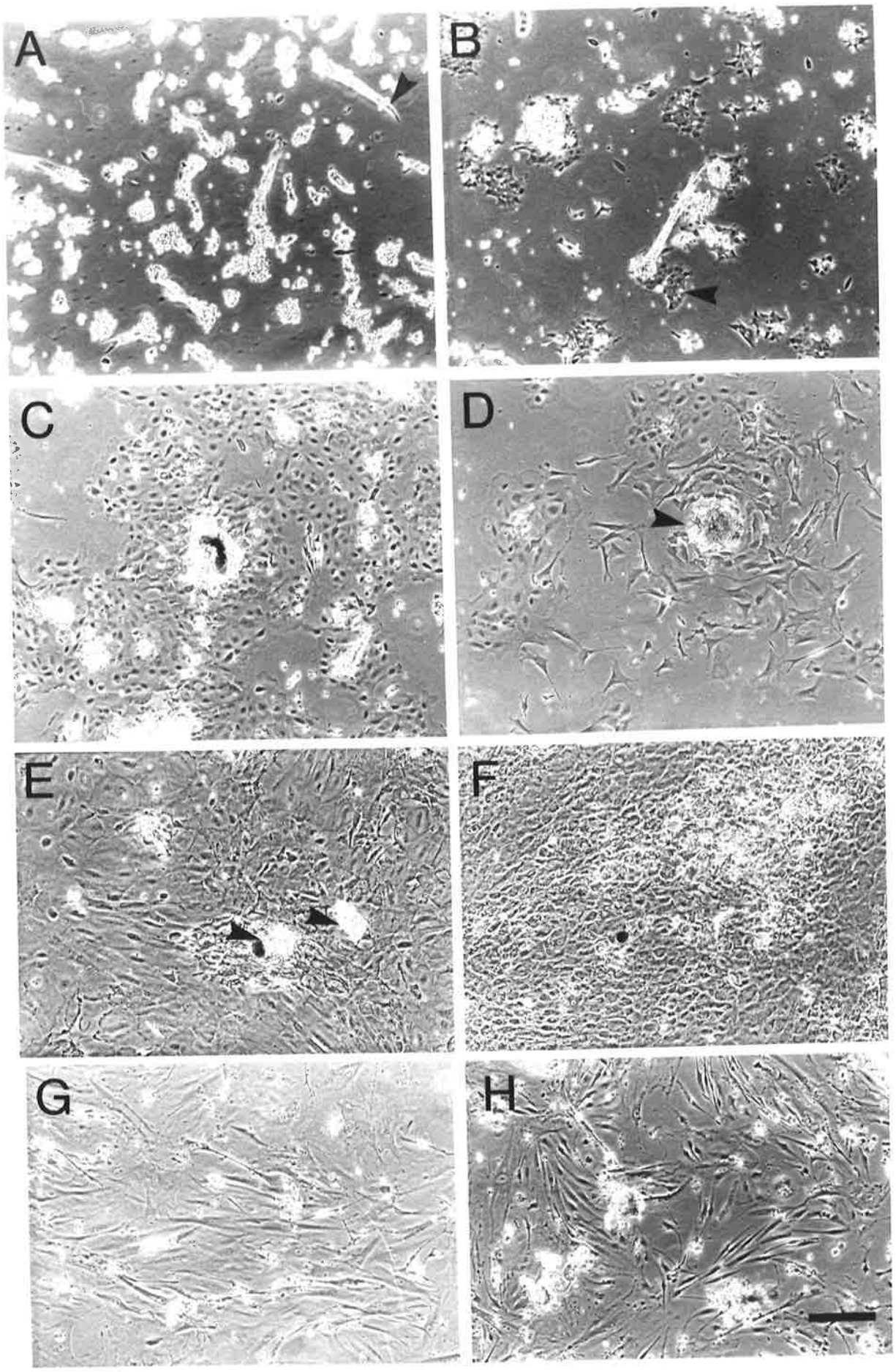
(D) Colonies of attached keratinocytes and dermal papilla cells are visible after 3 days in culture. Clumps of small, rounded cells (arrowhead) are maintained above adherent dermal papilla fibroblast colonies. Apart from previously keratinised hair shafts (which do not become attached), these cell clumps are the only remnants of follicle structure.

(E) A confluent pavement of keratinocytes and dermal papilla cells after 4 days in culture with clumps of small, rounded cells growing above them (arrowheads).

(F) A region of dead/differentiated keratinocytes after 4 weeks in culture with dermal papilla fibroblasts.

(G, H) Passage of follicle cultures results in the growth of mainly dermal papilla fibroblasts.

Phase-contrast microscopy. Bar, 200 μm.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C
D
E
F
G
H

to the dishes and there were visible outgrowths of epithelial cells from the bulb regions (figure 4.1B). After 48 hours, quite large colonies of epithelial cells had formed around the original follicles (figure 4.1C). Apart from previously keratinised fibres, the follicle structure had virtually disintegrated after 2-3 days in culture, with colonies of epithelial cells, dermal papilla cells and clumps of small, rounded cells which persisted above the dermal papilla colonies (figure 4.1D). The adherent dermal papilla and epithelial cells formed a confluent layer but the rounded cell clumps appeared unchanged, showing limited cell proliferation (figure 4.1E). After 2-3 weeks of culture the adherent epithelial cells formed regions of dead enucleate cells (figure 4.1F) and cultures appeared to be in decline with virtually no epithelial cell proliferation seen. Passage of cells upon confluence (after 3-4 days or 1-2 weeks depending on plating density) appeared to favour fibroblast growth (figure 4.1G & H) and unpassaged cultures were similar, with basically only dermal papilla fibroblasts persisting after prolonged culturing (6-8 weels).

Follicle-derived cells were held in culture and passaged for more than 6 months with no evidence of cell immortalisation. Cell immortalisation is reported to be a slow, multistage process often taking several months with cells possibly undergoing a "crisis" period when cell proliferation is balanced by cell death before a small focus of cells is immortalised (Shay and Wright, 1989). However, for immortalisation of the follicle cells to occur in culture, cortical cells must be viable and TAg expression is necessary. In order to investigate whether cells expressing TAg were present in this culture system a time course experiment was performed. Follicle cells were analysed for TAg expression after 1-6 days in culture by immunofluorescent staining (figure 4.2). As expected, staining was seen in the cortex of keratinised hair shafts (figure 4.2A) and in some cells of the rounded cell clumps that formed as the follicle structure disintegrated with culturing (figure 4.2B, D-F). TAg expression was never seen in the adherent cells of the culture. TAg expression was strong after overnight culture of the isolated follicles but was reduced after 3-4 days in culture (figure 4.2E & F) and appeared non-existent after 5-6 days in culture.

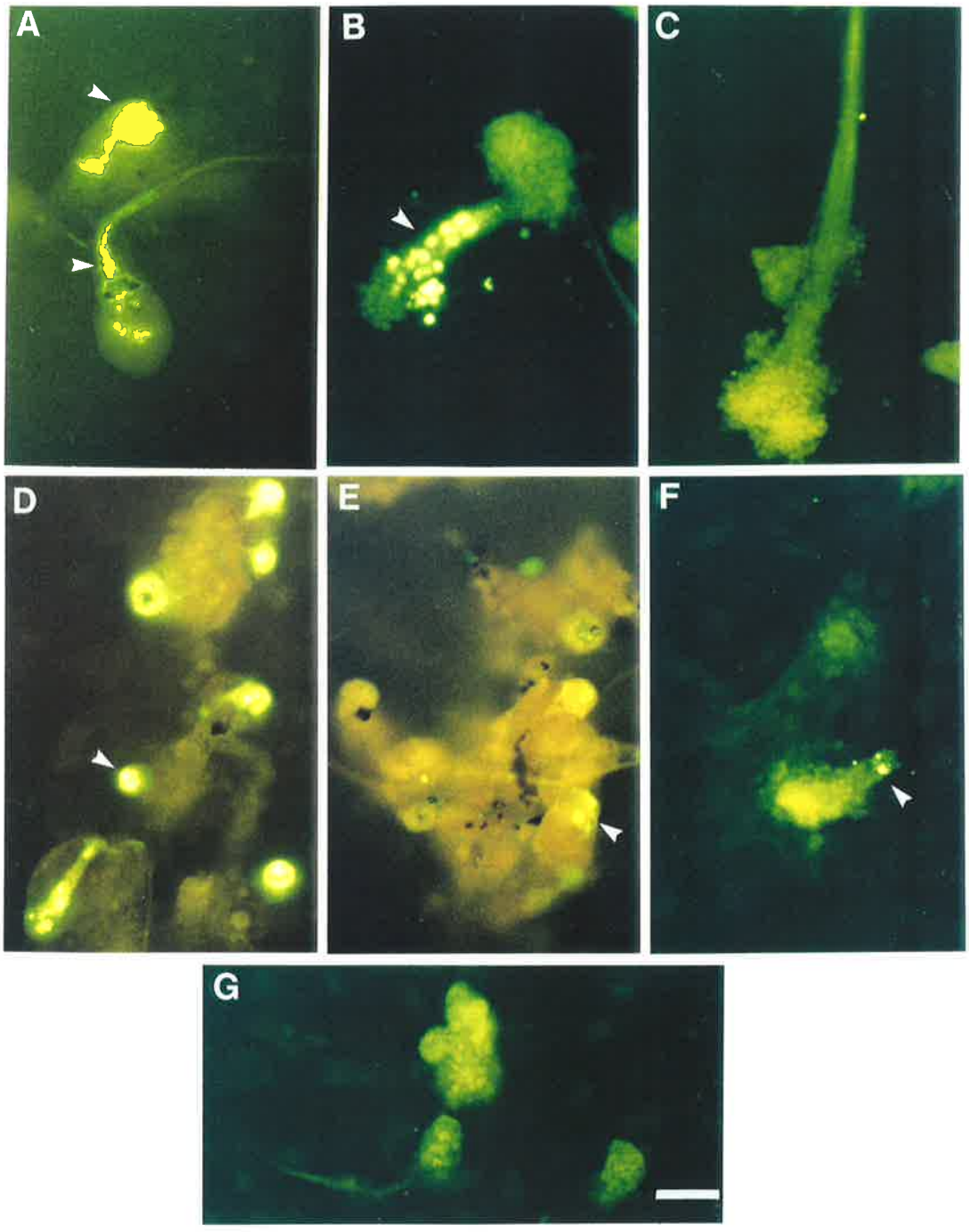
4.2.2 *In situ* hybridisation analysis

The apparent lack of cell immortalisation both *in vivo* and in culture, together with the loss of detectable TAg expression in cultured follicles by day 5 of culture, suggested that TAg expression may have been targeted too late in follicle differentiation and that the cells may have exited permanently from the cell cycle and therefore could not be immortalised.

To examine whether cells expressing TAg under the control of the *K2.10* promoter were still undergoing cell division the expression patterns of histone *H3* and *K2.10* were analysed by *in situ*

Figure 4.2 Immunofluorescent staining for SV-40 TAg expression in cultured *K2.10-TAg* follicle cells.

After 24 hours in culture strong TAg expression is observed in the follicle shafts (A, B). Staining is still obvious after 48 hours in culture (D), however, reduced staining is observed after 3 to 4 days in culture (E, F respectively). After 5-6 days in culture it is difficult to detect any specific staining for TAg (data not shown). Levels of background staining are shown (C, G) in which no α-TAg 1^{o} antibody was used. Note, no specific staining is detected in adherent cells but is only seen in hair fibres and cell clumps. Also note that the staining seen in the bottom follicle in (A) is not staining of follicle bulb cells, but staining of suprabulbar cortical cells which become swollen and rounded in culture. Examples of specific staining are indicated by arrowheads. Bar, 50 μm (A-C, F, G); 10 μm (D, E).


A
B
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hybridisation to mRNA. Histone H3 mRNA is synthesised during S phase of the cell cycle but is present in G_1 and G_2 and can therefore be used to detect cycling cells (Chou *et al.*, 1990). A comparison of histone *H3* with *K2.10* expression was performed on consecutive whisker follicle sections taken from mice transgenic for the sheep *K2.10* gene (Powell and Rogers, 1990). *K2.10* transgenic mice were used rather than *K2.10-TAg* mice because the high level of *K2.10* transgene expression in that particular line of mice allowed easy detection, whilst I was unable to detect TAg mRNA by *in situ* hybridisation in *K2.10-TAg* transgenic follicles.

Expression of histone *H3* was seen in the bulb region of the follicle up to the top of the dermal papilla (figure 4.3A, C, D & F). The proliferative zone identified by histone *H3* expression is consistent with the region of cell division in the follicle bulb identified by other workers using ^{3}H-thymidine or bromo-deoxyuridine labelling (Downes *et al.*, 1966; Epstein and Maibach, 1969; Tezuka *et al.*, 1990). K2.10 mRNA was detected in the cortex of the follicle, appearing above the tip of the dermal papilla, 2-3 cells above the level of histone *H3* expression. Histone *H3* expression in *K2.10-TAg* transgenic follicles was the same as observed in *K2.10* transgenic follicles i.e. expression did not appear above the dermal papilla (figure 4.3C & F). Despite the expression of TAg in the cells higher up in the follicle in the *K2.10-TAg* transgenic mice, no cellular proliferation was seen in those cells as evidenced by the lack of histone *H3* expression (figure 4.3C & F).

4.2.3 Alternative promoters for targeting TAg to the follicle

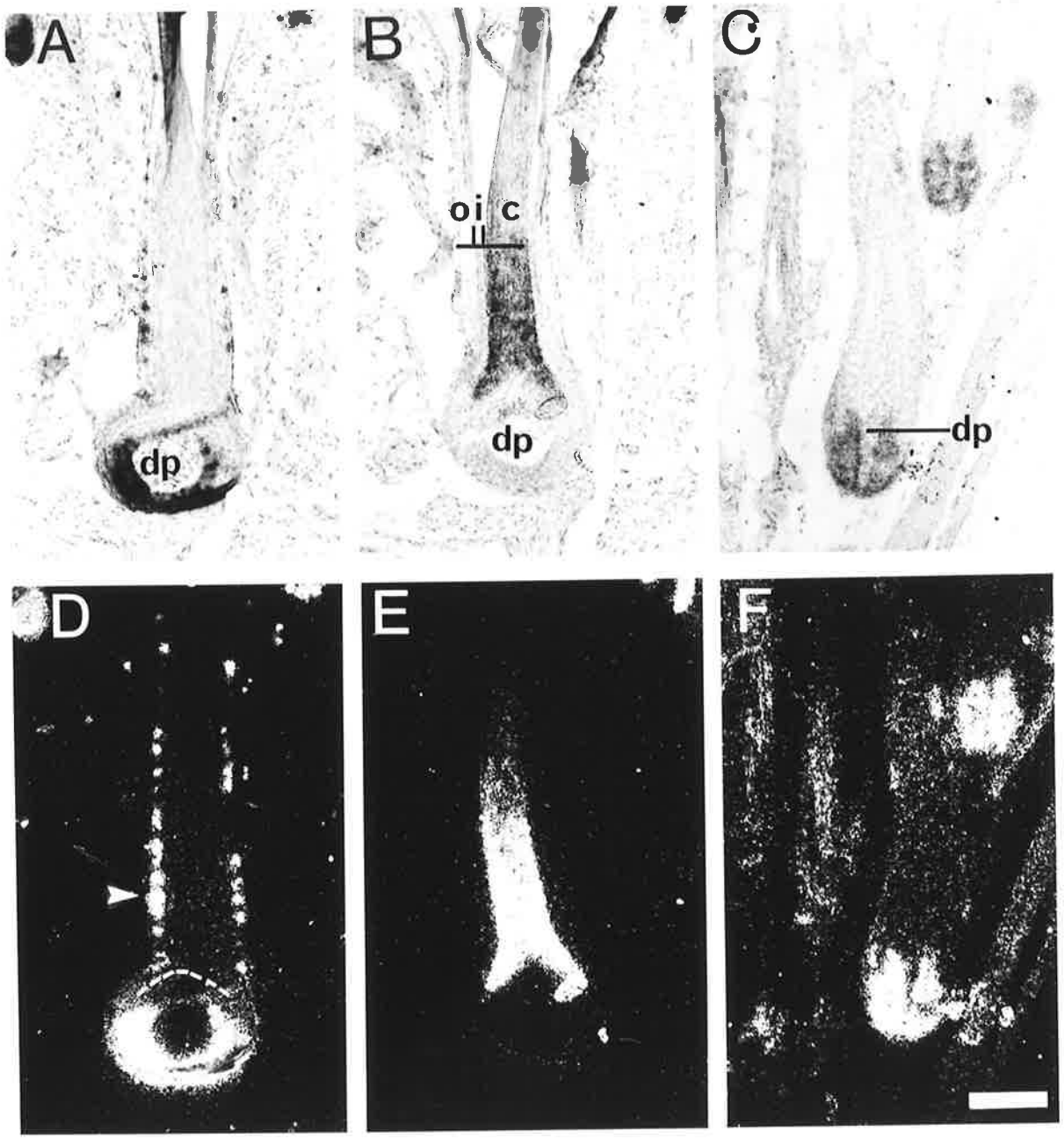
It appears that the *K2.10* promoter targeted TAg expression too late in cortical cell differentiation for immortalisation to occur. Therefore, in order to drive follicle expression of TAg at an earlier stage of cell differentiation, the use of several other promoters was investigated; these included two other hair keratin promoters and a more general promoter, the metallothionein Ia promoter.

(i) Sheep *K2.12* promoter

It is known by protein analysis that 4 pairs of IFs are expressed in the hair cortex (Crewther *et al.*, 1980; Heid *et al.*, 1986). This laboratory has characterised genes encoding 3 of the hair keratin type II IFs (Powell *et al.*, 1992; Powell and Beltrame, 1994) and a partial cDNA clone of a fourth (B. Powell, unpublished data). Expression patterns of these genes has been shown to differ, with *K2.9* and *K2.10* being expressed early in cortical differentiation (2-3 cells above the dermal papilla as shown) and *K2.11* being expressed later (Powell *et al.*, 1992). Since hair keratin type II IF mRNA is detectable below the cells expressing *K2.9* and *K2.10* (Powell *et al.*, 1992) and

Figure 4.3 Comparison of histone *H3* expression with expression from the *K2.10* promoter in the hair follicle by *in situ* hybridisation.

Consecutive whisker follicle sections from *K2.10* transgenic mice were probed for *H3* expression (A, D) and *K2.10* expression (B, E). Note that expression of *K2.10* is first detectable in cells 2-3 cell layers above the top of the dermal papilla and above follicle bulb cells showing *H3* expression (D; dashed line). (C, F) *H3* expression in *K2.10-TAg* transgenic pelage follicles. Note that *H3* expression does not continue past the top of the dermal papilla. Expression is shown as dark grains under brightfield microscopy (A-C) and as white grains under darkfield (D-F). Also note regions of *H3* expression in the ORS of the follicle (D; arrowhead). The apparent "wing" of *K2.10* expression in the upper right bulb in (E) is artefactual due to folding of the tissue which can be seen in (B). dp, dermal papilla; o, outer root sheath; i, inner root sheath; c, cortex and cuticle. Bar, 100 μm.


A
dp
B
oi
c
dp
C
dp
D
E
F

macrofibrils are also evident in these cells (Chapman and Gemmell, 1971), it was proposed that this represents expression of a fourth type II keratin, *K2.12* (Powell *et al.*, 1992). A partial cDNA sequence of the putative sheep *K2.12* gene had previously been isolated and *in situ* hybridisation to sheep wool follicles localised expression to the follicle cortex, with expression first detectable in the cells around the tip of the dermal papilla (figure 4.4; B. Powell, unpublished data).

In order to isolate the *K2.12* gene a comparison of the putative cDNA sequence and the other type II genes was performed to identify a region that was unique to *K2.12*. The partial cDNA encodes 286 amino acids of the central α-helical domain of the type II keratin protein (Powell *et al.*, 1992). DNA sequence comparison showed a high degree of identity between all the type II hair keratins, however, the *K2.12* sequence is more divergent (90% similarity between *K2.12* and other the hair type II genes compared with 95-96% similarity between *K2.9*, *K2.10* and *K2.11*) especially in exon 4 (figure 4.5). A 91 bp fragment spanning this more variable region was used as a probe to screen a pWE15 sheep genomic cosmid library. The library screening was performed under conditions which favoured probe binding to the *K2.12* cDNA above the other type II keratin genes (figure 4.6). From the first round screening of 3-5 genome equivalents, 1 duplicate positive was obtained (3-4 other faint possible positive clones were seen but these were not present in duplicate; data not shown). The single duplicate positive clone was purified and mapped by restriction digestion with various enzymes and Southern blotting using the 91 bp *K2.12* probe fragment (figure 4.7). The library screening and initial Southern blot were performed by C. McLaughlan.

Southern blots of the cosmid were then probed with conserved hair keratin type II sequences (see 2.1.12) in order to verify that a type II gene was present. The probes spanning exons 4, 5 and 6 and the C-terminal region probe hybridised to vector sequences only and the N-terminal region probe gave no signal at all (data not shown). Isolation and sequencing of a 600 bp Sac I fragment that hybridised with the 91 bp *K2.12* probe (figure 4.7) confirmed that this was not the *K2.12* gene as it showed no similarity to the cDNA sequence and was, in fact, only homologous to the probe over a 22 bp region at the 5' end of the probe (figure 4.8). The fragment sequenced showed no significant homology to any keratin genes nor any other sequence in the database.

(ii) Sheep *K1.15* promoter

During the course of this work a sheep keratin type I IF gene, s*K1.15*, was isolated in our laboratory which is expressed in the follicle bulb cells that line the dermal papilla (Whitbread, 1992). These cells are a unique subpopulation of bulb cells in the proliferative zone of the follicle and therefore appeared to be a good target for cell immortalisation using the TAg approach. However,

Figure 4.4 ***In situ*** **hybridisation showing** ***K2.12*** **expression in a merino sheep wool follicle.**

(A) Bright field photograph which indicates the position of the dermal papilla (arrowhead, dp).

(B) Dark field photograph of the same follicle showing the expression pattern of *K2.12* using a riboprobe derived from the 91 bp *K2.12* probe fragment (see boxed region in figure 4.5). The horizontal dashed line indicates the approximate lower boundary of *K2.12* expression which is readily detectable well below the apex of the dermal papilla as indicated. Bar, 30 μm. (*In situ* hybridisation was performed by B. Powell and A. Nesci).

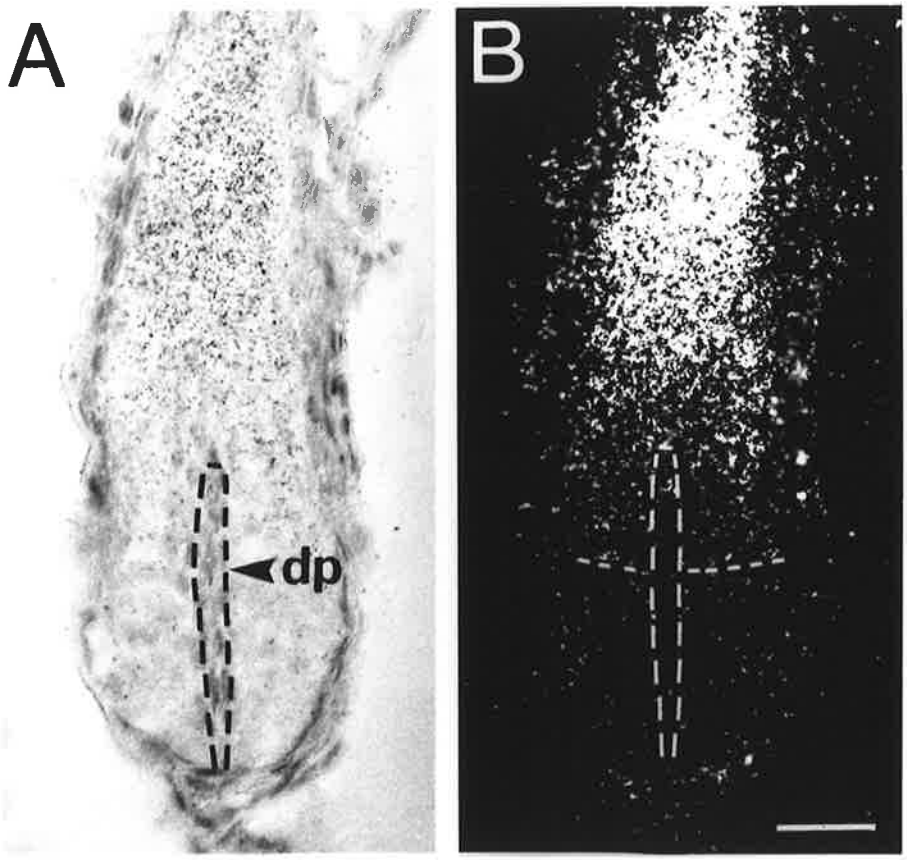

A
dp
B

Figure 4.5 Hair keratin IF type II sequence comparison.

Sequence comparison of the sheep*K2.12* cDNA sequence (B. Powell, unpublished data) with the known nucleotide sequences for the sheep*K2.9* (Powell *et al.*, 1992), *K2.11* (Powell and Beltrame, 1994) and *K2.10* (B. Powell and K. Ward, unpublished data) genes. The exon boundaries are conserved between genes as marked. Asterisks (*) represent identical nucleotide sequence with that of *K2.12* and differences are shown. The boxed region spanning exons 4 and 5 represents the *K2.12* probe used to screen a sheep cosmid library for the *K2.12* gene (see 4.2.3i).

Figure 4.6 Dot blot analysis to determine the conditions for screening the sheep pWE15 cosmid libary with the *K2.12* probe in order to favour binding to *K2.12* sequences over other keratin IF sequences.

Dilutions of 50, 100 and 200 ng of *K2.9* and *K2.10* clones were blotted as indicated together with 00 ng of *K2.12* cDNA. The pWE15 cosmid vector was also spotted onto the filter. The filter was hybridised vith the 91 bp *K2.12* cDNA probe fragment spanning exons 4 and 5 (see figure 4.5) and was washed at ·. final stringency of 2X SSC, 0.1% SDS at 65°C. Under these conditions the probe was only seen to hybridise to the *K2.12* cDNA and not the other, highly related hair keratin IF clones. *K2.12* is the partial sheep cDNA clone, *K2.9* gene is a cosmid clone containing the sheep *K2.9* gene together with 2 other hair-related keratin IF genes, *K2.9* cDNA is the sheep cDNA clone, and *K2.10* gene is the sheep *K2.10* gene.

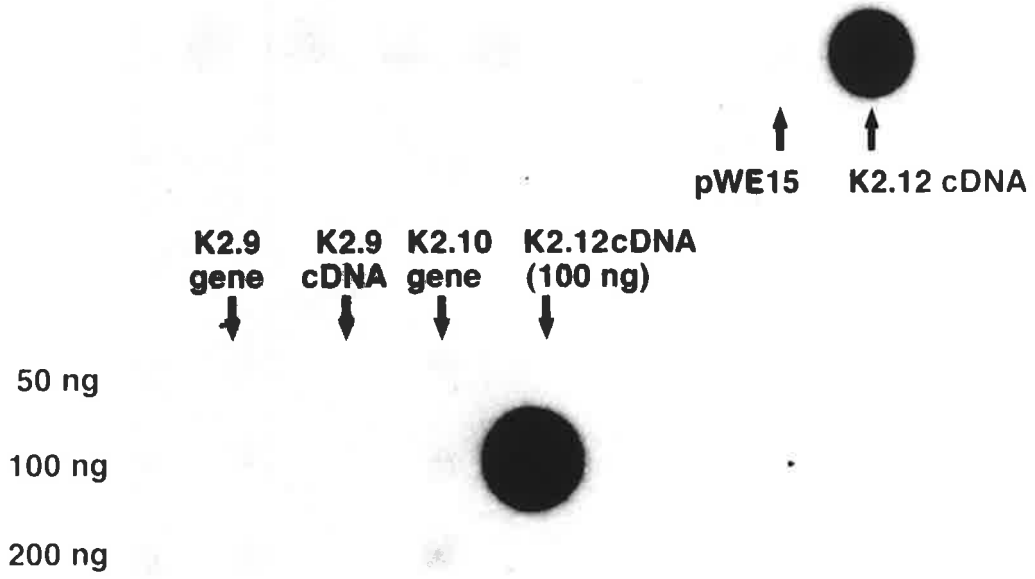
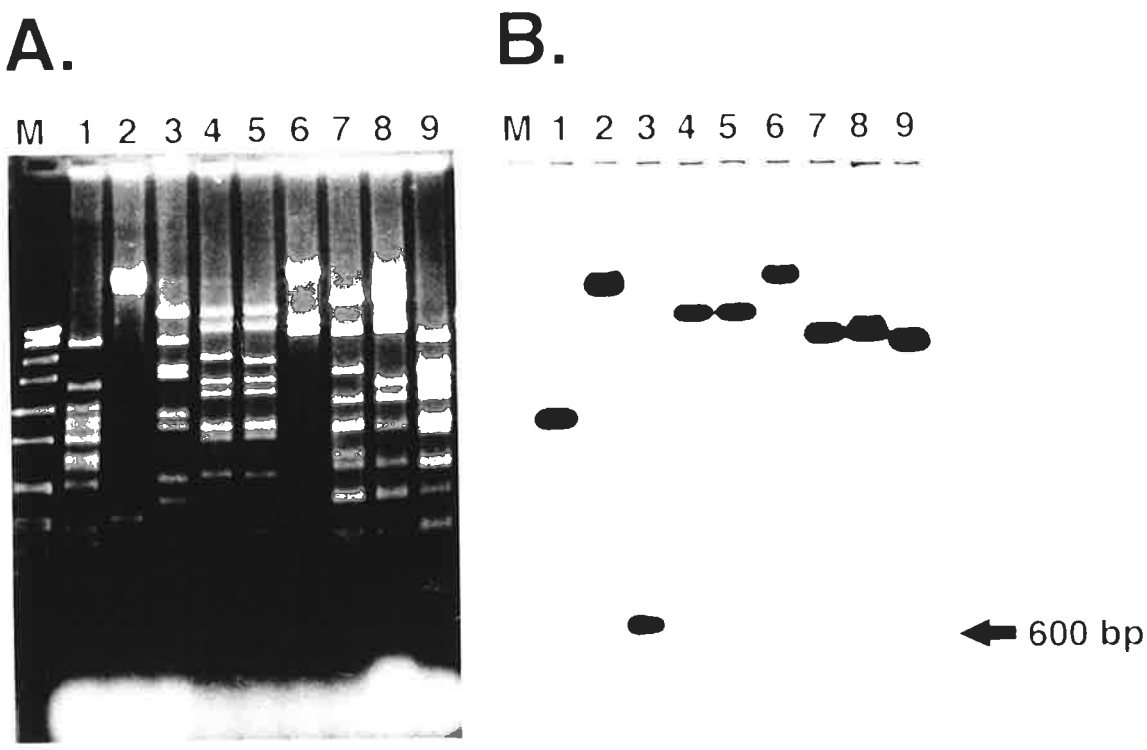

pWE15
K2.12 cDNA
K2.9 gene
K2.9 cDNA
K2.10 gene
K2.12cDNA (100 ng)
50 ng
100 ng
200 ng

Figure 4.7 Southern blot analysis of the cosmid clone isolated from a sheep pWE15 library using a probe derived from the sheep *K2.12* cDNA.

(A) The cosmid DNA was digested with the following enzymes: Pst I (lane 1), Kpn I (lane 2), Sac I (lane 3), Eco RI (lane 4), Eco RI and Bam HI (lane 5), Bam HI (lane 6), Bam HI and Hind III (lane 7), Hind III (lane 8), Eco RI and Hind III (lane 9) and fractionated on a 1% agarose gel together with Eco RI-digested SPP1 markers.

(B) The DNA was then transferred to Zetaprobe GT membrane. The filter was probed with a 91 bp *K2.12* cDNA fragment (see figure 4.5), washed at a final stringency of 2X SSC, 0.1% SDS at 65^{o}C and exposed to autoradiographic film at -80^{o}C. The 600 bp Sac I fragment which hybridised to the probe was isolated and sequenced (see figure 4.8).


A.
M 1 2 3 4 5 6 7 8 9
B.
M 1 2 3 4 5 6 7 8 9
600 bp

A

SacI
GAGCTCACGGCAGACCTCTGTGTTCCCTGTGCCAACGGGGAGGCGCTGGTGGGCGTTAGTCCC

TTGACGAGGGAGGGCAGGTGGGTCTGTGGAGAATACTGGAGCCCCTGTGGCCTAGCCACCTTC

CCTTTTTCCCCTAATTGCACTATACTTGCTCTCGCTGCAGTCCGGAGATACCCAAGACCTCCG

TGGGGTCATGACCAGAGACTTAGCAAGTCTCGCCTTATCCGTGGAGCTTGACAGGGAGAAGTG

TGGCCGTTGTCTGGCATCCGTAGCTCATTTCTTCCTCCTTTCCTCGCGTTGTGTTACTCTGAA

CCGTTCTGTTTTTATTTACCAAAGTACTGTACTTGGCTATTGCAGTGTTCCAAAACCAAATGT

TTTCTTTTGTTGTGTCTTTAACCTTCAATACTTGGTGCAATAGAAGCTGCAGAGATGTGCCAC

TTTATCTATGAACTGGAGTTTTGTATACCAATAAATTCTAGTTTAAAGACAGTTTCGAGTCGT

CTATGTCACTGTCTGACTGCTGTTTCATGGCACGAGATGGAGAGCTGCCGGTCCTTCTCAGAG

ACTTTGCAGGAAACTGGGCCTGTTCTGCCCAGGGACAGTCGTTTGGGTCTCACTGAGCTC *SacI*

B

5'CCTGGAGGCCAACGTGGAGGCGCTGGTGGAGGAGTCTAATTTCCTGAAGCGCCTCTATGAT

GAGGAGATCCAGATCCTCAATGCCCACATCTCAG3'

Figure 4.8

(A) Sequence of the 628 bp Sac I fragment from the cosmid clone isolated from the *K2.12* library screening. The only homology between the isolated clone and the *K2.12* probe used to screen the sheep cosmid library is the 22 bp region that is underlined.

(B) Sequence of the *K2.12* probe derived from the *K2.12* cDNA sequence (see figure 4.5). The 22 bp region that hybridised to the isolated cosmid clone is underlined.

as *K1.15* is also expressed in other tissues such as tongue, oesophagus, epidermis and follicle ORS, a temperature-sensitive mutant of TAg, *tsA58* (Jat and Sharp, 1989), was chosen for this construct.

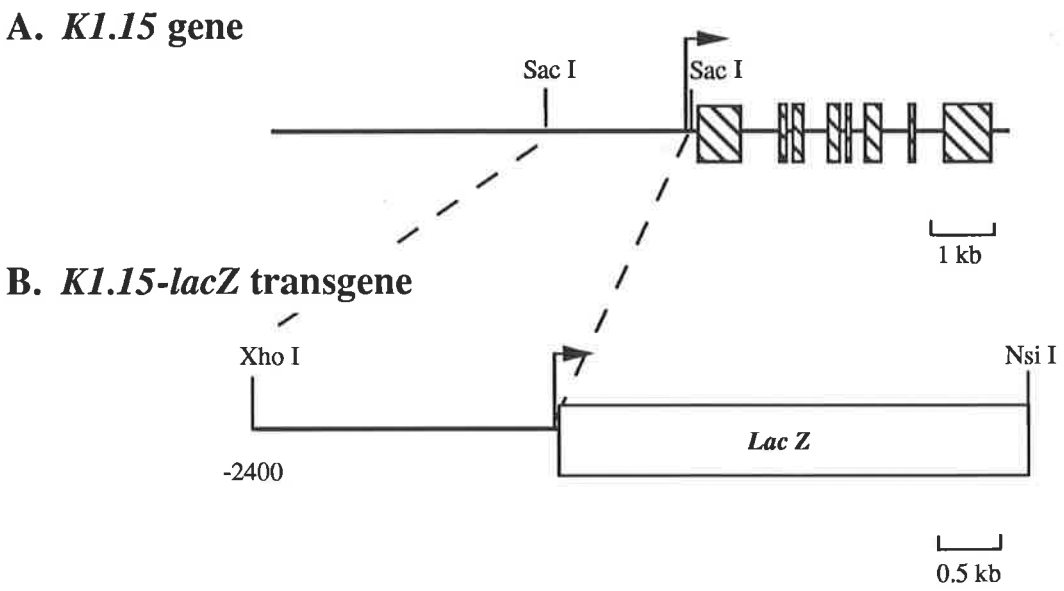
Two constructs were prepared for transgenesis, *K1.15-lacZ* and *K1.15-TAg*. Both constructs contained 2.5 kb of immediate 5' promoter sequence from *K1.15* linked to either *lacZ* or *TAg (tsA58)* (figure 4.9). The 2.5 kb *K1.15* promoter fragment had been shown to direct reporter gene expression in Chinese hampster ovary cells, but at a very low level (Whitbread, 1992), and attempts to produce a larger construct were unsuccessful (see Discussion 4.3.4ii). As it was uncertain whether the 2.5 kb promoter fragment would be sufficient to drive expression of the reporter genes in the follicle bulb cells as desired, the *K1.15-lacZ* construct was initially used to generate transgenic mice. This construct would allow rapid and easy detection of expression by the sensitive X-gal staining method (see 2.2.5.2).

Eight transgenic mice were generated with *K1.15-lacZ* (figure 4.10), however, none of these mice were shown to express β-galactosidase in the epidermis or hair follicles. Thus, the *K1.15*-TAg construct was not used for transgenesis as it was unlikely to be appropriately expressed. Recently, a 5 kb *K1.15* promoter fragment has successfully targeted *lacZ* expression to the follicle (B. Powell and A. Nesci, personal communication) and it is planned to use it to drive *tsA58*-TAg expression in transgenic mice in the near future.

(iii) s*Mt-Ia* promoter

Another readily available promoter was the sheep metallothionein Ia promoter (Peterson and Mercer, 1986) which has been used to express sheep growth hormone in transgenic mice (Shanahan *et al.*, 1989). Bacterial cysteine biosynthesis enzymes have also been expressed in transgenic mice under the control of this promoter and expression has been detected in hair follicle bulb cells (K. Ward, personal communication). Follicle bulb cell expression of the mouse metallothionein I gene has also been described (Karasawa *et al.*, 1991).

A transgene construct, *MtIa-TAg*, was prepared containing 860 bp of *MtIa* promoter upstream of the temperature-sensitive TAg mutant, *tsA58*, (figure 4.11A). Of 80 mice born from microinjection, Fo-80 was the only transgenic mouse (figure 4.11B). Unfortunately, this mouse died and was discarded before analysis of transgene expression could be performed. Transgenesis with *MtIa-TAg* has been discontinued at this stage.



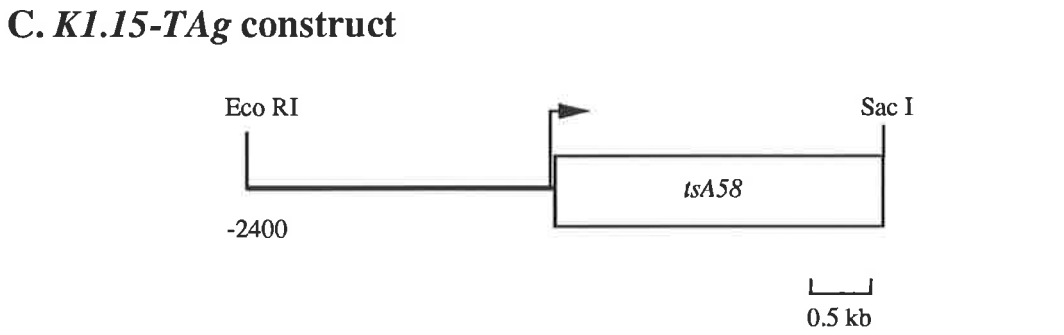


Figure 4.9 Generation of *K1.15* transgenesis constructs.

(A). A diagrammatic representation of the sheep *K1.15* gene showing the Sac I promoter region fragment (2451 bp) used in the generation of the following constructs. This fragment spans the region from -2390 to +61 in the 5' UTR of the *K1.15* gene.

(B). Generation of the *K1.15-lacZ* construct. The 3.85 kb Hind III/Bam HI fragment from pCH110 encoding the *lacZ* reporter gene was cloned into a pGEM7Zf(+) Hind III/Bam HI vector. The *K1.15* Sac I promoter fragment was endfilled and cloned into the Sma I site in the polylinker of the pGEM7Zf(+)/*lacZ* vector upstream of the *lacZ* gene. The resultant *K1.15-lacZ* transgene fragment (6.3 kb) was excised by digestion with Xho I and Nsi I. (C). Generation of the *K1.15-TAg* construct. A 3 kb Kpn I/Bam HI fragment of SV40 *tsA58* containing the SV-40 early region genes was cloned into the Kpn I/Hind III sites of pGEM7Zf(+). Digestion with Kpn I and Sfi I removed the SV-40 ori region (303 bp). The Kpn I/Sfi I ends of the vector were blunted and the endfilled *K1.15* Sac I promoter fragment was cloned in. The resultant *K1.15-TAg* construct (5.1 kb) was excised by digestion with Eco RI and Sac I.

Figure 4.10 Dot blot analysis of *K1.15-lacZ* transgenic mice.

Genomic tail DNA from mice #1-63 (5 μg), together with transgene copy number controls, were blotted onto Zetaprobe membrane under alkaline conditions. The filter was probed with the *lacZ* gene fragment (3.85 kb) and washed at a final stringency of 0.1X SSPE, 0.1% SDS. The following mice were identified as *K1.15-lacZ* transgenics: #4, #8, #18, #25, #26, #27, #28 and #51. DNA loading variations were normalised by re-probing the filter with a mouse *K14* gene probe (data not shown). (Dot blot performed by A. Nesci).

K1.15-lacZ transgenesis dot blot

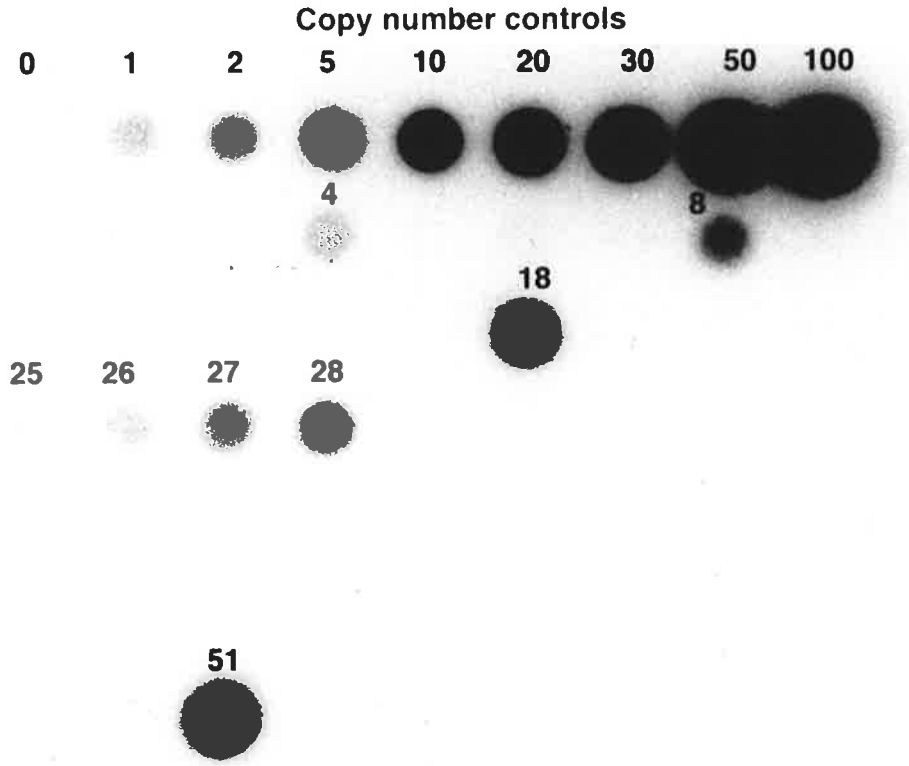
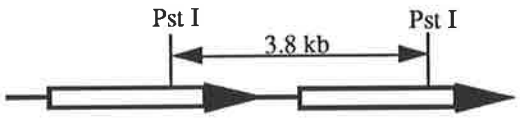
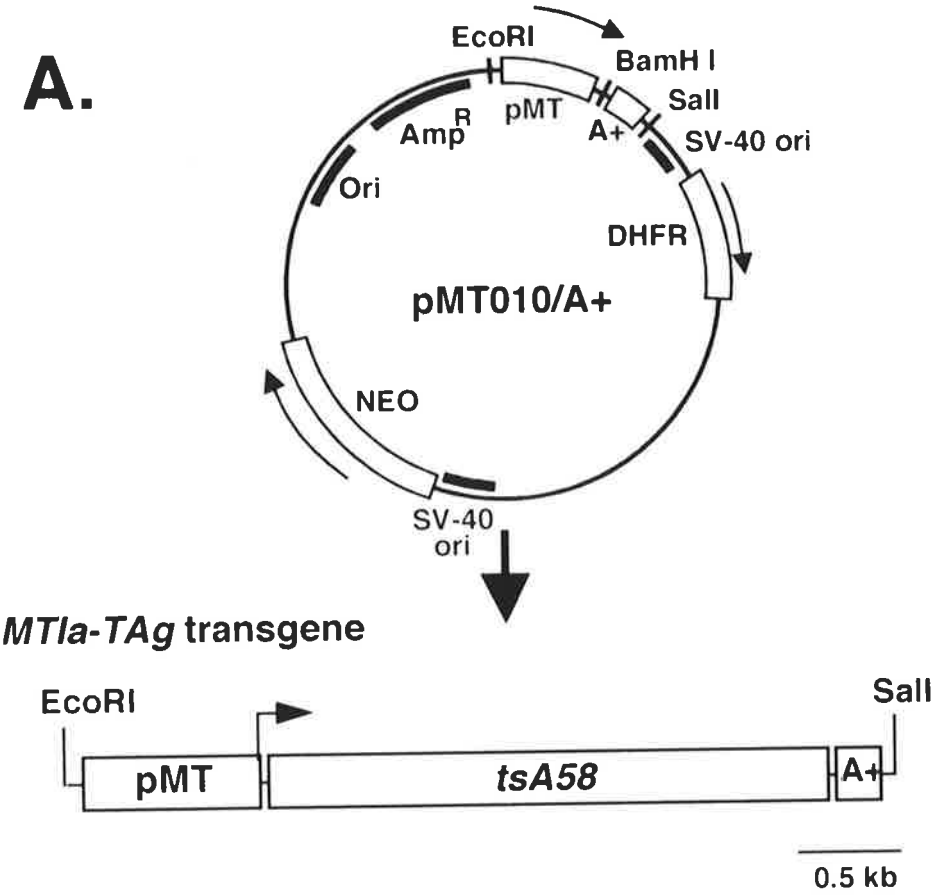


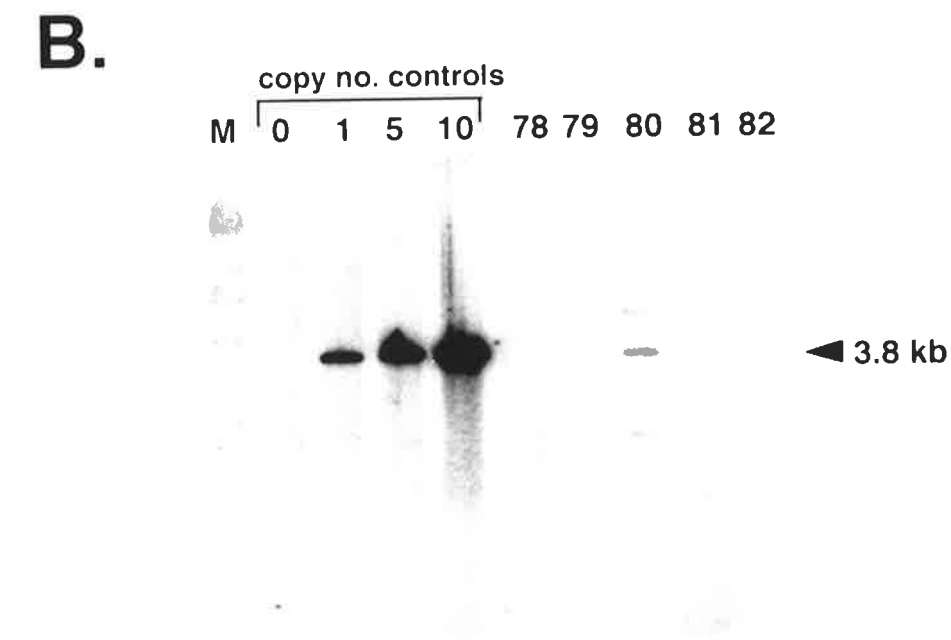
Figure 4.11 ***MTIa-TAg*** **transgenesis.**

(A) Construction of the *MTIa-TAg* transgene. The Bgl I/Bam HI fragment (2.7 kb) of SV-40 *tsA58* was endfilled and cloned into the unique Bam HI site of pMT010/A+ (Choo *et al.*, 1986). The transgene fragment was excised from the vector by digestion with Eco RI and Sal I. The resultant transgene (3.8 kb) contained the sheep *MTIa* promoter (860 bp) upstream of the *tsA58* early region encoding both large (T) and small (t) antigens and including the polyadenylation signal. Although unnecessary, the *MTIa* polyadenylation fragment was also included due to the convenience of the Sal I restriction site.

(B) Southern blot analysis of the *MTIa-TAg* transgenic mouse. Pst I digested mouse tail genomic DNA (10 μg) from mice #78-#82 was separated on a 1% agarose gel and transferred to Zetaprobe GT membrane. Copy number controls equivalent to 1, 5 and 10 copies of the transgene in 10 μg of genomic DNA were prepared from transgene fragment mixed with 10 μg of Pst I digested non-transgenic DNA. The blot was probed with radiolabelled *TAg* fragment and washed in 2X SSPE, 0.1% SDS at 65^{o}C and exposed to a phosphor screen. Only mouse #80 was shown to be transgenic and the transgene appeared to be integrated in a head-to-tail arrangement indicated by the 3.8 kb fragment consistent with this arrangement (see diagram below). Whilst more than one copy of the transgene was integrated (as shown by the transgene arrangement), the copy number suggests that the mouse is chimeric because there is less than 1 copy equivalent per genome.




A.
EcoRI
BamH I
Sall
pMT
A+
SV-40 ori
Amp R
Ori
DHFR
pMT010/A+
NEO
SV-40
ori
MTIa-TAg transgene
EcoRI
Sall
pMT
tsA58
A+
0.5 kb


B.
copy no. controls
M
0
1
5
10
78
79
80
81
82
3.8 kb

4.3 Discussion

4.3.1 Is cortical cell immortalisation possible?

The lack of follicle cell immortalisation in the transgenic mice raised several possibilities; firstly, is cortical cell immortalisation possible and, if so, why was this approach unsuccessful?

Although rare, tumours containing differentiated hair cells do occur (for reviews see Headington, 1976; Grubendorf-Conen, 1990). Benign pilomatricoma and the less common pilomatrix carcinoma (Zagarella *et al.*, 1992) arising from the follicle bulb cells exhibit differentiated hair cell characteristics, particularly those of cortical cells (Headington, 1976). Immunocytochemical and biochemical detection of hair-specific keratins indicates that the majority of pilomatricoma cells resemble the cortical cell lineage although some cells may undergo squamous cell differentiation which may be related to the ORS (Moll *et al.*, 1988b). Benign pilomatricomas do not become vascularised and undergo spontaneous involution. It has been suggested that the duration of growth of the cells in the tumour mimics the normal anagen time of the follicle from which it was derived (Headington, 1976). (It should be noted that anagen in human scalp hair follicles may last as long as 6-10 years (see Forslind, 1990) compared with 14 days for mouse follicles). It has also been reported that a hair follicle bulb cell tumour arose in a mouse in which the *p53* tumour-suppressor gene was knocked-out (Jacks *et al.*, 1994). Unfortunately, no further information about the differentiation state of the tumour cells, nor about the possibility of producing a cell line from any subsequent hair tumours, was able to be obtained from the authors.

Considering that the follicle bulb cells are one of the most highly proliferating cell populations of the body and given the large number of follicles that are present, the frequency of follicle cell transformation, both naturally occurring and in p53-mutant mice, appears very low. However, the occurrence of pilomatricomas and pilomatrix carcinomas indicate that it should be possible to immortalise hair follicle cells which express differentiated cortical cell markers such as hair-specific keratins.

The mitotic activity of hair follicle bulb cells is quite high and it is estimated that the doubling time of these cells may be as short as 13 hours in mouse follicles (Bullough and Laurence, 1958). Therefore, in the case of *K2.10-TAg* mice it is possible that the rapid follicle bulb cell proliferation and consequent migration of differentiating cortical cells up the follicle would not allow sufficient time for immortalisation to occur before the cells were extruded with the mature hair without access to nutrients. It is known that cell immortalisation is a multistep process which may require several

months to occur, whilst the growing phase of a mouse hair follicle is 14 days (Hardy, 1949). In order to investigate whether cell immortalisation would occur *in vitro* follicles were isolated from 2-5 day old transgenic mice and cultured.

4.3.2 Culturing of follicle cells

Until recently, culturing of hair follicles and follicle-derived cells has met with very limited success, with most cultures being of ORS cells (for review see Schaart *et al.*, 1990). However, successful maintenance and growth of whole follicles in culture has been achieved (Philpott *et al.*, 1990, 1992), and the isolated follicles grow and produce a hair for up to 10 days in culture. Normal morphology is maintained and the growth rate mimics the *in vivo* rate (Williams *et al.*, 1993). The growth of germinative epithelial cells, a distinct cell population of the follicle bulb located at the base of the dermal papilla, has also been achieved (Reynolds and Jahoda, 1991; Reynolds *et al.*, 1993). The germinative epithelial cells are able to proliferate in culture and form follicle-like organotypic structures, but do not differentiate into the various cell types of the follicle (Reynolds *et al.*, 1993).

Given the limited growth period of the intact follicles and the lack of cell differentiation of the germinative epithelial cells, and that other follicle culture systems generally only achieve ORS growth, culturing of *K2.10-TAg* follicles was based on the premise that cells would already be "activated" by TAg expression such that they would be able to proliferate in culture until immortalisation could occur. Therefore, the method of follicle isolation and culture chosen was based on that of Rogers *et al.* (1987) due to the ease of follicle isolation and the high yield. Another consideration is that the culture of single, intact hair follicles essentially reproduces the *in vivo* situation of continuous outgrowth and differentiation, whereas the culture system used here is a "disintegrating" follicle culture in which the cells can disperse, allowing all cells continued access to nutrients.

The growth and morphology of *K2.10-TAg* follicle cells in this culture system was very similar to that seen in the cell recombination experiments of Reynolds and Jahoda (1991) in which various cell types of the follicle were isolated and cultured both separately and in combination with dermal papilla cells or skin fibroblasts. The predominant cell types were epithelial cells which formed pavement-like colonies, dermal papilla cells and tight clumps of small, rounded cells growing on dermal papilla cells. The hexagonal epithelial cells exhibited a morphology consistent with that of ORS cells, however, as follicle bulb cells also behave similarly in culture (Reynolds and Jahoda,

1991), these cells may be derived from both the ORS and the follicle bulb. The cell clumps which grow on top of dermal papilla colonies are likely to consist of ORS cells (which have been shown to persist as small, rounded cells when grown over papilla cells (Reynolds and Jahoda, 1991), follicle bulb cells and differentiating cells of the follicle (as TAg-expressing cells were identified in these clumps, if only for a short period). Since TAg expression is lost after 5-6 days in culture, these cells do not appear to undergo any proliferation.

Whilst cells were maintained in culture for 6-8 months, very little proliferation was seen after 2 months and fibroblasts (papilla cells and, in some cases, skin fibroblasts) predominated.

In order for cortical cell immortalisation to occur in culture, cells expressing TAg must be able to grow and proliferate. As the antibody staining shows a loss of TAg expression after 5-6 days of follicle culture it appears that cortical cell growth and differentiation is unable to be maintained under the culture conditions used. No proliferation of TAg-expressing cells was seen, as evidenced by the immunofluorescent staining time course assay. This therefore suggests that TAg expression in these cells was unable to overcome cellular quiescence and force them into a proliferative state. Expression of TAg in responsive cells generally results in an increased proliferative capacity and extension of lifespan, even in pre-immortal cells (for review see Hanahan, 1988; Shay *et al.*, 1993). TAg can overcome proliferation controls which operate in the G_1 phase of the cell cycle (for review see Pardee, 1989) and can stimulate DNA synthesis in quiescent cells (Dobbelstein *et al.*, 1992; Sompayrac and Danna, 1994) and in senescent human diploid fibroblast cells, although the senescent cells fail to undergo mitosis (Gorman and Cristofalo, 1985; Rubelj and Pereira-Smith, 1994).

4.3.3 Lack of follicle cell immortalisation

Whilst several TAg transformed cell types exhibit differentiation markers consistent with postmitotic cells (Mellon *et al.*, 1990; Peschon *et al.*, 1992; Suri *et al.*, 1993), it appears that TAg may need to be initially expressed in a cell that is capable of undergoing cell division in order for immortalisation to occur at a later stage of differentiation. For example, targeted expression of TAg to neuronal cells does not induce hyperplasia and tumorigenesis when under the control of the glucagon gene promoter (Efrat *et al.*, 1988b), however, when the gonadotropin-releasing hormone (*GnRH*) promoter (Mellon *et al.*, 1990) or the tyrosine hydroxylase *(TH)* promoter (Suri *et al.*, 1993) are used, tumours result. It is believed that glucagon expression in CNS neurons is restricted

to postmitotic cells, whilst the *GnRH* promoter is active when cells are still dividing. *TH*-targeted TAg expression also produces neuronal tumours despite the fact that *TH* expression is only observed in postmitotic neurons. The authors hypothesise that, even though TH is only expressed in postmitotic neurons, the presence of TH, and therefore TAg, so soon after the appearance of postmitotic neurons (within 1 day) may allow the cell to re-enter the cell cycle while maintaining *TH* expression. However, expression of TAg late in development, such as in the glucagon-TAg mice, may be incapable of driving the cell back into the cell cycle once it has been withdrawn from the cycle for a number of days (Suri *et al.*, 1993).

It is possible, however, to target TAg expression to cells that are withdrawing from the cell cycle and undergoing terminal differentiation as was seen in the ocular lens cells of γF-crystallin-TAg transgenic mice (Bryce *et al.*, 1993). Since γ-crystallin genes are expressed only in terminally differentiating lens fibre cells, resultant lens tumours suggest that developing fibre cells become competent for γ-crystallin expression before they withdraw irreversibly from the cell cycle.

In contrast, α-crystallin genes are initially expressed in proliferating lens epithelial cells before they commence terminal differentiation. The mouse αA-crystallin gene has also been used to target TAg expression in transgenic mice and produce lens tumours (Mahon *et al.*, 1987). Two phenotypically distinct tumours resulted (Nakamura *et al.*, 1989). The difference between the two tumour types lies in the temporal and spatial patterns of TAg expression in the developing lens cells. In αT1 cells TAg is expressed very early in development and subsequent tumours are poorly differentiated and fast growing, whereas TAg expression in αT2 cells occurs later and is restricted to differentiating cells resulting in well differentiated, slow growing tumours. These findings suggest that the state of differentiation of the target cells plays a role in oncogenesis (Nakamura *et al.*, 1989).

Given the potent effect of TAg on cellular proliferation, the lack of follicle hyperplasia in the transgenic mice suggested that the cells in which TAg was expressed were no longer capable of undergoing cell division and had permanently exited from the cell cycle. The *in situ* hybridisation study of sheep *K2.10* expression patterns in transgenic mouse follicles compared with the cell proliferation marker, histone *H3*, showed that cell proliferation had ceased before *K2.10* expression was detectable and was not re-established in hair cortical cells expressing the *K2.10-TAg* transgene. Therefore, it seems likely that TAg is unable to induce re-entry into the cell cycle at this point in the hair keratinocyte differentiation pathway. In order to produce a follicle-specific cell line through targeted transgenesis, TAg expression must be targeted earlier in follicle differentiation where cell division is still possible.

4.3.4 Other promoters for TAg targeting in the follicle

(i) *K2.12* promoter

As it was desirable to produce a cell line with differentiated cortical cell characteristics to enable expression studies of hair keratin genes, a cortical keratin promoter that was active earlier than *K2.10* in cortical cell development, such as *K2.12*, was thought to be ideal. *K2.12* is the earliest known hair type II keratin expressed in the follicle and the cells in which expression is first detected appear to be in the proliferative zone just below the tip of the dermal papilla (see figure 4.4). Thus, it was hoped that the *K2.12* promoter would be able to target TAg to cells at the stage where they were beginning to undergo terminal differentiation but were still capable of cell division, analogous to γF-crystallin expression in the lens (Bryce *et al.*, 1993).

Although this attempt to isolate the sheep *K2.12* gene using a library screening approach was unsuccessful, the alternative approaches of (a) isolating all hair keratin type II IF genes with a conserved probe and screening out known genes or (b) using 3' PCR-RACE to obtain a *K2.12* gene-specific 3' untranslated region probe for library screening were not pursued. However, it would be of some interest to obtain this gene. As an early marker of cortical cell differentiation it would be useful for the characterisation of future cell lines. A gene-specific 3' untranslated region probe would confirm that the expression pattern seen is due to *K2.12* (and not an as yet unidentified related gene) and analysis of the promoter region may identify elements that govern the timing of expression of this gene. Finally, the *K2.12* gene promoter may still prove to be the ideal promoter for targeting TAg to cortical cells and might allow cortical cell tumours to be isolated and cultured rather than trying to achieve immortalisation under culture conditions.

(ii) *K1.15* promoter

The isolation and characterisation of the sheep *K1.15* gene provided the opportunity to target the unique population of follicle bulb cells (Whitbread, 1992). The expression pattern of Histone *H3* (see figure 4.3) indicates that these cells lie in the proliferative region of the follicle and, therefore, expression of TAg would be expected to result in immortalisation of these cells. *K1.15*-expressing cells line the basement membrane which separates the dermal papilla from the follicle bulb (L. Whitbread and B. Powell, unpublished observations) and it is likely that these cells are involved in dermal-epidermal interactions necessary for follicle induction and growth. Expression of the sheep *K1.15* gene in transgenic mice also occurred in the cells lining the dermal papilla of pelage follicles

(Whitbread, 1992). However, expression in transgenic whisker follicles was localised to a small cluster of cells adjacent to the dermal papilla at the base of the follicle bulb. Whilst these cells may not correspond to the germinative epithelial cells identified by Reynolds and Jahoda (1991) [since germinative epithelial cells do not contain any appreciable amounts of "soft" keratin (Reynolds and Jahoda, 1991), and *K1.15*-expressing cells appear to be localised slightly higher up than germinative epithelial cells] their location suggests that they play a specialised role in the follicle and may be the basal cells of the follicle. Thus, immortalisation of these cells could provide insights into the complex interactions between follicle keratinocytes and dermal papilla cells, as well as the differentiation potential of these cells and their role in follicle growth.

Transgenic mice expressing *K1.15* were generated using a 15 kb fragment encompassing the gene and including 8 kb of 5' flanking sequence and 600 bp of 3' flanking sequence (see figure 4.9) (Whitbread, 1992). In order to use the 15 kb *sK1.15* gene fragment to direct the expression of other genes (such as TAg) in transgenic mice, several unsuccessful attempts were made to introduce a unique cloning site into the 5' UTR of the gene using site-directed mutagenesis (data not shown). Therefore, in an attempt to define a shorter 5' flanking sequence that would act as a suitable promoter to drive the expression of other genes, expression of a 2.5 kb proximal promoter fragment was shown to drive expression of a reporter gene in Chinese hampster ovary cells, albeit at a very low level (Whitbread, 1992).

Since the activity of this promoter fragment was not certain in transgenic mice, it was argued that the *K1.15-lacZ* construct should initially be tested as it was thought that the *lacZ* reporter gene would provide a rapid and sensitive means of analysis of expression in the skin and follicle. However, none of the 8 transgenic mice obtained showed any expression of *lacZ* in the skin or hair follicles suggesting that the promoter fragment was inactive or not active at significant levels in these tissues. (No other tissues, such as oesophagus, tongue or intestine, were tested for expression because follicle expression was of primary interest). It has since been reported that *lacZ* may not be the ideal reporter gene for use in transgenesis (Cui *et al.*, 1994) and that *lacZ* sequences may contain elements which act as weak transcriptional silencers (Paldi *et al.*, 1993). Therefore, using the alternative construct, *K1.15*-TAg, may have yielded a more definitive result. However, recent results showing good follicle expression of *lacZ* in transgenic mice using a 5 kb 5' promoter fragment of *K1.15* (B. Powell and A. Nesci, personal communication) indicate that this longer promoter should be successful in targeting TAg to the cell population of interest.

(iii) *Mt-Ia* promoter

Four distinct metallothionein isoforms have been identified in sheep (sMT-Ia, sMT-Ib, sMT-Ic and sMT-II) (Peterson *et al.*, 1988) whilst only 2 genes (*MT-I* and *MT-II*) are present in the mouse. Detection of *sMt-Ia* expression in hair follicle bulb cells of transgenic mice (K. Ward, personal communication) indicated that this may also be a suitable promoter for targeting TAg expression and obtaining immortalised hair follicle cell lines. The expression of the murine metallothionein I gene has been well characterised in the follicle with expression localised to the ORS and matrix cells of the hair bulb (Karasawa *et al.*, 1991). Antibody staining of follicles for metallothionein (Karasawa *et al.*, 1991) appears to coincide with Histone *H3* expression seen in the follicle bulb (see figure 4.3). It would be expected that TAg expression in these cells could therefore lead to the production of a number of immortalised cell lines with different characteristics; such as undifferentiated follicle bulb cells, and cells exhibiting differentiation characteristics of inner root sheath, cortical or medullary cells.

The extremely low rate of transgenesis (one transgenic out of 80 pups born), the low transgene copy number of the only transgenic and its unexpected death at approximately 6 weeks old may suggest some problem with producing viable mice with the *sMtIa-TAg* construct. However, without being able to analyse the single transgenic mouse for cause of death or transgene expression no conclusions can be drawn. The expression pattern of the *sMt-Ia* promoter has been analysed to some extent in *sMtIa*-growth hormone transgenic mice (Shanahan *et al.*, 1989). Low level expression is seen in the brain at days 17-18 of gestation, but not in kidney or liver. Neonatal mice show transgene expression in the brain, liver, kidney and intestine at day 1 which disappears from all tissues except brain after 3-5 days. Low expression is detectable in brain, testis, lung and occasionally ovary and kidney in adult mice. It appears unlikely that transgene expression should be lethal considering that the temperature-sensitive TAg mutant, *tsA58*, was used and this is active at 33-35^{o}C and inactive at higher temperatures. Also, several lines of mice have been produced which express *tsA58* under the control promoters such as *H-2K^{b}* which is active in virtually all tissues (Jat *et al.*, 1991).

Transgenesis with this construct was discontinued due to the late stage of the project but it is still an option to pursue for the production of useful follicle cell lines via transgenic mice.

4.3.5 Final remarks

No immortalised follicle cell lines were obtained during the course of this work. Further transgenesis with TAg constructs has been discontinued or taken on by other workers due to the time constraints of this thesis. As no follicle cell line was developed, other techniques were employed for the analysis of the *K2.10* promoter, such as *in vitro* gel shift assays and deletion analysis using transgenic mice. These are discussed in the following chapters.

Chapter Five

Analysis of the sheep *K2.10* gene promoter using transgenic mice

5.1 Introduction

The hair follicle is a complex and dynamic structure which provides an interesting model for the study of cellular proliferation and differentiation. Cellular differentiation in the follicle cortex involves activation of specific keratin gene families at different stages of cortical cell differentiation such that a hierarchy of gene expression is established (Powell *et al.*, 1991, 1992).

Keratins are the structural proteins of the hair and form the bulk of the synthesised protein in terminally differentiating keratinocytes. However, whilst the expression patterns of these genes in the follicle have been mapped (Bertolino *et al.*, 1990; McNab *et al.*, 1990; Kaytes *et al.*, 1991; Powell *et al.*, 1991; Fratini *et al.*, 1993; Huh *et al.*, 1994; Jenkins and Powell, 1994; Winter *et al.*, 1994), little is known about their control.

Studying the regulation of hair follicle genes may provide insights into the mechanisms controlling keratinocyte differentiation. Identification of important hair follicle regulatory elements could improve understanding of hair growth *per se* as well as various environmental and nutritional effects on hair growth [for example, the effect of increased dietary cysteine on wool growth (Fratini *et al.*, 1994)] and factors controlling fibre strength, length and diameter.

To date, no follicle-specific regulatory elements or factors have been identified, although the expression of several transcription factors such as Skn-1a/Skn-1i (Andersen *et al.*, 1993), BMP-2 (Lyons *et al.*, 1990) and c-fos (Fisher *et al.*, 1991) has been shown in the follicle, and putative binding sites for known factors have been identified by sequence comparisons (Powell *et al.*, 1991). Identification of hair-specific regulatory elements and factors has been hampered by the lack of follicle cell lines or other cell lines which support hair keratin gene expression. To overcome this, transgenic mice and *in vitro* techniques have therefore been used as investigative tools in the work described here. The current experiments attempt a more detailed analysis of the sheep *K2.10* gene

promoter by making several deletion constructs of the proximal promoter region, linking these to the *lacZ* reporter gene and looking for hair follicle expression in transgenic mice.

Whilst generation of transgenic mice expressing sheep *K2.10* (Powell and Rogers, 1990) shows that the 14.5 kb *K2.10* gene fragment spanning the entire gene is sufficient to target high level expression to the hair follicle cortex following the pattern of the endogenous gene, expression in *K2.10-TAg* transgenic mice shows that 2.8 kb of 5' promoter sequence is also sufficient to direct correct spatio-temporal gene expression in the follicle. This proximal promoter sequence contains a number of consensus binding sites for general transcription factors (figure 5.1) and several putative regulatory regions that have been identified as highly conserved sequences in the hair keratin genes. The function of the most highly conserved such element within this promoter region named HK-1 (hair keratin factor-1) (Powell *et al.*, 1991) is also examined. This putative regulatory element was identified due to the high conservation of the 8 bp HK-1 sequence, CTTTGAAG, found in 8 hair keratin genes, including both mouse and sheep genes.

5.2 Results

5.2.1 *K2.10(400)-lacZ* construct

The *K2.10(400)-lacZ* transgene contained 400 bp of proximal 5' *K2.10* promoter (-350 to +53) linked to the E.*coli lacZ* reporter gene (figure 5.2) and was microinjected into fertilised mouse embryos. Twelve transgenic mice were identified by dot blot (data not shown) and Southern blot analyses: #3, #7, #10, #13, #15, #16, #17, #23, #31, #41 #46 and #50 (figure 5.3). Transgene copy numbers varied from less than 1 copy (low copy number chimeric mice) to approximately 200 copies.

Transgenic mice were analysed for expression of β-galactosidase in dorsal skin biopsies by staining for enzymic activity using X-gal (see 2.2.5.2). Only four mice, #3, #7, #31 and #46, were shown to express β-galactosidase (figure 5.4). Expression was directed to the cortex of the hair follicle in each case and was usually seen over the entire cortex. The percentage of pelage follicles expressing β-galactosidase ranged from virtually all follicles of mouse #31 to approximately 20% of follicles of mice #7 and #46.

However, despite readily detectable β-galactosidase expression in the follicles of these transgenic mice, none of their transgenic offspring showed any follicle expression of the transgene (data not shown). Mice #3.4 and #3.9 appear to have inherited a mutated transgene locus. No transgene

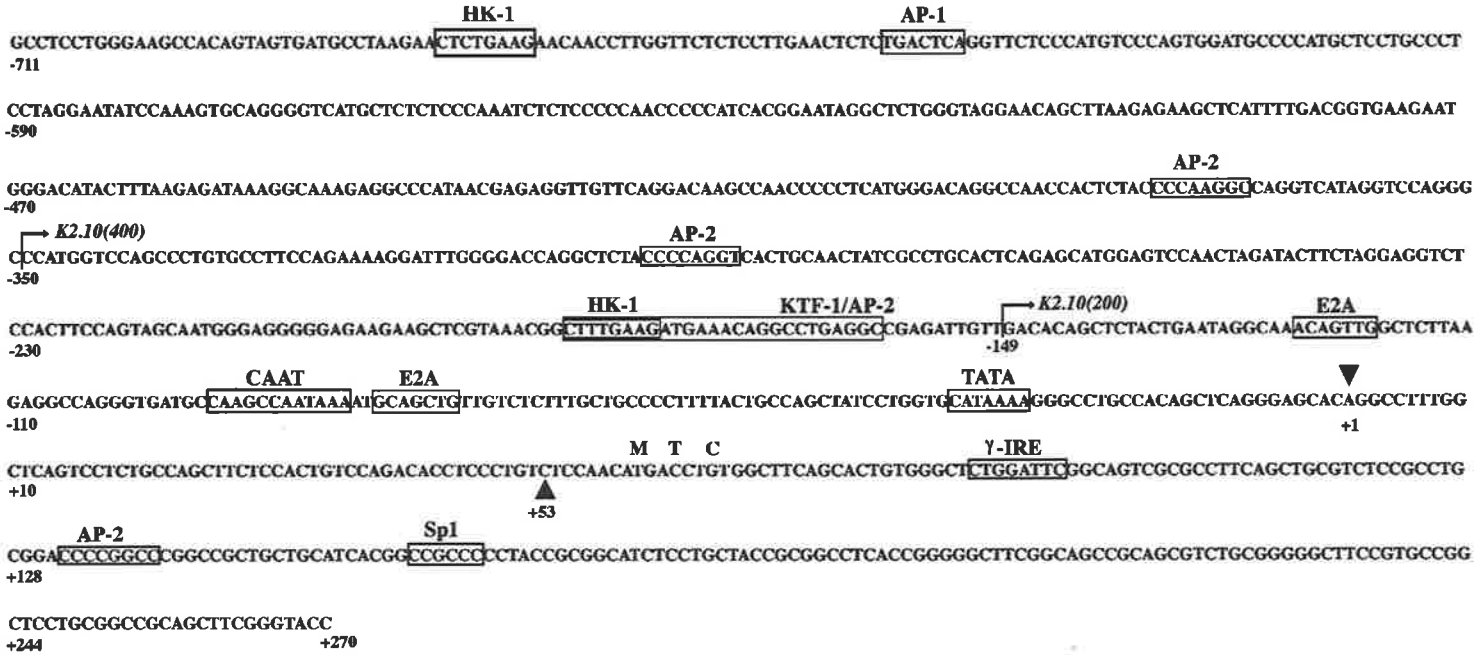


Figure 5.1 Sequence of the sheep *K2.10* gene proximal promoter and part of exon 1 showing putative transcription factor binding sites. AP-1, AP-2, HK-1, Sp1, E2A, γ-interferon responsive element (γ-IRE), CAAT and TATA motifs are boxed. The CAP site at +1 marks the start of transcription and +53 is the point at which a Sal I site was engineered in the 5' UTR such that reporter genes could be cloned into this unique site. Bent arrows indicate the 5' ends of the *K2.10(400)* and *K2.10(200)* promoter deletions as labelled (see 5.2.1 and 5.2.2). M, T, C (methionine, threonine and cysteine) are the first three translated codons in exon 1. (The remainder were omitted for clarity of the figure). Modified from Powell *et al.,* (1991).

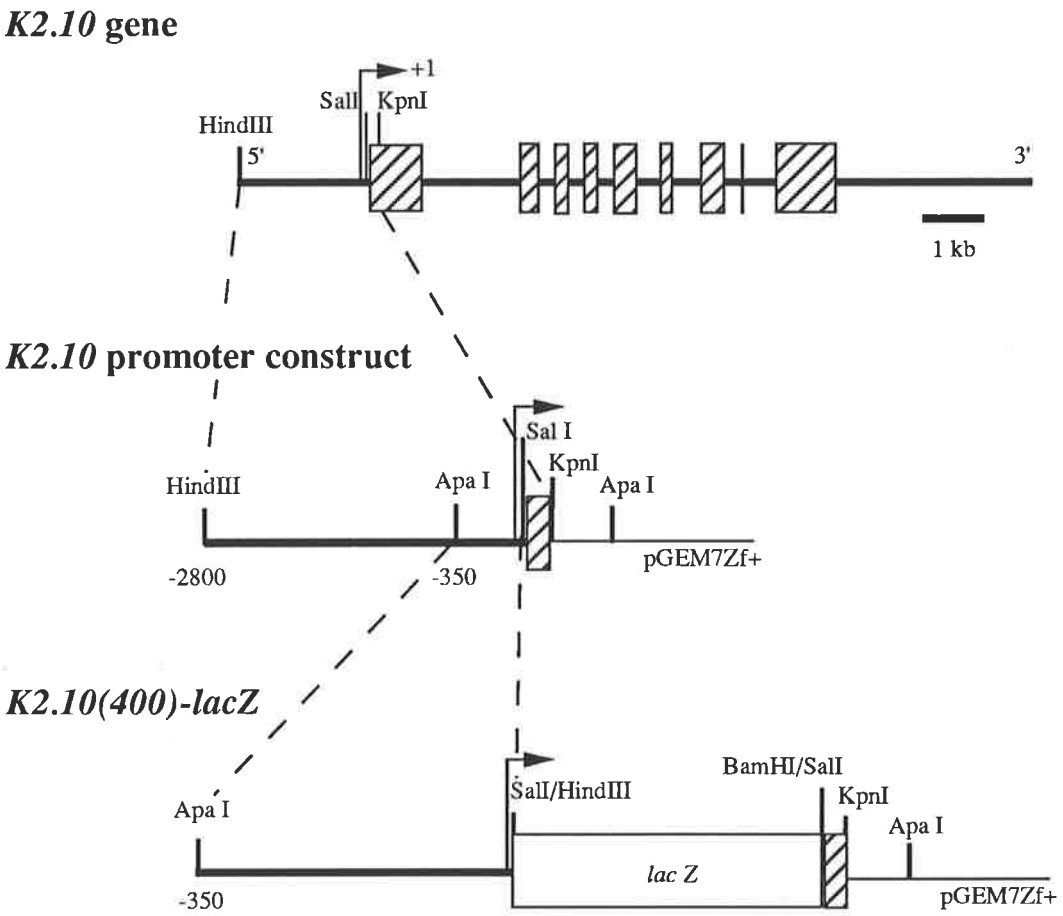


Figure 5.2 Generation of the *K2.10(400)-lacZ* transgene construct.

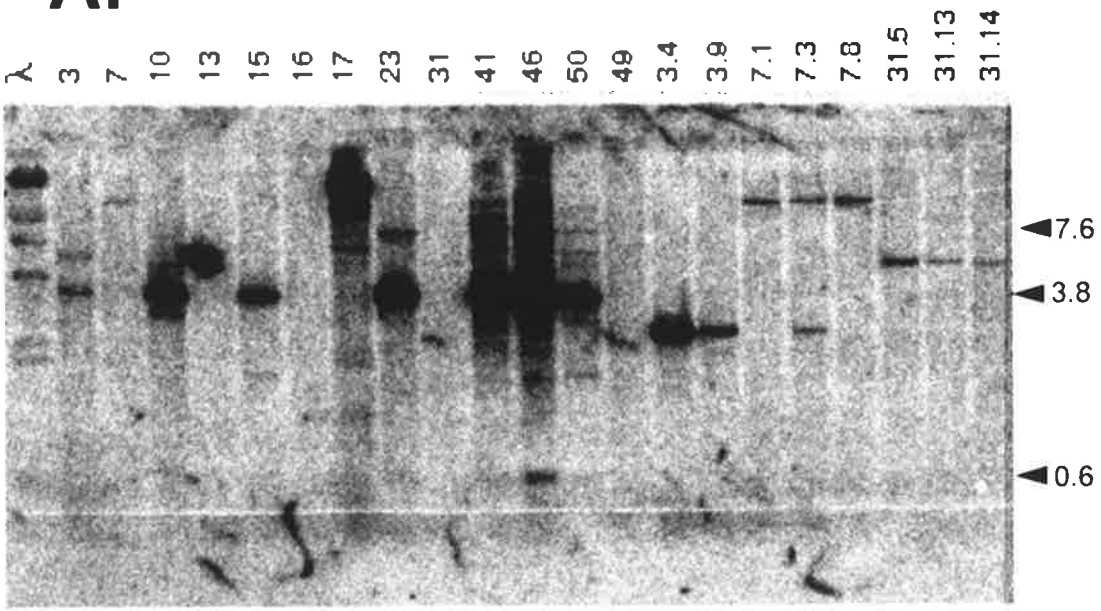
K2.10(400)-lacZ was constructed using the pGEM7Zf(+) vector which contained the *K2.10* promoter fragment (3 kb, including 200 bp of exon 1 sequence) into which a Sal I site had been engineered at position +53. The Hind III/Bam HI *lacZ* fragment (3.85 kb) from pCH110 (Pharmacia) was cloned into this Sal I site after endfilling. The construct was then digested with Apa I to yield the *K2.10(400)-lacZ* transgene fragment (4.45 kb) containing ~400 bp of *K2.10* proximal promoter from +53 to -350. *K2.10* gene sequence is shown as a thick line, hatched boxes represent exon sequence and the site of transcription initiation is indicated by a bent arrow. Vector sequence, pGEM7Zf(+), is shown as a thin line. *K2.10* promoter construct and *K2.10(400)-lacZ* are not drawn to scale.

Figure 5.3 Southern blot analysis of *K2.10(400)-lacZ* transgenic mice.

(A) Genomic mouse tail DNA (10 μg) was digested with Eco RI and separated on a 1% agarose gel. The DNA was transferred to Zetaprobe GT membrane under alkaline conditions. The filter was hybridised with radiolabelled *lacZ* probe (3.85 kb Hind III/Bam HI *lacZ* gene fragment) and washed at a final stringency of 2X SSPE, 0.1% SDS at 65°C. The filter was then exposed to a phosphor image screen. Mice #16 and #13 were shown to be transgenic by dot blot analysis (data not shown), however, the sensitivity of the Southern was not good enough to confirm this, suggesting the possibility that they are low copy number chimeric mice. Mouse #49 is a non-transgenic control. Mice 3.4 and 3.9 are F_1 offspring of mouse #3, mice 7.1, 7.3 and 7.8 are F_1 offspring of #7 and 31.5, 31.13 and 31.14 are F_1 offspring of #31.

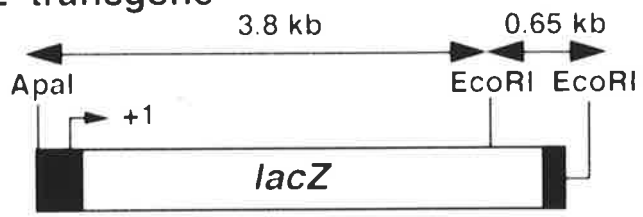
(B) A diagrammatic representation of the *K2.10(400)-lacZ* transgene fragment and possible arrangements of multiple copy insertions in the mouse genome. Eco RI digestion of a head-to-tail array results in a diagnostic band of 3.8 kb. This arrangement is seen in mice #3, #10, #15, #23, #41, #46 and #50. A 7.6 kb fragment is diagnostic of head-to-head arrangements and this is seen in mice #23, #41, #46 and #50 (Note that the 7.6 kb band present in tracks corresponding to #41 and #46 is more clearly seen on a lighter exposure of this gel; data not shown). The band sizes displayed by mice #7 and #31 are indicative of single copy number transgenics. These bands are also seen in their F_1 offspring. (The difference in band intensity between #7 and #7.3 and #7.8 is due to loading inaccuracies). Note that there is also a smaller band (2.8 kb) present in #7.3, suggesting that #7 was chimeric for the second insertion site that appears to contain a deletion or rearrangement of the transgene. The banding pattern of #17 suggests either incomplete digestion (although the DNA appeared to be cut to completion) or transgene rearrangements. Mice #3.4 and #3.9 display a single band of 2.8 kb which is not detectable in the founder animal. This indicates that either #3 was chimeric or that a specific transgene rearrangement has occurred. The band size suggests there has been a deletion in the transgene locus similar to that present in mouse #7.3.

A.

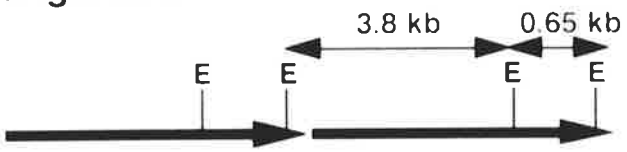


B.

K2.10(400)-lacZ transgene



Head-to-tail arrangement



Head-to-head arrangement

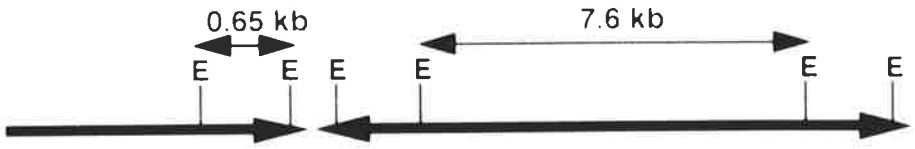
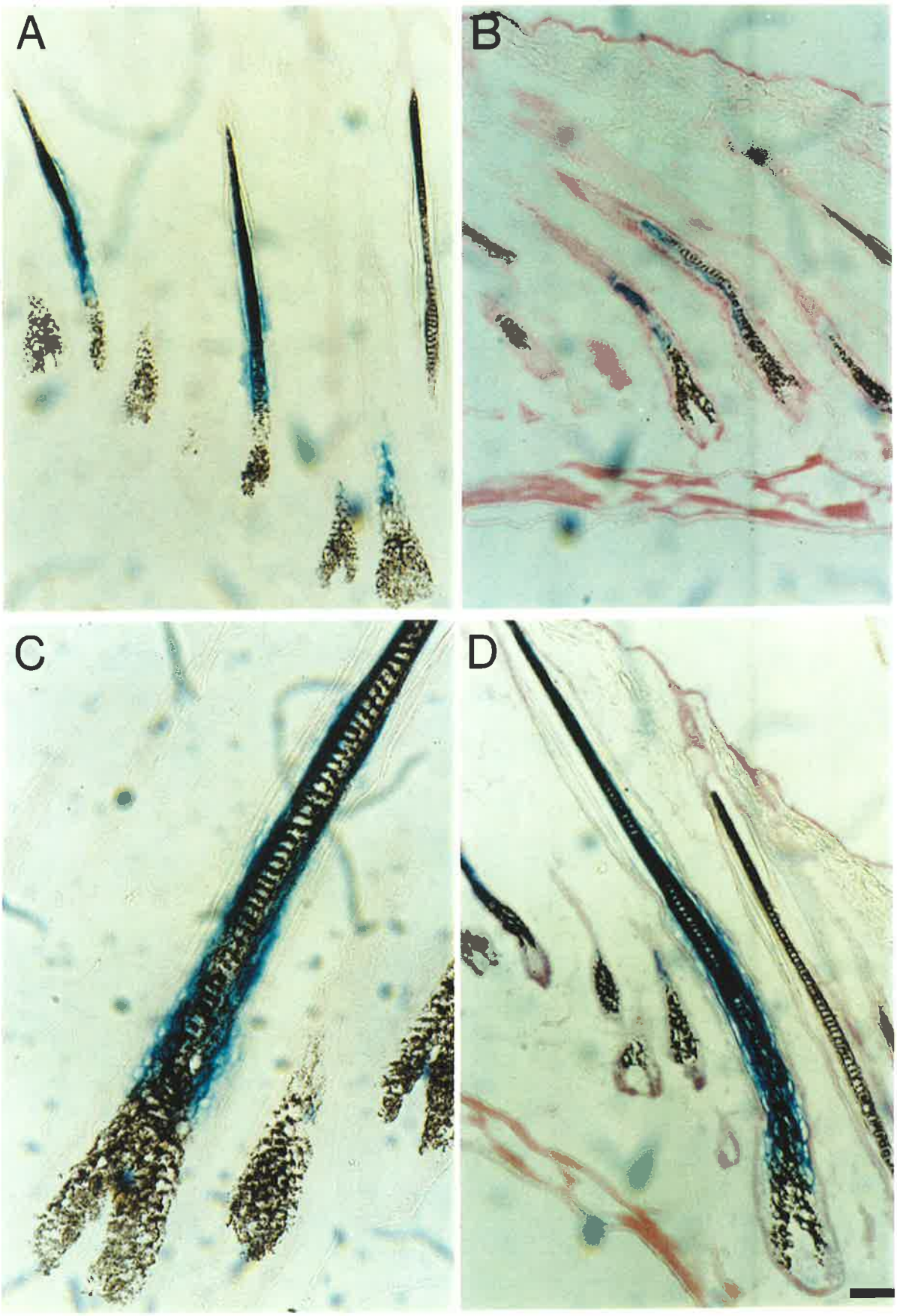
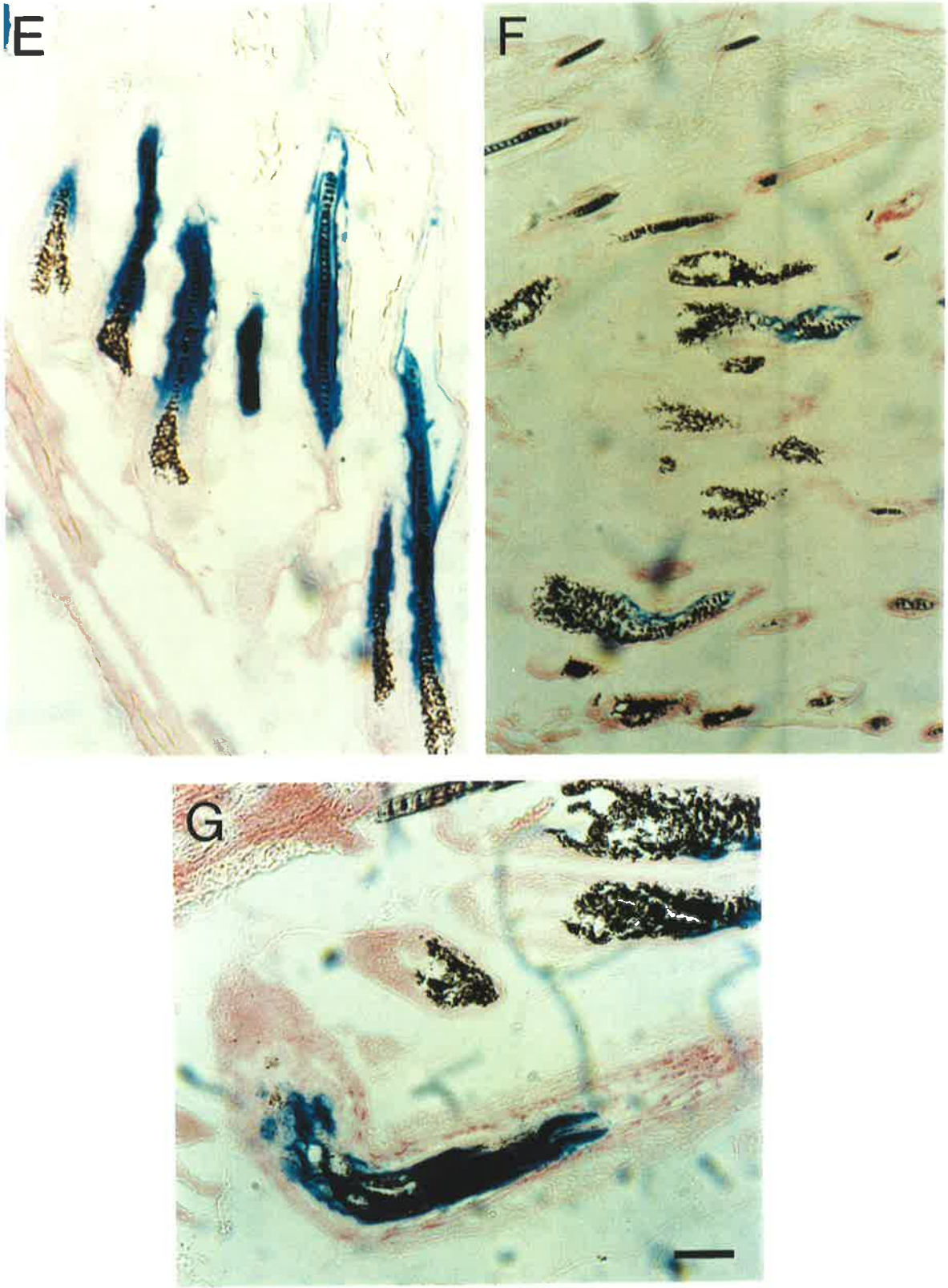


Figure 5.4 Expression of *K2.10(400)-lacZ* in the skin of transgenic mice.

(A,C), skin from transgenic mouse #3; (B,D), skin from transgenic mouse #46; (E), skin from transgenic mouse #31; and (F,G), skin from transgenic mouse #7 all show β-galactosidase staining (blue staining) localised to the cortex of the hair follicle. No expression is seen in the epidermis. Sections were counterstained with eosin. Bar, (A,B,D,E,F) 100 μm; (C,G) 50 μm.


A
B
C
D


E
F
G

rearrangements were seen in #7 and #31 offspring, although mouse #7.3 appears to have also inherited a second locus in which the transgene is mutated (figure 5.3). Mouse #46 was not mated.

Whole embryo stainings for β-galactosidase activity on e16.5 transgenic embryos derived from #7-line also failed to show any specific staining in developing whisker follicles or other tissues, although staining of intestinal villi was detected in both wild type and transgenic foetuses (figure 5.5).

5.2.2 *K2.10(200)-lacZ* construct

A second deletion construct, *K2.10(200)-lacZ*, containing only the immediate 200 bp of 5' *K2.10* promoter (-149 to +53) linked to the *lacZ* reporter gene was analysed in transgenic mice (figure 5.6). This construct lacked all of the conserved sequences identified in the promoter that may act as putative control elements (see figures 5.1 and 5.6A), but contained the CAAT and TATA elements thought necessary for minimal promoter function. Of 115 mice born, 17 transgenic mice were identified by dot blot analysis (data not shown) and this was confirmed by Southern blot (figure 5.7). Transgene copy numbers ranged from 5-10 copies up to approximately 100 copies (mouse #102).

Dorsal skin biopsies from these mice were analysed for β-gal activity but no expression of *K2.10(200)-lacZ* was evident in the hair follicles or skin of these mice (data not shown).

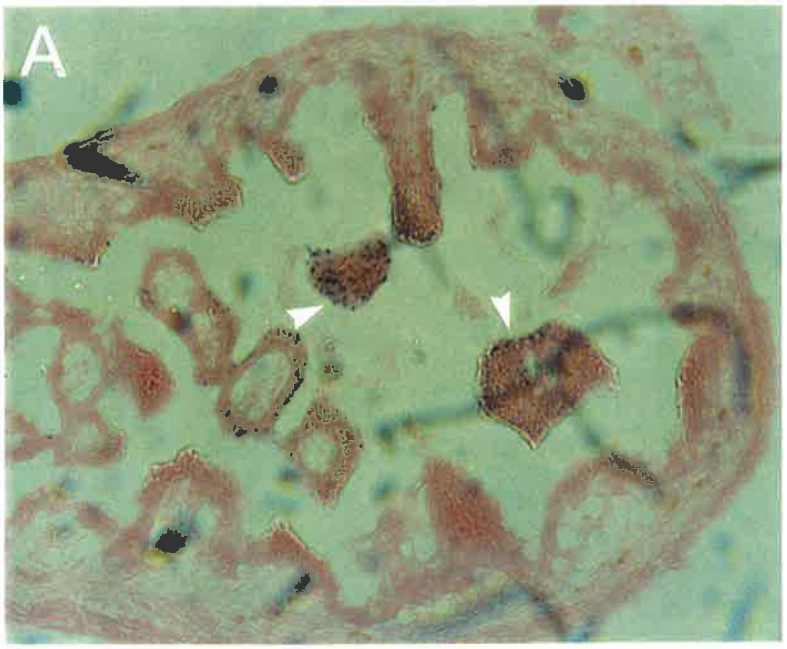
5.2.3 K2.10(400ΔHK-1)-lacZ construct

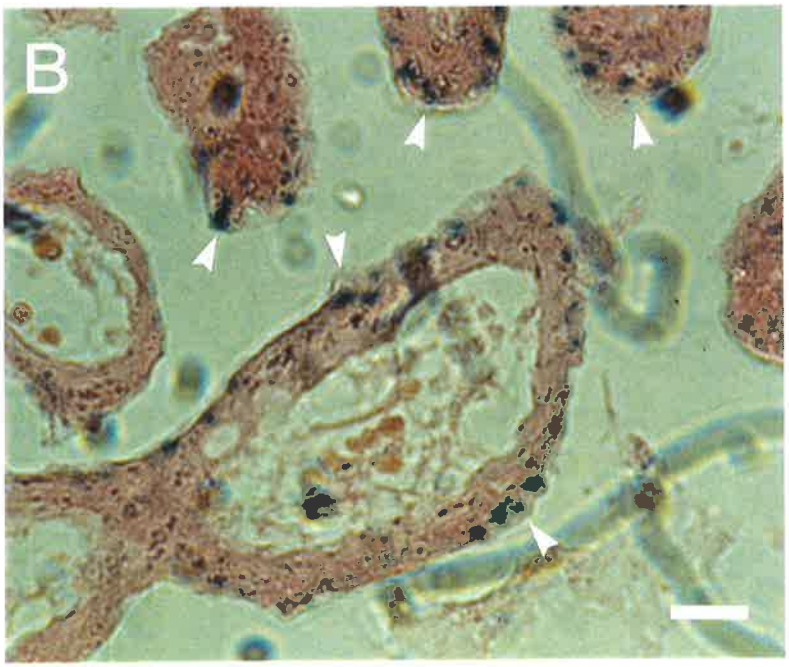
The *K2.10(400ΔHK-1)-lacZ* construct was generated to investigate a possible role of the putative HK-1 element at -180 of the *K2.10* promoter (see figure 5.1). Preliminary binding studies showed that the sequence was capable of binding a protein(s) from hair follicle nuclear extracts (see 6.2.2) and the high conservation of the sequence between different hair keratin genes from both sheep and mouse suggested that this element may play a regulatory role in gene expression.

K2.10(400ΔHK-1)-lacZ was generated by site-directed mutagenesis of the HK-1 element of *K2.10(400)-lacZ* using oligo ΔHK-1mut (2.1.11). The HK-1 sequence was mutated from CTTTGAAG to CGTCTAGG disrupting the proposed consensus sequence. The resultant construct (figure 5.8) was used to generate 13 transgenic mice which were identified by Southern blot (figure 5.9). Transgene copy numbers ranged from approximately 10 copies (mouse #50) to 100 copies

Figure 5.5 Staining of β-galactosidase activity in the intestinal villi of foetal mice.

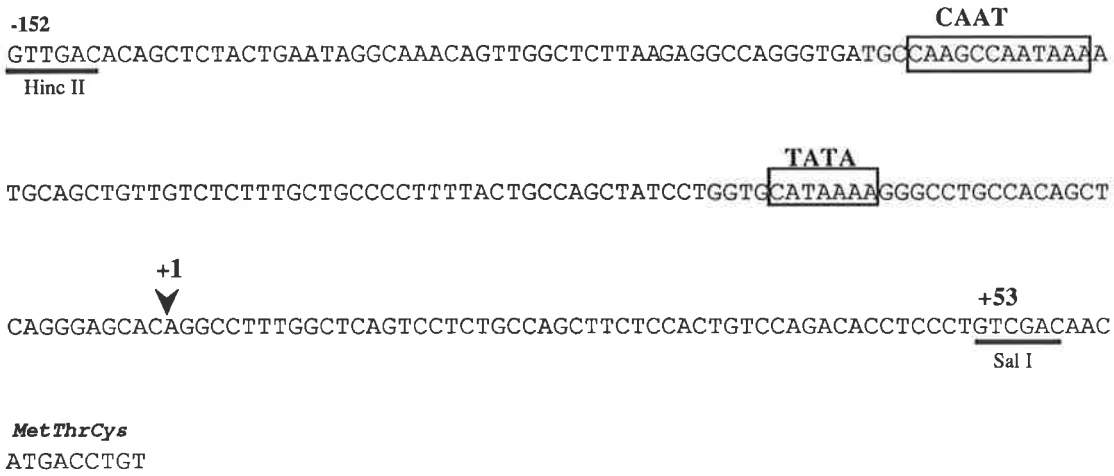
Both transgenic and non-transgenic e16.5 mouse embryos stained for β-galactosidase activity in intestinal villi (arrowheads). No other staining was observed in e16.5 *K2.10(400)-lacZ* transgenic embryos. Bar, (A) 100 μm; (B) 25 μm.


A


B

A.

***K2.10(200)* promoter**



B.

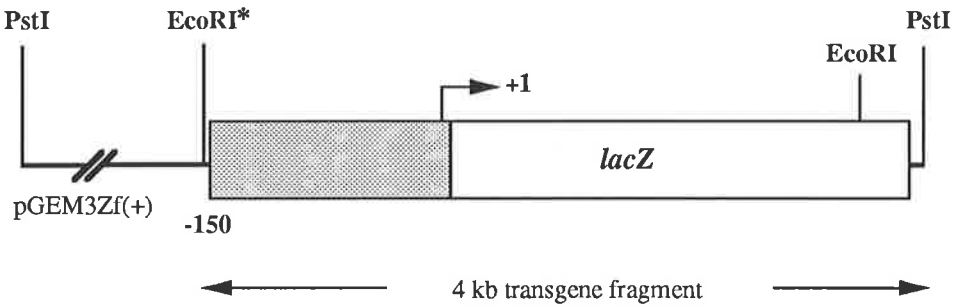


Figure 5.6 Generation of the *K2.10(200)-lacZ* transgene fragment.

(A). Sequence of the *K2.10* promoter used to generate the transgene. The Hinc II site at -152 and the position of the engineered Sal I site at +53 are shown. The CAAT and TATA motifs are boxed and the CAP site is denoted as +1.

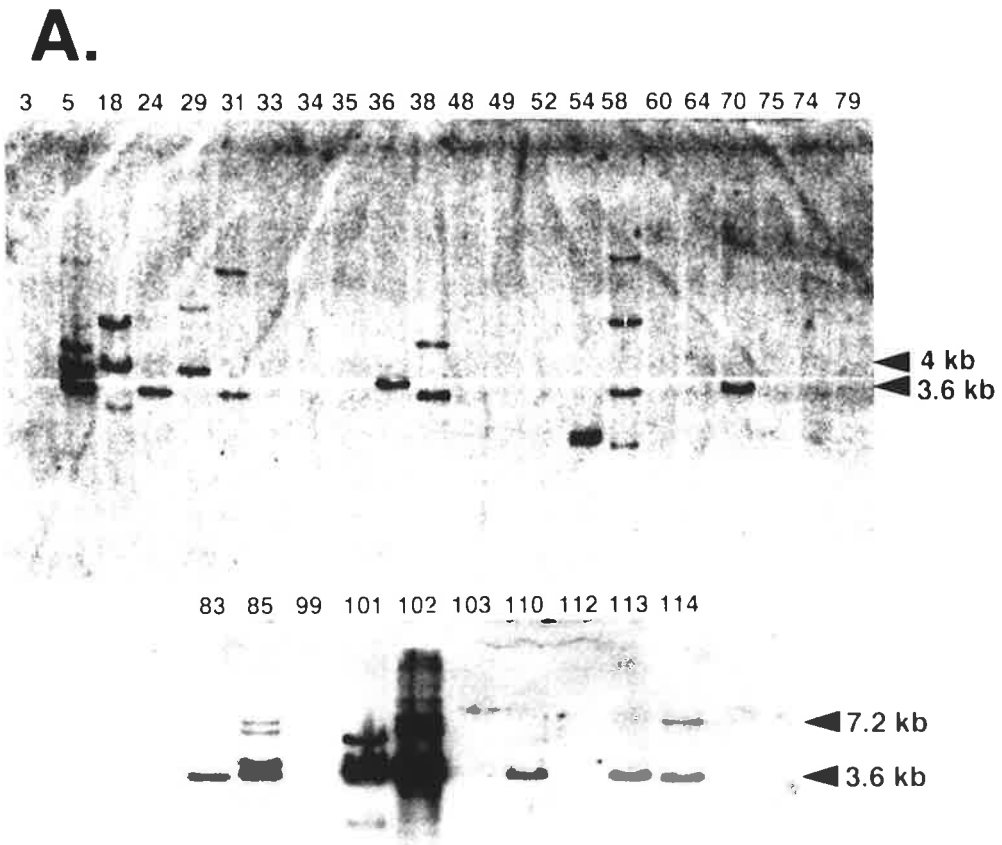
(B). In order to generate the *K2.10(200)-lacZ* construct, the 2.8 kb *K2.10* promoter fragment (containing the engineered Sal I site) was digested with Sal I and then Hinc II. The resultant 200 bp fragment was cloned into a Sal I/Sma I digested pGEM3Zf(+) vector. The 3.85 kb*lacZ* gene (an endfilled Hind III/Bam HI fragment) was subsequently cloned into the endfilled Sal I site at +53 of the *K2.10* promoter. Excision of the transgene fragment was achieved by digestion with Pst I followed by partial digestion with Eco RI. The 4 kb transgene construct contained 200 bp of 5' proximal *K2.10* promoter (from -149 to +53) linked to the *lacZ* reporter gene. Note: this construct lacked the 200 bp of *K2.10* exon 1 sequence which was present in all other *K2.10* constructs. The *K2.10* promoter region is represented by the stippled box, pGEM3Zf(+) vector sequence is represented as a thin line and the transcription start site is indicated by a bent arrow. The asterisk denotes the 5' terminal Eco RI site of the transgene fragment which may not be regenerated upon transgene insertion into the mouse genome unless the Eco RI/Pst I transgene fragments insert in a head-to-head arrangement (see figure 5.7). Not drawn to scale.

Figure 5.7 Southern blot analysis of *K2.10(200)-lacZ* transgenic mice.

(A) Mouse tail genomic DNA (10 μg) was digested with Eco RI and separated on a 1% agarose gel. The DNA was transferred to Zetaprobe GT membrane, the filter was hybridised with radiolabelled *lacZ* probe (3.85 kb Hind III/Bam HI *lacZ* gene fragment) and washed at a final stringency of 2X SSPE, 0.1% SDS at 65°C. The filter was then exposed to a phosphor image screen.

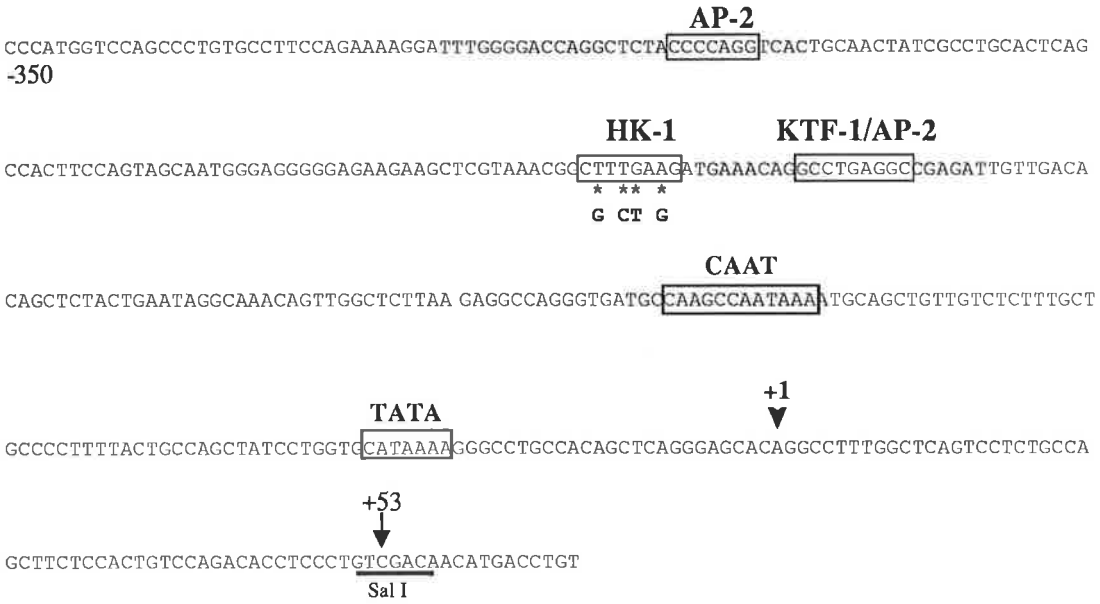
(B) A diagrammatic representation of the arrangement of multiple transgene copies integrated into the mouse genome at a single locus. As the transgene construct was produced with Eco RI/Pst I ends, regeneration of the terminal Eco RI site could only occur in head-to-head arrangements and digestion with Eco RI would result in a diagnostic fragment of 3.6 kb as shown. If the Eco RI site is not regenerated a 7.2 kb fragment would be expected. Tail-to-tail arrangement produces a 900 bp diagnostic Eco RI fragment which is not detectable on this Southern blot. Head-to-tail arrays generate a diagnostic 4 kb Eco RI fragment.

Mouse #29 shows a 4 kb EcoRI band indicative of multicopy transgene insertion in a head-to-tail arrangement. Mouse #5 also shows a 4 kb fragment together with a 3.6 kb band diagnostic of head-to-head arrays. Mice #24, #31, #38, #58, #70, #83, #85, #101, #102, #110, #113 and #114 all contain a 3.6 kb Eco RI fragment. A 7.2 kb band is also present in mice #85, #102 and #114 which suggests a head-to-head arrangement of some transgenes in which the terminal Eco RI site is not regenerated. The Southern pattern of other mice #18, #36 and #58 may be due to incomplete DNA digestion (although the DNA appeared to be fully digested), single-copy transgene insertion into one or multiple sites or due to DNA rearrangements (as must have occurred in mouse #54).


A.
3 5 18 24 29 31 33 34 35 36 38 48 49 52 54 58 60 64 70 75 74 79
4 kb
3.6 kb
83 85 99 101 102 103 110 112 113 114
7.2 kb
3.6 kb
B.
Tail-to-tail, head-to-head array
E
E
E
E
0.9 kb
3.6 kb
7.2 kb
Head-to-tail array
E
E
4 kb

A.

***K2.10(400)* promoter sequence**



B.

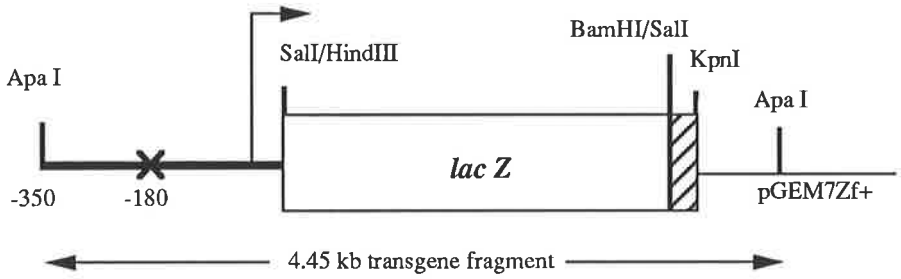


Figure 5.8 Generation of the *K2.10(400ΔHK-1)-lacZ* construct.

(A). *K2.10* promoter sequence spanning -350 to +69 showing the mutations introduced into the HK-1 element at -180. Putative AP-2 sites and the CAAT and TATA motifs are boxed. The KTF-1/AP-2 site is so-called due to the striking similarity with the Xenopus KTF-1 site (Snape *et al.*, 1990,1991). The transcription start site is indicated at +1 and the engineered Sal I cloning site is shown at +53.

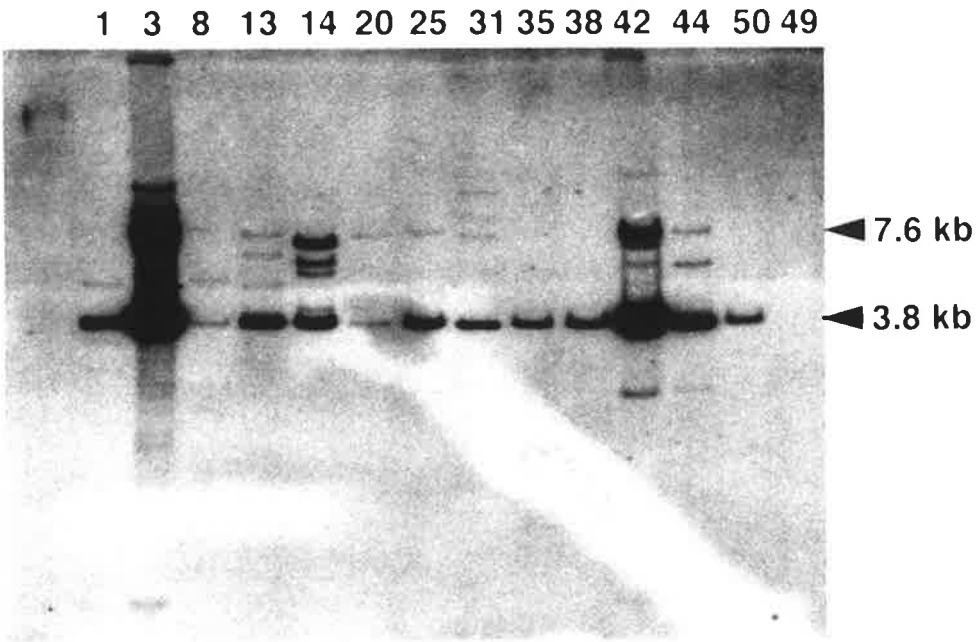
(B). In order to generate the*K2.10(400ΔHK-1)-lacZ* construct, site-directed mutagenesis was performed on the *K2.10* promoter fragment (3 kb) in pGEM7Zf(+) (see 2.2.1.11). The HK-1 sequence was mutated from CTTTGAAG to CGTCTAGG using the mut-HK-1 38mer oligo (see 2.1.11) and the mutation was confirmed by sequencing. The Hind III/Bam HI *lacZ* gene fragment (3.85 kb) from pCH110 was cloned into the engineered Sal I site at +53 of the *K2.10* promoter after endfilling. The construct was then digested with Apa I to yield the*K2.10(400ΔHK-1)-lacZ* transgene fragment (4.45 kb) containing 400 bp of *K2.10* proximal promoter from +53 to -350 and a mutated HK-1 element at -180. *K2.10* promoter sequence is represented as a thick line and the mutated HK-1 sequence as a cross, exon 1 sequence (200 bp) is represented as a hatched box, the transcription start site is indicated by the bent arrow and pGEM7Zf(+) vector sequence is shown as a thin line. Not drawn to scale.

Figure 5.9 Southern blot analysis of *K2.10(400ΔHK-1)-lacZ* transgenic mice.

(A) Mouse genomic DNA (10 μg) was digested with Eco RI, separated on a 1% agarose gel and transferred to Zetaprobe GT membrane. The filter was hybridised with radiolabelled *lacZ* probe (3.85 kb Hind III/Bam H1 *lacZ* gene fragment) and washed at a final stringency of 2X SSPE, 0.1% SDS at 65°C.

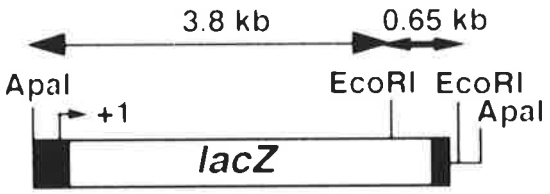
(B) A diagrammatic representation of possible arrangements of multiple copies of the transgene integrated into the mouse genome. The head-to-tail arrangement results in a diagnostic 3.8 kb Eco RI fragment, whilst the head-to-head arrangement generates a 7.6 kb Eco RI fragment. All *K2.10(400ΔHK-1)-lacZ* transgenic mice appear to contain transgenes in the head-to-tail array as indicated by the 3.8 kb band. Mice #3, #13, #14, #31, #42, #44 and possibly #20 and #25 also contain some transgene copies in a head-to-head array. The multiple banding pattern seen for mice #3 and #42 is likely to be due to transgene integration into more than one locus, but could also be explained by incomplete digestion of the DNA or transgene rearrangements. DNA from a non-transgenic mouse, #49, was used as a negative control.

A.

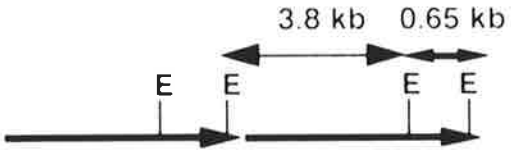


B.

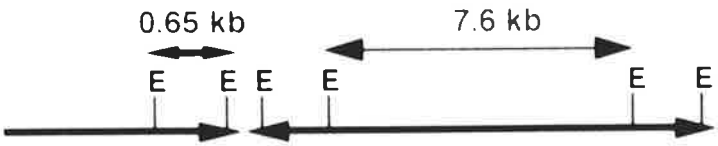
K2.10(400 \HK-1)-lacZ transgene



Head-to-tail arrangement



Head-to-head arrangement



(mouse #3) and all mice contained at least 2 copies in a head-to-tail array (resulting in the 3.8 kb Eco RI band described in figure 5.8). Of these transgenic mice, only #14 and #38 showed *lacZ* expression by X-gal staining of skin (figure 5.10). Expression was confined to the hair follicle cortex, however, unlike *K2.10(400)-lacZ* mice, expression was patchy and only small pockets of cortical cells were stained. Both mice #14 and #38 showed expression in only 1-10% of pelage follicles.

5.3 Discussion

5.3.1 Expression of *K2.10(400)-lacZ*

Expression of *K2.10(400)-lacZ* in the skin of transgenic mice was confined to the hair follicle, specifically the follicle cortex. The expression seen in mouse #31 which also covered the cuticle and other layers of the follicle appears to be due to "bleeding" of the stain (as very strong staining was observed), rather than true expression in these cell types. Therefore, the regulatory elements required for cortical cell specificity of expression lie within the 400 bp 5' proximal promoter region of *K2.10*.

K2.10(400)-lacZ day 16.5 embryos were examined for β-gal expression to investigate whether the shorter promoter region resulted in deregulated expression in other tissues but transgene-specific expression was not detected. No adult tissues were analysed for expression apart from skin. β-galactosidase activity was observed in intestinal villi of both transgenic and non-transgenic embryos. Similar reports of endogenous β-gal staining have been documented, particularly in kidney epithelium and testis (Cui *et al.*, 1994).

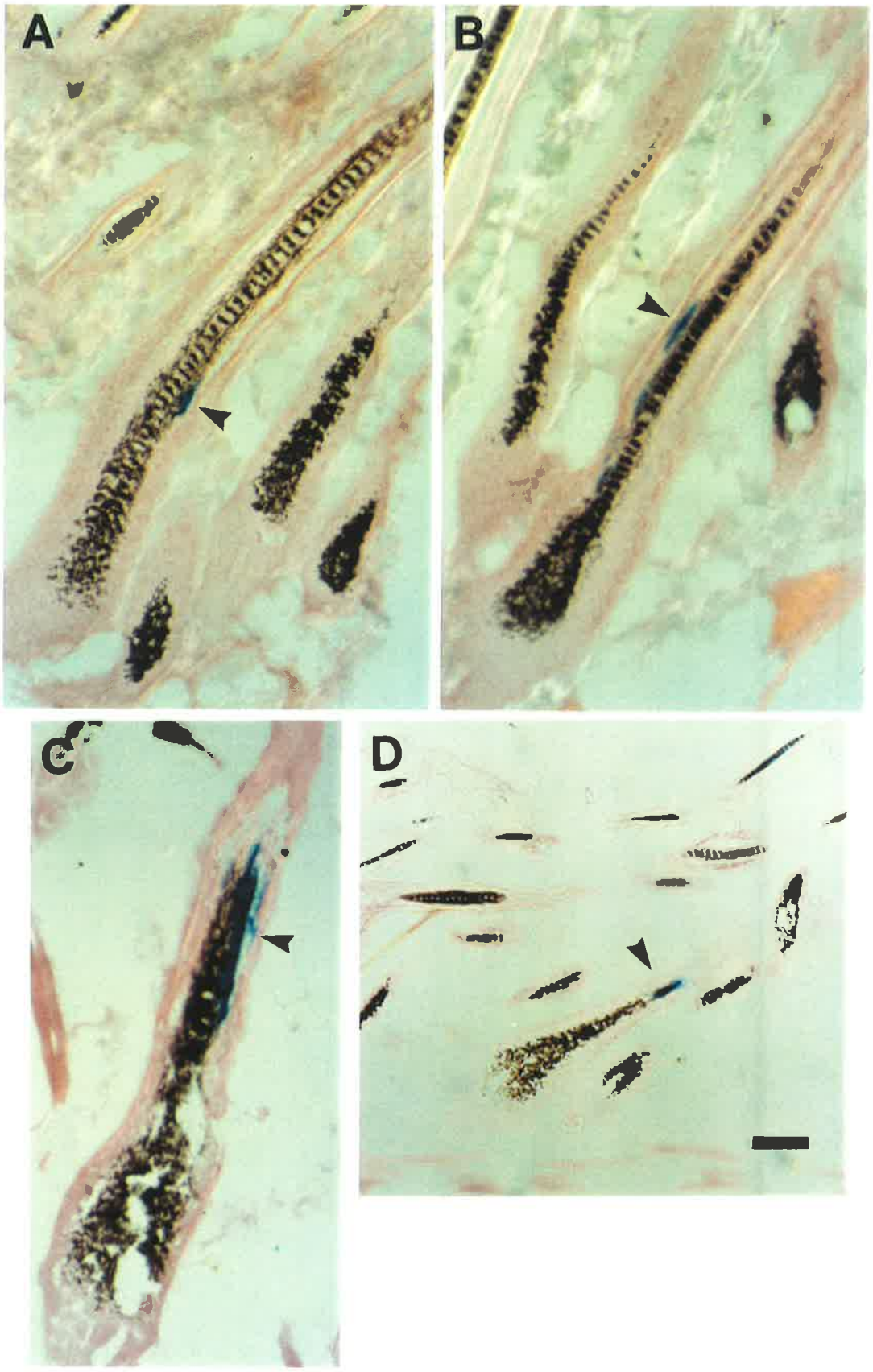
Adult progeny of expressing founder mice showed no detectable β-gal expression in the hair follicle. A similar finding in the adult offspring of *human β-actin-lacZ* transgenic mice has been reported (Cui *et al.*, 1994) and, in cultured cells expressing *lacZ*, heterogeneous expression is often observed in clonal populations (J. Beltrame, personal communication). The mechanism of this shutdown of *lacZ* expression is unknown.

5.3.2 Expression of *K2.10(200)-lacZ*

The 200 bp promoter region of *K2.10(200)-lacZ* contains the CAAT and TATA elements and the transcription initiation (CAP) site thought to be necessary for minimal promoter function, however,

Figure 5.10 Expression of *K2.10(400ΔHK-1)-lacZ* in the skin of transgenic mice.

(A,B) skin from transgenic mouse #14, (C,D) skin from transgenic mouse #38. Patchy β-galactosidase staining is localised to the hair follicle cortex (arrowheads). No staining of the epidermis was observed. Bar, (A,B,C) 50 μm; (D) 100 μm.


A
B
C
D

no expression was observed in the skin of any of the 17 *K2.10(200)-lacZ* mice. The lack of expression could be explained by a number of possibilities. Firstly, a low level of expression from the minimal promoter may be undetectable. Although the X-gal staining procedure is very sensitive, it is known that there is a threshold of enzyme concentration below which no blue staining is seen (Fire, 1992). A second possibility is that the weak minimal promoter is subject to 'silencing' by the *lacZ* gene. It is known that promoters such as *3-hydroxy-3-methylglutarylCoA reductase* (*HMG*) which direct ubiquitous and constitutive expression of the *CAT* reporter gene in transgenic mice do not produce the same expression pattern when the *lacZ* reporter gene is used (Paldi *et al.*, 1993). The mechanism which restricts *lacZ* expression in cells in which *HMG* should be active is unknown. However, it has been postulated that the *lacZ* gene may contain a sequence motif which acts as a weak transcriptional silencer in certain cell types (Paldi *et al.*, 1993). This would prevent expression from weak promoters, such as minimal promoters, possibly in a manner analogous to the neomycin resistance gene (*neo*) which contains a sequence that binds a transcription inhibitor factor present in some mammalian cells (Artelt *et al.*, 1991). The presence of a negative regulatory element in the 200 bp 5' *K2.10* promoter would provide another explanation for the lack of expression in the *K2.10(200)-lacZ* mice. It should also be noted that the *K2.10(200)-lacZ* construct lacks 200 bp of *K2.10* exon 1 sequence that was present in all other *K2.10* transgenes. Sequence analysis of this region reveals the presence of possible AP-2 and Sp1 binding sites and a γ-interferon responsive element (see figure 5.1), however, it is not known whether these motifs are functional, nor whether they had any affect on *K2.10-TAg*, *K2.10(400)-lacZ* and *K2.10(400ΔHK-1)-lacZ* transgene expression.

Interestingly, work on the human *K5* gene promoter has shown that as little as 90 bp of 5' proximal promoter is sufficient to direct *lacZ* expression in transgenic mice (Byrne and Fuchs, 1993). Expression levels were lower with the 90 bp promoter than when a larger (6 kb) promoter fragment was used and expression of the shorter transgene was largely restricted to stratified epithelia such as epidermis, hair follicles and tongue. However, whilst *K5* is normally expressed in mitotically active basal cells of stratified epithelia, the shorter construct was expressed in terminally differentiating cells in these tissues. *In vitro* gel shift analysis and methylation interference studies revealed several binding sites for proteins within this 90 bp region, including two Sp1 binding sites and three other sites which appear to bind as yet unidentified proteins (Byrne and Fuchs, 1993).

Please insert this ammendment at the end of paragraph 2, page 66, replacing the last sentence.

The apparent reduction in the percentage of transgenic mice expressing as the promoter fragment was shortened, and the fact that not all follicles showed expression in these mice, may also indicate that shorter promoters are more susceptible to "position-effects" in the genome, possibly due to the loss of isolating elements such as matrix attachment regions (Bonifer *et al.*, 1990; Phi-Van *et al.*, 1990) or due to the loss of distal enhancer elements. It has recently been shown that distal enhancers can suppress position-effect variegation and appear to act by protecting transgene constructs from repression by flanking chromatin (Walters *et al.*, 1996). These distal enhancer elements have little effect on the rate of gene transcription, but rather, they appear to influence the ability of a gene to remain transcriptionally active.

It has also been reported that tandem arrays of transgenes can cause heterochromatin formation in Drosophila, resulting in gene silencing similar to position-effect variegation (Dorer and Henikoff, 1994). Position-effect variegation has been observed in mice using a number of different transgenes (Robertson et al., 1995 and refs therein). Whilst this phenomenon is believed to result from the influence of surrounding heterochromatin or compacted centromeric DNA, it is not yet clear why this effect varies between otherwise identical cell types within a given tissue.

Gene silencing is associated with methylation and loss of DNase I hypersensitivity at the promoter (Walters *et al.*, 1996), although it is unclear whether heterochromatin formation or methylation is the primary step in silencing. Methylation is known to interfere with the binding of a number of transcription factors, including AP-2 (Comb and Goodman, 1990), and to inhibit transcription initiation (for review see Eden and Cedar, 1994). It would have been of interest, therefore, to compare the methylation patterns of the *K2.10* promoter constructs in follicle-derived DNA from mice expressing a range of reporter gene activities in order to investigate the role of methylation in the expression and silencing of these genes. It is also likely that methylation is associated with the lack of transgene expression in the progeny of expressing founder mice. In F_1 mice transgenes have been transmitted via the germline, having undergone meiosis. It is believed that genomic imprinting patterns are established during this process and that methylation is involved in subsequent gene inactivation (for review see Efstratiadis, 1994). Given the apparent propensity for these transgenes to become silenced in Fo mice, it is possible that germline transmission has resulted in complete inactivation of these genes.

5.3.3 Expression of *K2.10(400ΔHK-1)-lacZ*

Mutation of the HK-1 element in *K2.10(400ΔHK-1)-lacZ* transgenic mice did not affect the specificity of expression, as cortical expression was preserved. It is therefore unlikely that this element is involved in determining follicle-specific expression. However, the expression pattern in the cortex was significantly reduced with only small patches of expression observed. This result raises the possibility that the HK-1 element (which has been shown to bind a protein(s); see 6.2.2) may act as an enhancer sequence such that mutation of the element reduces expression levels. Alternatively, the mutations introduced in the ΔHK-1 motif may not have abolished trans-acting factor binding completely resulting in some low-level follicle-specific transactivation. This seems unlikely considering the extensive disruption of the highly conserved HK-1 sequence, however, gel shift analyses (see 6.2.2) suggests that HK-1 flanking sequences also play a role in protein binding to this element. The mutated HK-1 sequence present in the *K2.10(400ΔHK-1)-lacZ* construct has not yet been tested for the ability to bind hair follicle nuclear proteins. The fact that two independent Fo mice exhibited the same level and pattern of transgene expression supports the argument that the HK-1 element acts as an enhancer. Another possible explanation for the low level of transgene expression which must be considered is a position effect due to the site of transgene integration. The low number of expressing transgenics obtained (i.e. two Fo mice) makes it difficult to determine whether the apparent decrease in expression is due to the transgene insertion site and position-effect variegation (Henikoff, 1990) or due to the mutation of the HK-1 motif.

It is interesting to note that reduction of the *K2.10* promoter from 3 kb to 400 bp has resulted in progressively fewer expressing transgenic mice. The large *K2.10* transgene (14.5 kb with 2.8 kb of 5' promoter; Powell and Rogers, 1990) was active in virtually all transgenic mice (B. Powell, personal communication). The *K2.10-TAg* construct with 3 kb of *K2.10* promoter was active in ten out of twenty transgenic mice, whilst *K2.10(400)-lacZ* was expressed in only four out of the twelve mice obtained. Comparable expression levels were seen with all of these constructs, although not all of the follicles in *K2.10(400)-lacZ* mice showed expression. The apparent reduction in the percentage of transgenic mice expressing as the promoter fragment was shortened, and the fact that not all follicles showed expression in these mice, may also indicate that shorter promoters are more susceptible to "position-effects" in the genome, possibly due to the loss of isolating elements such as matrix attachment regions (Bonifer *et al.*, 1990; Phi-Van *et al.*, 1990).

The reported "silencing" effects of the *lacZ* reporter gene make this an unreliable choice for future hair keratin promoter expression studies in transgenic mice given the results obtained in this work.

In situ hybridisation with radioactive cRNA probes would enable the detection of any suitable reporter gene with stable messenger RNA. An alternative reporter system may be the green fluorescent protein (GFP) from *Aequorea victoria* (Chalfie *et al.*, 1994). Cells expressing GFP fluoresce under UV light and do not require any other co-factors or substrates for detection. This system is attractive because it appears to be highly sensitive and stable, and would allow simple and rapid analysis of samples (Wang and Hazelrigg, 1994).

5.3.4 Summary of expression of *K2.10* promoter deletion constructs

This crude deletion analysis of the *K2.10* promoter in transgenic mice demonstrated that 400 bp of *K2.10* 5' promoter contains the sequences sufficient for hair cortical cell-specific expression. Mutation of the putative regulatory element, HK-1, did not abolish cortical cell expression nor deregulate expression in other cell types present in the skin or hair follicle. A significant decrease in expression levels was observed in *K2.10(400ΔHK-1)-lacZ* mice; expression was confined to small patches of cortical cells and less than 10% of follicles showed any expression. As only two expressing *K2.10(400ΔHK-1)-lacZ* Fo mice were produced it is impossible to say whether the expression level is due to position effects of transgene insertion, the unpredictable nature of *lacZ* expression in adult transgenic tissues (Cui *et al.*, 1994) or due to the disruption of an enhancer element (HK-1). Specific binding of a hair follicle nuclear protein(s) to this element supports the hypothesis that the HK-1 element may be an enhancer, although it appears unlikely to be necessary to determine tissue specificity (see chapter 6). Lack of expression in the skin of *K2.10(200)-lacZ* mice showed that 200 bp of proximal promoter was not sufficient for detectable expression.

In conclusion, a more detailed analysis of the *K2.10(400)* promoter region is required to define the regulatory elements involved in expression. The following chapter describes the analysis of the 400 bp *K2.10* promoter fragment for protein binding sites using the gel shift binding assay.

Chapter Six

Analysis of the sheep *K2.10* gene promoter using gel mobility shift assays

6.1 Introduction

In the previous chapter, mouse transgenesis was used to show that 400 bp of the sheep *K2.10* gene promoter was sufficient to confer follicle-specific expression and that the HK-1 element, a putative regulatory element based on sequence conservation, was a possible enhancer sequence.

Further analysis of the HK-1 element is discussed in this chapter where oligonucleotides containing the HK-1 sequence were tested in gel mobility shift assays (GSA) for the ability to specifically bind protein(s) present in hair follicle nuclear extracts. A sequence with striking similarity to the KTF-1/AP-2 site found in Xenopus keratin genes (Snape *et al.*, 1990, 1991) is located adjacent to the HK-1 motif. This putative element was also analysed for the ability to bind hair follicle nuclear proteins.

In an attempt to identify binding sites for other proteins which may be involved in the transcriptional regulation of the gene, oligonucleotides spanning the 400 bp promoter region were synthesised and analysed in gel shift assays with mouse hair follicle nuclear extracts.

6.2 Results

6.2.1 Analysis of nuclear extracts from mouse hair follicles

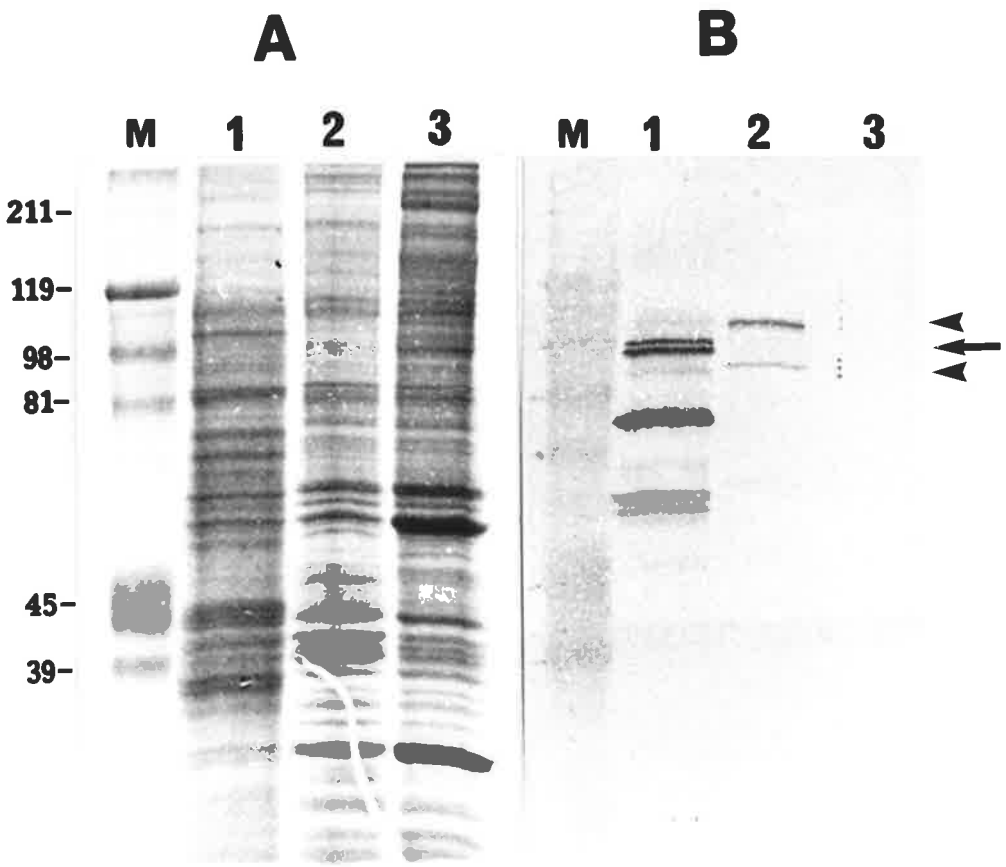
Mouse hair follicle nuclear extracts were prepared from 3-5 day old mouse pups (see 2.2.4.4ii). In order to determine whether nuclei from differentiated follicle cells were among those isolated from cells of the lower portion of the follicle, a Western blot analysis was performed on mouse hair follicle nuclear extracts obtained from *K2.10-TAg* transgenic mice (figure 6.1). The 97 kD TAg protein was detected as two bands of approximately 100 and 90 kD in follicles from *K2.10-TAg* transgenic mice. The presence of TAg nuclear protein in these extracts indicates that they contain protein from nuclei of differentiated cortical cells in which the *K2.10* gene is expressed.

The hair follicle nuclear extract was also tested for binding ability in gel shift assays using an AP-1 oligo (see 2.1.11). This oligo had been used previously in gel shift analyses with purified Fos and Jun proteins (Risse *et al.*, 1989). As *c-fos* expression has been shown in the follicle matrix

Figure 6.1 Western blot analysis of mouse hair follicle nuclear extracts.

(A) Fractionation of proteins on a 10% SDS-polyacrylamide gel. Lane 1 contains crude COS-1 cell extract (100 μg), lane 2 contains hair follicle nuclear extract (50 μg) from *K2.10-TAg* transgenic mice and lane 3 contains hair follicle nuclear extract (50 μg) from normal mice. Duplicate tracks of each sample were fractionated on the gel. One half of the gel was stained with Coomassie blue (A), and the identical, duplicate tracks were transferred to nitrocellulose (B). M, pre-stained protein molecular weight markers (5 μg) of indicated sizes (kD).

(B) Western blot of COS-1 cell and hair follicle protein extracts probed with SV-40 TAg mAb (Pab419). Proteins from gel (A) were transferred to nitrocellulose by electroblotting and the filter was incubated with TAg mAb (diluted 1:200). Primary antibody was visualised by the binding of 2^{o} conjugate (anti-mouse IgG-alkaline phosphatase diluted 1:1000) and detection of alkaline phosphatase activity (see 2.2.4.3). Bands corresponding to large T antigen (~97 kD; arrow) are seen in COS-1 cell extracts (lane 1). Lane 2 shows two bands which bind TAg mAb corresponding approximately to 100 kD and 90 kD proteins (arrowheads). Although these bands do not appear to correspond to the expected size (97 kD), faint bands of a similar size are also detected in COS-1 cell extracts but are not present in follicle nuclear extracts prepared from non-transgenic control follicles and, therefore, appear to represent TAg. One explanation for these size variations may be differing post-translational modifications including phosphorylation, glycosylation and adenylation (Fanning and Knippers, 1992). Bands below 60 kD appear to be due to antibody cross-reactivity to non-TAg epitopes as similar bands are present in all lanes, although at greatly reduced levels in lanes 1 and 2 (which may not be visible in this reproduction). The origin of the strong band of approximately 75 kD in COS-1 cell extracts (lane 1) is unknown, but may correspond to a modified form of TAg, or may be due to antibody cross-reactivity with an epitope present in COS-1 extracts.


A
B
M
1
2
3
M
1
2
3
211-
119-
98-
81-
45-
39-

(Fisher *et al.*, 1991), this oligo could be used to examine the hair follicle nuclear extracts for AP-1 binding. Figure 6.2 shows that a specific AP-1 complex is formed when the oligo is incubated with nuclear extracts as binding is almost abolished by as little as a 10-fold molar excess of unlabelled AP-1 oligo but not with the non-specific oligo, NS-2 (for sequence see 2.1.11).

It should be noted that all gel shift assays were performed as described in 2.2.1.17, using ^{32}P-kinased oligonucleotide probes. Non-specific competitor oligos used throughout this work were analysed to ensure that they contained no sequence similarity to the oligo probe.

6.2.2 Analysis of the *K2.10* HK-1 element

A 20 bp oligonucleotide spanning the sheep *K2.10* HK-1 motif (see 2.1.11) appears to bind specific protein(s) when incubated with mouse hair follicle nuclear extracts (figure 6.3A,B). Two specific complexes are formed: a lower mobility complex and a less abundant higher mobility species which is nevertheless specific and present as a doublet in gel shift assays. The relative intensity of this complex compared with the lower mobility complex varies between different preparations of follicle nuclear extracts but the complex was invariably present in gel shift assays performed with this oligo. The specific HK-1 complexes are unaffected by the presence of non-specific competitor oligos (such as NS-1) but are abolished upon competition with excess unlabelled HK-1 oligo. Non-specific complexes are generally characterised by the ability of excess non-specific competitor oligos to abolish or greatly reduce complex formation. In order to further show that hair follicle nuclear proteins were binding to the putative HK-1 8 bp motif, a mutant 20-mer HK-1 oligo, ΔHK-1, was generated in which the HK-1 sequence was changed from CTTTGAAG to ACGATCTG, destroying the inverted repeat present in this motif. This mutant oligo was unable to compete for HK-1 binding at 100-fold molar excess of unlabelled ΔHK-1, whilst 100-fold excess of unlabelled HK-1 oligo completely abolished radiolabelled HK-1 complex formation (figure 6.3B). However, ΔHK-1 appears to weakly bind an HK-1-specific complex (figure 6.4A, lanes 1-6) and ΔHK-1 can also compete for HK-1 binding at a concentration of 1000-fold excess (figure 6.4B).

The 8 bp HK-1 sequence has been identified in a number of hair keratin genes (see table 1.1), and some of these sites contain 1-2 nucleotide differences from the proximal *K2.10* sequence. To examine whether 1-2 nucleotide changes in the HK-1 consensus sequence affected protein binding, a series of variant HK-1 oligos (HKv-2 to HKv-8; see 2.1.11) was generated in which 1 or 2

Figure 6.2 Analysis of mouse hair follicle nuclear extracts for AP-1 binding activity in gel shift assays.

Radiolabelled AP-1 oligo probe incubated with hair follicle nuclear extracts forms an AP-1-specific retarded complex (arrow) which is unaffected by non-specific competitor oligo (NS-2; lanes 3-5), but is lost upon competition with excess unlabelled AP-1 oligo (lanes 6-8). Lane 1 contains free probe only; Dashes (-) indicate that no competitor oligo is present. 10X, 100X, 1000X refer to molar excess amounts of competitor oligo.

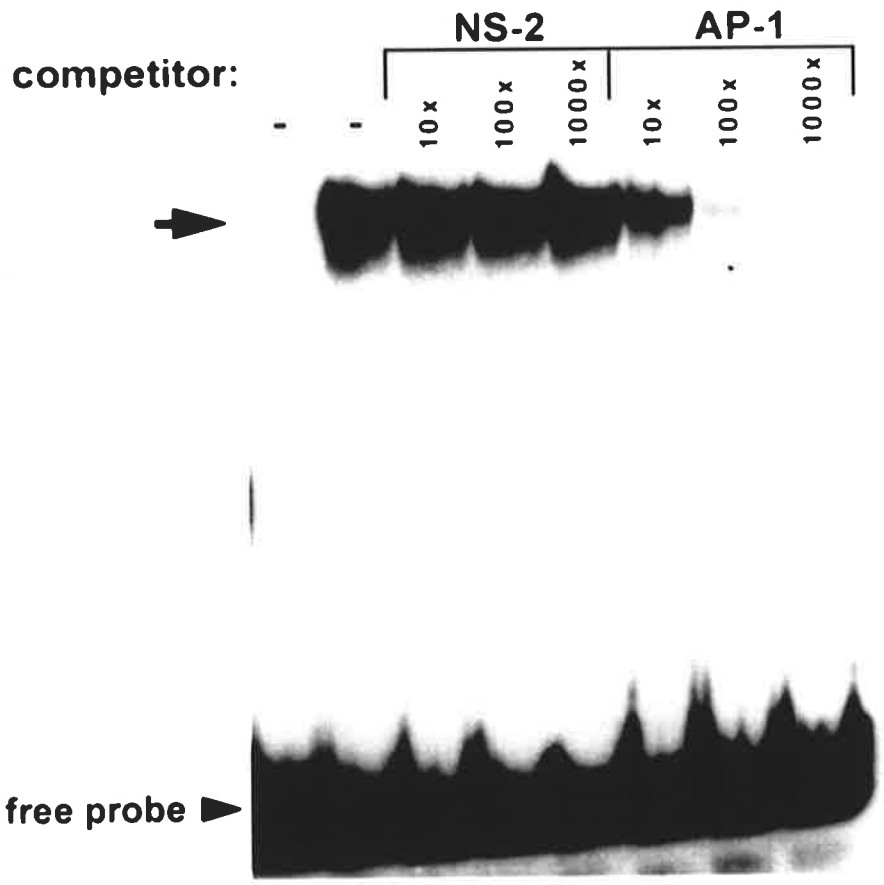

competitor:
NS-2
AP-1
-
-
10x
100x
1000x
10x
100x
1000x
free probe

Figure 6.3 Gel shift analysis of HK-1 oligo binding to hair follicle nuclear proteins.

(A) Radiolabelled HK-1 oligo probe incubated with hair follicle nuclear extracts produces two specific retarded complexes in GSAs (arrowheads). The higher-mobility complex often appears as a doublet (see B). Excess non-specific oligo (NS-1) is unable to compete for complex formation (lanes 3-5) even at 1000-fold molar excess, whilst excess HK-1 oligo abolishes radiolabelled complex formation (lanes 6-8). Lane 1 contains free probe only; lane 2 contains probe and nuclear protein extract.

(B) Analysis of the 8 bp HK-1 motif in the formation of retarded complexes. The HK-1 oligo was mutated in the 8 bp consensus sequence to create ΔHK-1 (see 2.1.11 and below). This oligo has a greatly reduced ability to compete for HK-1 binding. Radiolabelled HK-1 complex formation (arrowheads) is abolished in the presence of 100-fold molar excess of unlabelled HK-1 oligo (lane 6) but is virtually unaffected by 100-fold excess ΔHK-1 (lane 4). The non-specific complex (NS) appears greatly reduced in the presence of excess non-specific oligo and can be competed out with poly(dIdC) (data not shown). The high-mobility non-specific bands (unlabelled) are also able to be competed out with non-specific oligos at 1000-fold excess (data not shown). Note the variation in the bandshift profile between (A) and (B) due to different nuclear extract preparations. Lane 1 contains free probe only; lane 2 contains probe and nuclear extract only. NS, non-specific complex; 10X, 100X, 1000X refer to molar excess amounts of unlabelled competitor oligo.

HK-1 oligo sequence (upper strand only):	5'-AACGG CTTTGAAG ATGAAAC-3'
ΔHK-1 oligo sequence (upper strand only):	5'-AACGG ACGATCTG ATGAAAC-3'

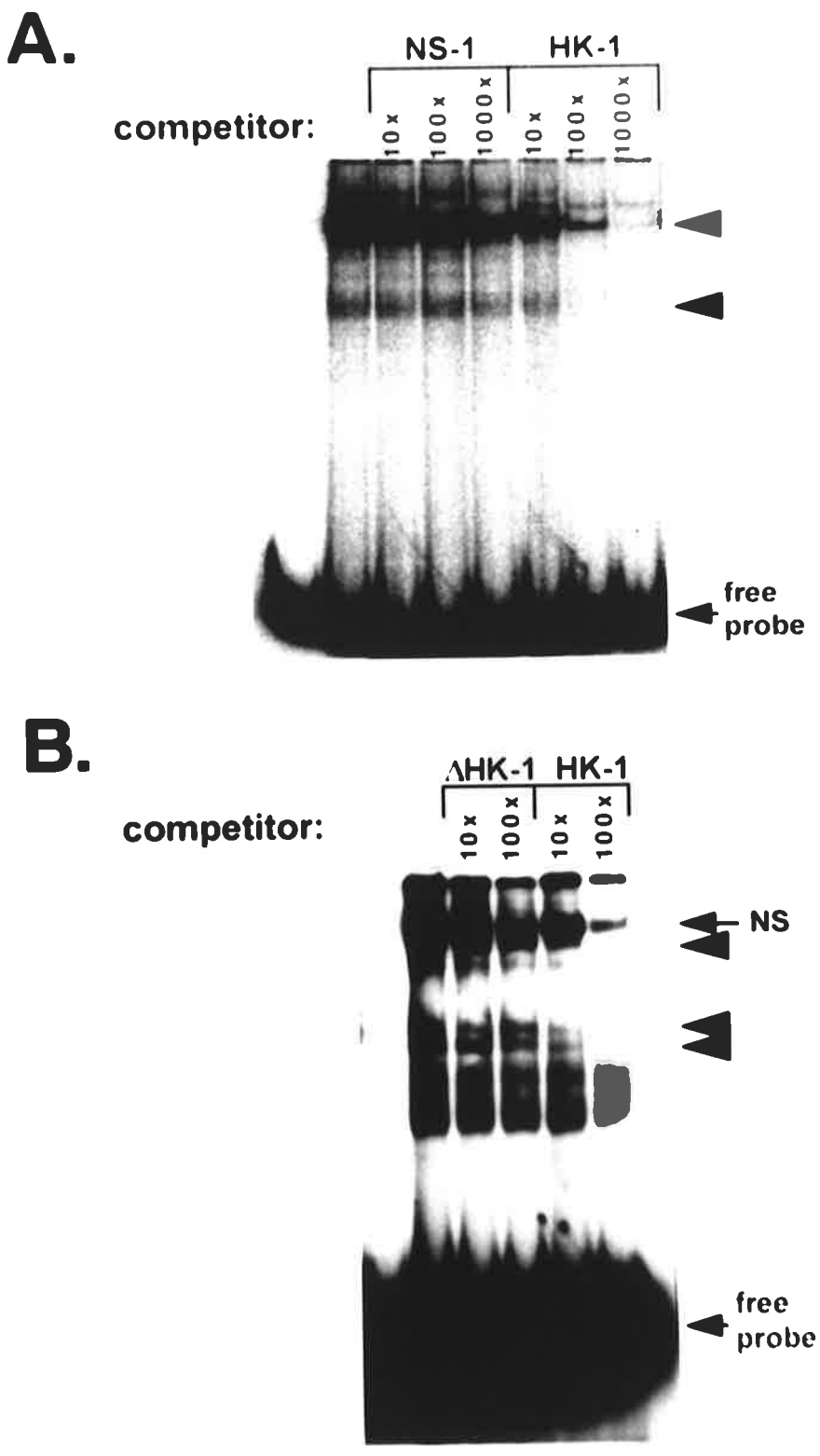
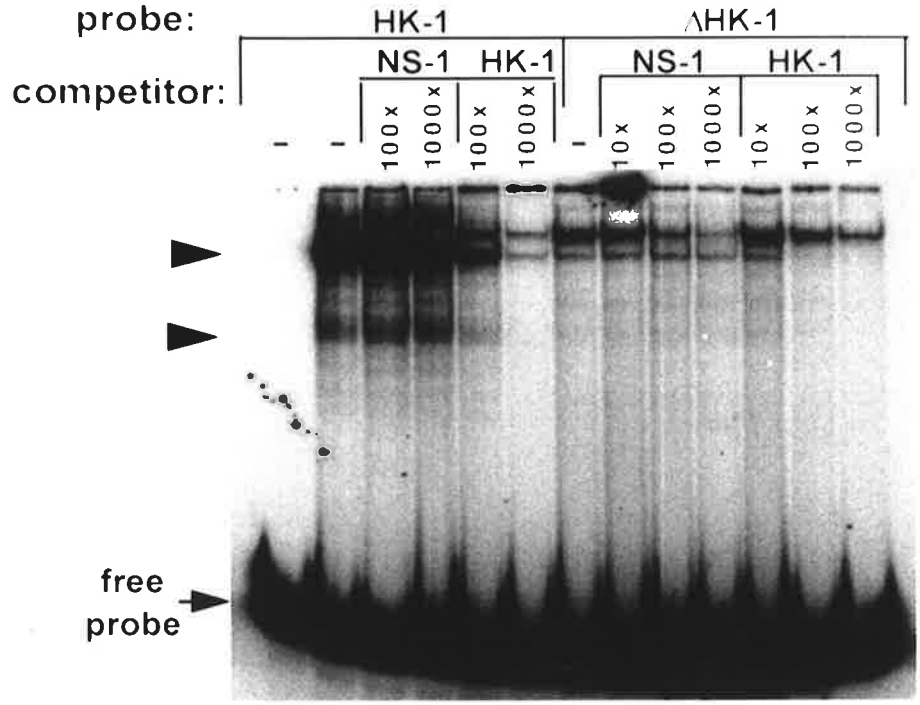

A.
NS-1
HK-1
competitor:
10x
100x
1000x
10x
100x
1000x
free probe
B.
ΔHK-1
HK-1
competitor:
10x
100x
10x
100x
NS
free probe

Figure 6.4 Analysis of protein binding by ΔHK-1 oligo.

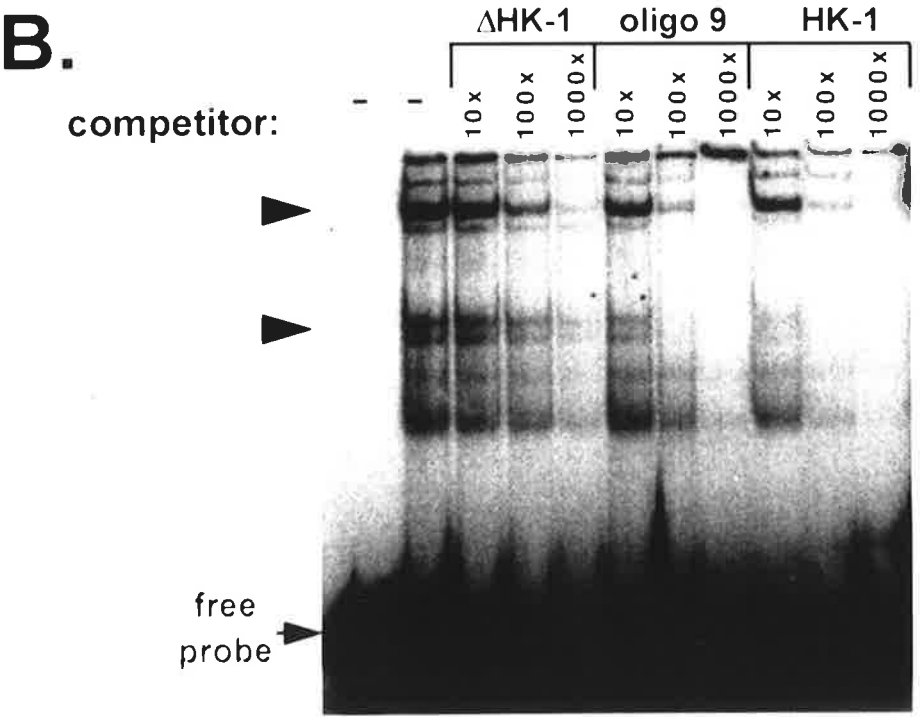
(A) ΔHK-1 forms HK-1-specific complexes with hair follicle nuclear extracts. Lanes 2-6 show retarded complexes formed with the radiolabelled HK-1 oligo probe and hair follicle nuclear extracts. The HK-1-specific complexes are indicated (arrowheads) and are not competed out with non-specific competitor (NS-1; lanes 3 and 4), but are almost abolished with excess unlabelled HK-1 oligo (lanes 5 and 6). Lane 7 shows the retarded complexes formed with the ΔHK-1 oligo probe. The upper band is non-specific. The lower band appears to be an HK-1-specific complex which is unaffected by non-specific competitor (NS-1, lanes 8-10) but is abolished in the presence of excess unlabelled HK-1 oligo (lanes 11-13). Binding to the ΔHK-1 oligo is significantly reduced compared with binding to the HK-1 oligo. Lane 1 contains free probe only (arrow); lane 2 contains probe and nuclear extracts. Dashes (-) indicate the absence of competitor oligo.

(B) GSA with radiolabelled HK-1 oligo probe and hair follicle nuclear extracts. HK-1-specific retarded complexes are indicated (arrowheads). Excess ΔHK-1 oligo (lanes 3-5), oligo 9 (a 35-mer oligo which spans the HK-1 sequence, see figures 6.9A and 6.11; lanes 6-8) and HK-1 oligo (lanes 9-11) all compete for HK-1-specific complex formation. However, competition with the ΔHK-1 oligo is at least 10-fold less efficient than oligos containing the wildtype 8 bp HK-1 sequence. Lane 1 contains free probe only (arrow); lane 2 contains probe and protein extract. 10X, 100X, 1000X refers to molar excess amounts of competitor oligo.

A.


probe:
HK-1
ΔHK-1
competitor:
NS-1
HK-1
NS-1
HK-1
– – 100x 1000x 100x 1000x – 10x 100x 1000x 10x 100x 1000x
free probe

B.


ΔHK-1
oligo 9
HK-1
competitor:
– – 10x 100x 1000x 10x 100x 1000x 10x 100x 1000x
free probe

nucleotide changes were introduced into the *K2.10* HK-1 oligo (figure 6.5A). A 20 bp oligo spanning the HK-1 site of the sheep *K2.11* promoter was also generated (HKv-1). The *K2.11* gene is activated later in cortical cell differentiation than *K2.10* so it was of interest to determine whether any difference in HK-1 binding activity between the 2 oligos was observed. These variant oligos, HKv-1 to HKv-8, were analysed for their ability to bind follicle extracts (figure 6.5B). All of the variant oligos, except HKv-7, were capable of forming an HK-1-like complex (as determined by competition studies with HK-1 and non-specific oligos; data not shown), although the binding affinities differed and were reduced relative to the *K2.10* HK-1 complex. The strongest complexes were formed with HKv-8 (which corresponds to the HK-1 motif found in the mouse *HKA1* gene) followed by HKv-2 (containing the *K2.11* HK-1 motif flanked by *K2.10* sequence), HKv-3 (containing the second, more distal *K2.10* HK-1 motif) and HKv-1 (an oligo derived from the sheep *K2.11* gene). Only weak binding was seen with oligos HKv-5 and HKv-6.

To explore whether the protein(s) binding the HK-1 oligo was exclusive to the hair follicle or a more generally expressed factor, a preliminary series of gel shift binding studies using the HK-1 oligo and nuclear extracts from a number of different cell lines and rat tissues was performed (figure 6.6). Nuclear extracts from COS-1 (monkey kidney), CHO (Chinese hamster ovary), HeLa (human epithelioma), HepG2 (human hepatocarcinoma), Ptk2, (potoroo kidney), JK-1 (human erythroleukaemia), HL60 (promyelocytic leukaemia) and C_2C_{12} (mouse myoblast) cell lines were assayed for binding to the HK-1 oligo. Specific retarded complexes were formed with extracts from CHO and HeLa cells (figure 6.6), but no specific binding was evident using the other extracts (data not shown). Nuclear extracts from rat testis, spleen, liver, brain, kidney, muscle and heart were also tested for HK-1 binding. Specific retarded complexes were produced using testis and brain extracts (figure 6.7). No specific HK-1 binding was found with the other extracts (data not shown).

6.2.3 Analysis of the *K2.10* KTF-1/AP-2 element

A 23 bp oligonucleotide spanning the KTF-1-like motif present in the sheep *K2.10* gene promoter (see figure 5.1) was synthesised (see 2.1.11) and analysed for the ability to bind nuclear proteins from mouse hair follicle nuclear extracts. Figure 6.8 shows that the KTF-1 oligo forms a specific, retarded complex in gel shift assays with hair follicle extracts. A similar specific complex is also formed when the KTF-1 oligo is incubated with AP-2 extracts, indicating that the *K2.10* KTF-1 motif is capable of binding AP-2.

Figure 6.5 Gel shift analysis using variant HK-1 oligos.

(A) Sequence of HK-1 and variant HK-1 oligos HKv-1 to HKv-8. HKv-1 oligo spans the HK-1 site of the sheep *K2.11* promoter. HKv-2 to HKv-7 and ΔHK-1 oligos contain various nucleotide substitutions (underlined) in the *K2.10* 8 bp HK-1 sequence. HKv-8 contains an A to G substitution outside of the 8 bp sequence which is found in the HK-1 motif of the mouse *HKA1* gene (see table 1.2). HKv-3 contains a T to C substitution which corresponds to the sequence of the more distal HK-1 site in the *K2.10* promoter.

(B) GSA using the above series of variant oligos as radiolabelled probes with mouse hair follicle nuclear extracts. Lane 1 contains free probe only; lanes 2 and 12 contain HK-1 probe; lanes 3-10 contain HKv-1 to HKv-8 probes respectively; lane Δ contains ΔHK-1 probe. The HK-1-specific complexes are labelled (arrowheads). Note that oligos HKv-1 to HKv-6 all form the lower-mobility HK-1 complex (visible on a darker exposure; data not shown), but at a lower abundance than the HK-1 and HKv-8 oligos. ΔHK-1 oligo also forms an HK-1-specific complex, but at a lower abundance than oligos HKv-1 to HKv-6. Oligo HKv-7 does not form any specific complexes. The non-specific (NS) retarded complex is labelled (arrow).

A. HK-1 Variant Oligo Series

HK-1	5'-AACGG	CTTTGAAG	ATGAAAC-3'
HKv-1	TGGCC	CTTTGAGG	ACAAAAA
HKv-2	AACGG	CTTTGAGG	ATGAAAC
HKv-3		CTCTGAAG	
HKv-4		ATTTGAAG	
HKv-5		CGTTGAAG	
HKv-6		CTTACAAG	
HKv-7		CTTTGCAG	
HKv-8		CTTTGAAG	G
ΔHK-1		ACGATCTG	

B.

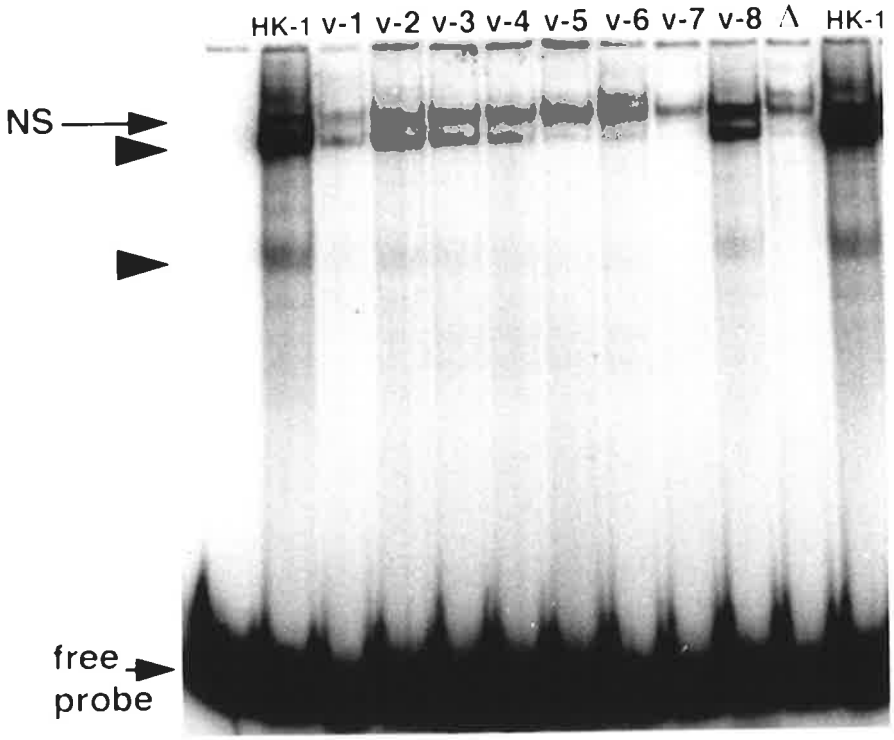


Figure 6.6 Analysis of HK-1 binding with CHO and HeLa cell nuclear extracts.

(A) GSA with radiolabelled HK-1 oligo probe and CHO cell nuclear extract . Lane 1 contains probe incubated with hair follicle nuclear extract (5 μg) to produce HK-1 retarded complexes (arrow and arrowhead). HK-1 probe incubated with CHO cell extract (5 μg) produces an HK-1-specific complex (arrow; lane 2). HK-1 complex formation is unaffected by competition with non-specific oligo (NS-1; lanes 3-5), but is abolished in the presence of 100-fold excess unlabelled HK-1 oligo (lanes 6-8). Note that the abundance of the CHO cell HK-1 complex is greatly reduced compared with the hair follicle complex. Also note that the higher-mobility complex (arrowhead) is not detected with CHO cell extracts.

(B) GSA with radiolabelled HK-1 oligo probe and HeLa cell nuclear extract (2.5 μg). A specific retarded complex is formed with HeLa cell nuclear proteins (arrow) which is unaffected by competition with non-specific oligo (KTF-1; lanes 3-5), but is greatly reduced in the presence of excess HK-1 oligo (lanes 6-8). Lane 1 contains free probe only. Dashes (-) indicate that no competitor oligo is present. 10X, 100X, 1000X refer to molar excess amounts of unlabelled competitor oligo.

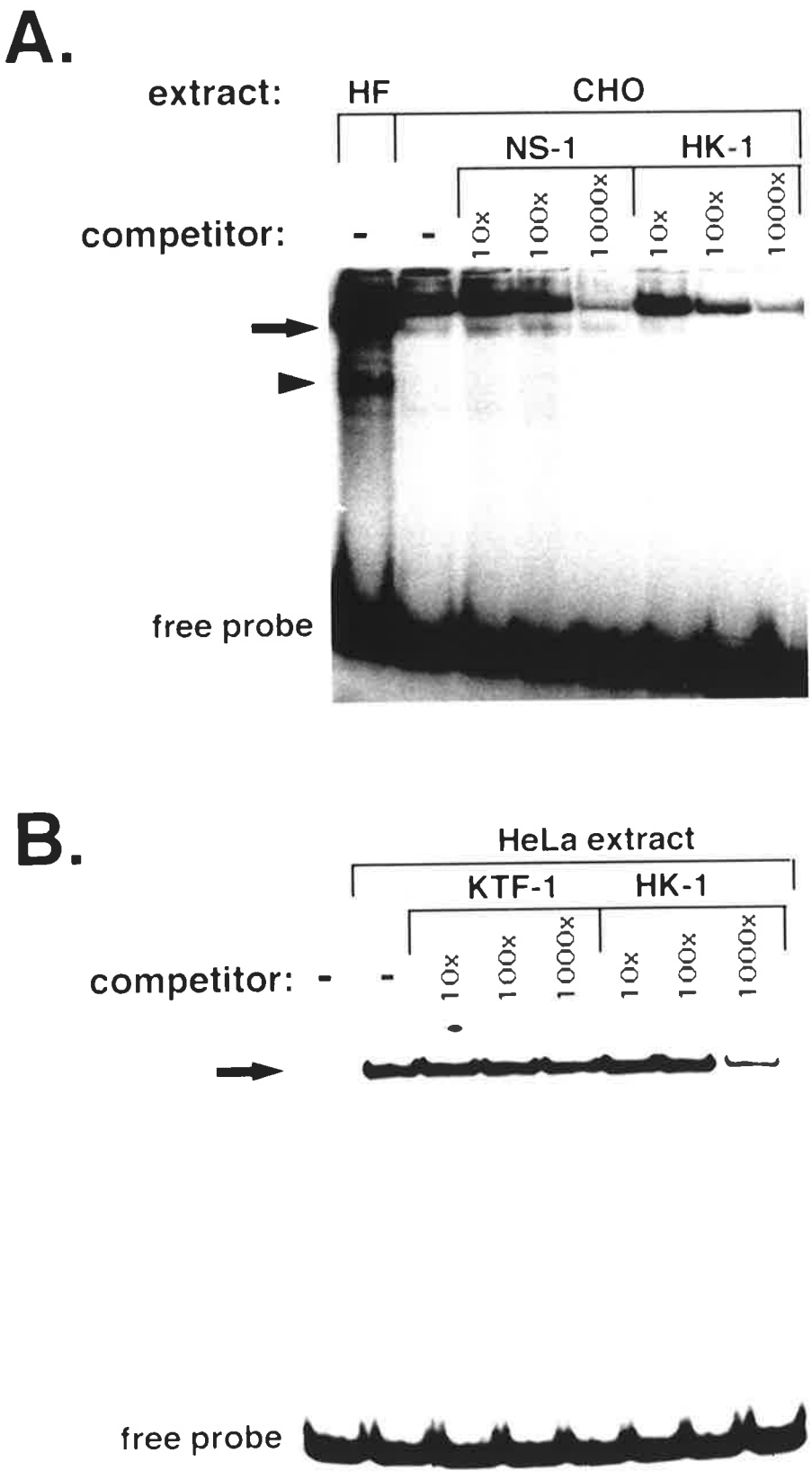

A.
extract:
HF
CHO
NS-1
HK-1
competitor:
-
-
10x
100x
1000x
10x
100x
1000x
free probe
B.
HeLa extract
KTF-1
HK-1
competitor:
-
-
10x
100x
1000x
10x
100x
1000x
free probe

Figure 6.7 Analysis of HK-1 binding with rat brain and testis nuclear extracts.

(A) GSA with radiolabelled HK-1 oligo probe and rat brain nuclear extract. Lane 1 contains HK-1 probe with hair follicle extract (5 μg) producing HK-1-specific retarded complexes (arrowheads). Lanes 2-8 contain HK-1 probe and brain nuclear extract (5 μg). An HK-1-specific retarded complex is formed with brain extracts (arrow) which is unaffected by non-specific competitor oligo (NS-1; lanes 3-5) but is abolished in the presence of excess unlabelled HK-1 oligo (lanes 6-8).

(B) GSA with radiolabelled HK-1 oligo probe and rat testis nuclear extract. Lane 1 contains free probe only. Lane 2 contains HK-1 probe with hair follicle nuclear extracts and the HK-1-specific retarded complexes are indicated (arrowheads). Lanes 3-9 contain HK-1 probe with testis extract (5 μg). A specific retarded complex is formed with HK-1 probe and testis extracts (arrow) which runs as a doublet. Non-specific oligo (NS-1) is unable to compete for specific complex formation (lanes 4-6). Excess unlabelled HK-1 abolishes complex formation (lanes 7-9). Note that the testis-HK-1 complex does not migrate with the hair follicle complex. Dashes (-) indicate that no competitor oligo is present. 10X, 100X, 1000X refer to molar excess amounts of unlabelled competitor oligo.

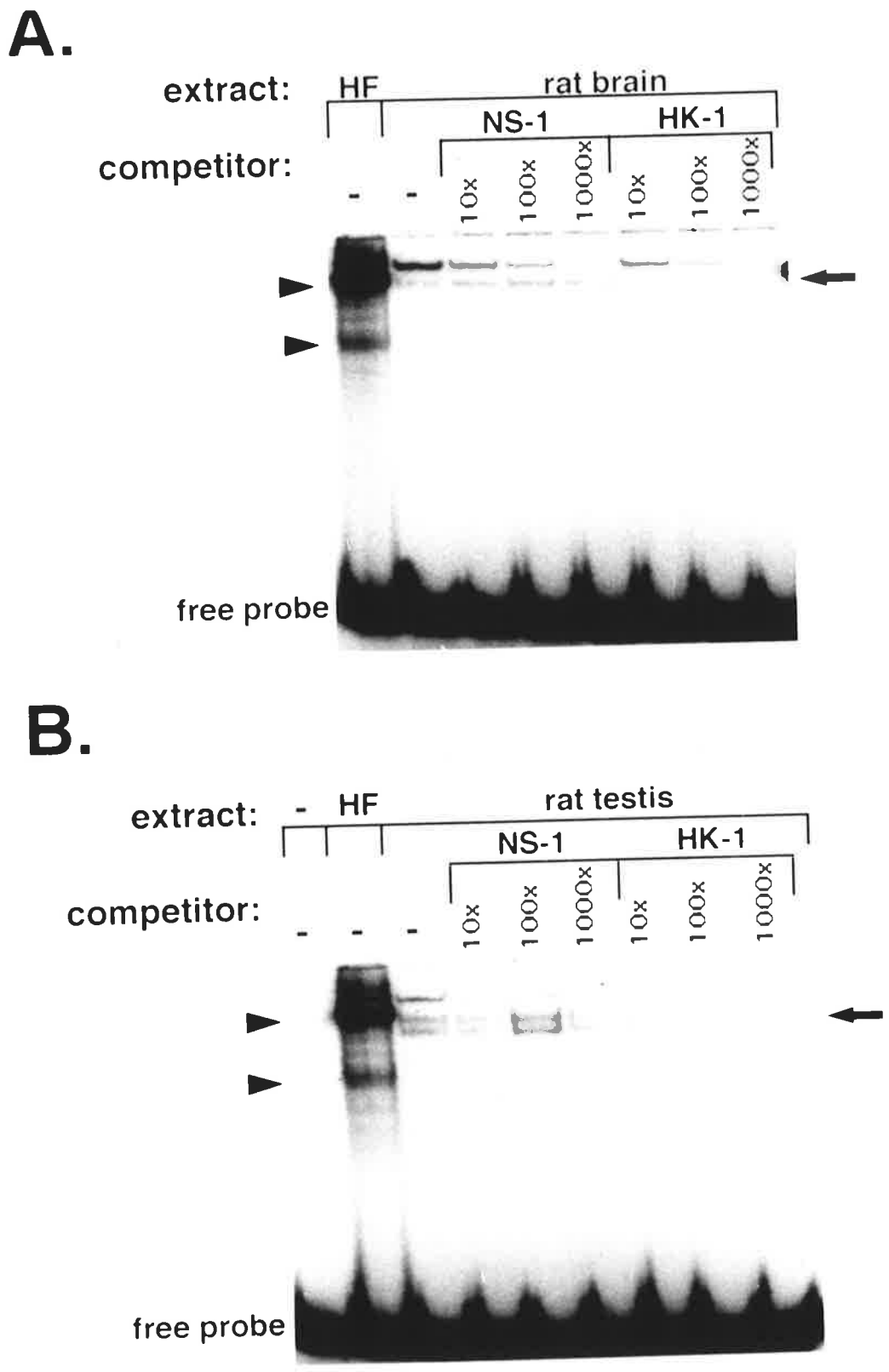
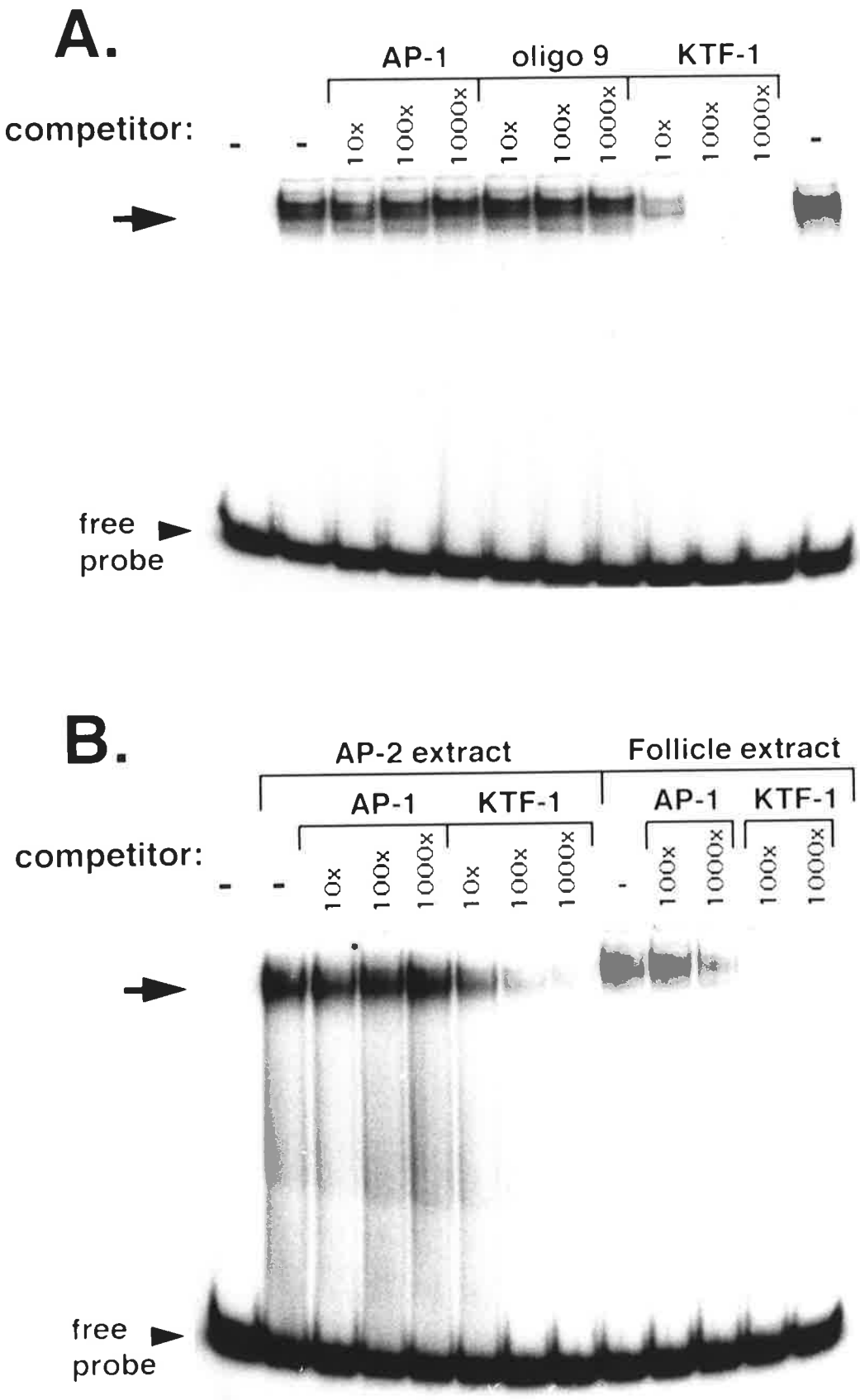

A.
extract:
HF
rat brain
NS-1
HK-1
competitor:
-
-
10x
100x
1000x
10x
100x
1000x
free probe
B.
extract:
-
HF
rat testis
NS-1
HK-1
competitor:
-
-
-
10x
100x
1000x
10x
100x
1000x
free probe

Figure 6.8 Analysis of the KTF-1/AP-2 element using gel shift binding assays.

(A) GSA using radiolabelled KTF-1 oligo probe and hair follicle nuclear extracts. A specific retarded complex is formed (arrow; lanes 2 and 12). Excess non-specific competitor oligo (AP-1) is unable to affect KTF-1 complex formation (lanes 3-5). Oligo 9 (a 35-mer oligo which spans the HK-1 and KTF-1 sites; see figure 6.9A) is also unable to affect KTF-1 complex formation (lanes 6-8), although binding is abolished in the presence of excess unlabelled KTF-1 oligo (lanes 9-11). Note that oligo 9 lacks 4 bp at the 3' end which are present in the KTF-1 oligo (see figure 6.9A). Lane 1 contains free probe only (arrowhead). Dashes (-) indicate that no competitor oligo is present.

(B) Gel shift analysis of AP-2 extract binding to KTF-1 oligo. A specific retarded complex (arrow) is formed with KTF-1 oligo probe and AP-2 extract (1.25 μg; lane 2) which is unaffected by excess non-specific competitor oligo (AP-1; lanes 3-5) but is competed out with excess unlabelled KTF-1 oligo (lanes 6-8). A similar complex is formed with KTF-1 probe and hair follicle nuclear extracts (5 μg; lanes 9-13). Note, however, that the hair follicle protein complex migrates slightly more slowly than the complex formed with AP-2 extract. Also note that AP-2 extract is produced by over-expression of the human AP-2 protein in bacteria. Lane 1 contains free probe only (arrowhead). Dashes (-) indicate that no competitor oligo is present. 10X, 100X, 1000X refer to molar excess amounts of unlabelled competitor oligo.


A.
AP-1
oligo 9
KTF-1
competitor:
-
-
10x
100x
1000x
10x
100x
1000x
10x
100x
1000x
-
free
probe
B.
AP-2 extract
Follicle extract
AP-1
KTF-1
AP-1
KTF-1
competitor:
-
-
10x
100x
1000x
10x
100x
1000x
-
100x
1000x
100x
1000x
free
probe

A series of overlapping 35-mer oligonucleotides spanning the 400 bp *K2.10* promoter was generated for gel shift studies (see 6.2.4). One of these oligos, oligo 9, encompassed both the HK-1 and KTF-1 motifs (see figure 6.9A). This oligo formed a number of specific complexes with mouse hair follicle nuclear extracts (figure 6.9B)- complexes 1 and 2 appear to correspond to the HK-1 complexes as binding is lost upon competition with unlabelled HK-1 oligo; the higher mobility complex 3 appears to be specific for oligo 9 as it is not able to be competed with KTF-1 oligo or with overlapping flanking oligos, oligo 8 and oligo 10 (figure 6.10A). As the KTF-1 oligo could not compete for any oligo 9 complexes it was of interest to see whether oligo 9 could compete for KTF-1 binding. However, it was found that oligo 9 was unable to compete for KTF-1 complexes (figure 6.8) and was also unable to bind AP-2 protein (figure 6.10B). Thus, it appears that the 4 bp lacking at the 3' end of oligo 9, which are present in the KTF-1 oligo, are required for AP-2 binding to this site. As the entire KTF-1/AP-2 consensus is present in oligo 9, it is unlikely that the 4 bp region is sequence-specific requirement, but merely that the extra length is required for stable binding. Analysis of the AP-2 binding site in the human *K14* gene (Leask *et al.*, 1990) has shown that mutation of the sequence flanking the binding site has no effect on protein binding.

6.2.4 Oligonucleotide dissection of the *K2.10* proximal promoter

A series of double-stranded, overlapping 35-mer oligonucleotides which span the region from -350 to +5 were synthesised (figure 6.11). Gel mobility shift assays were carried out with mouse hair follicle nuclear extracts using these oligonucleotides as probes.

Oligo 1 forms retarded complexes which run as 2-3 bands in GSAs. Complex formation appears to be competed out more efficiently with excess cold oligo 1 than with non-specific oligos (figure 6.12A) suggesting that a specific complex is formed with hair follicle nuclear proteins. Oligo 2 also forms a specific retarded complex in GSAs (figure 6.12B). This complex is able to be competed out with oligo 1, but not with oligo 3, suggesting that the binding site in both oligos occurs in the overlapping region between oligos 1 and 2. Oligo 3 appears to form a specific retarded complex which cannot be competed out with oligo 2 (figure 6.13A), whilst oligo 4 does not appear to bind any specific factors present in follicle nuclear extracts (figure 6.13B).

Oligo 5 forms a specific, high-mobility complex which is only competed out with 1000-fold excess of unlabelled specific ("self") oligo and not completely with non-specific competitors (figure 6.14A). Oligo 6 forms a similar high-mobility complex (figure 6.14B) and both oligos 5 and 6

Figure 6.9 Binding of mouse hair follicle nuclear extracts to *K2.10* oligo 9.

(A) A diagrammatic representation of oligo 9 sequence in relation to the HK-1 and KTF-1 oligos. Oligo 9 is a double-stranded 35-mer oligo spanning -190 to -155 of the *K2.10* promoter and contains the HK-1 and KTF-1/AP-2 binding motifs (underlined and labelled). Note that oligo 9 lacks 4 bp at the 3' end which are present in the KTF-1 oligo.

(B) Gel shift analysis of oligo 9 with hair follicle nuclear extracts. Three specific retarded complexes are formed (complexes 1-3, arrows). Other retarded bands are non-specific. Complex 1 is present at a low abundance and complexes 2 and 3 appear as doublets. Complexes 1, 2 and 3 are unaffected by non-specific competitor oligo (oligo 2 [see figure 6.11]; lanes 3-5). Oligos 10 and KTF-1 also do not compete for complex formation with oligo 9 (lanes 6-8 and 9-11 respectively). However, excess unlabelled HK-1 oligo can compete for complexes 1 and 2 indicating that these are HK-1-specific complexes (lanes 15-17). Complex 3 can only be competed out with excess unlabelled oligo 9 (lanes 18-20) suggesting that this complex is unique to oligo 9 and not present in HK-1 or KTF-1 oligos. Lane 1 contains free probe only. Dashes (-) indicate that no competitor oligo is present. 10X, 100X, 1000X refer to molar excess amounts of unlabelled competitor oligo.

A.

HK-1 AP-2

OLIGO 9 5′-AACGGCTTTGAAGATGAAACAGGCCTGAGGCCGAG-3′

HK-1 OLIGO 5′-AACGGCTTTGAAGATGAAAC-3′

KTF-1 OLIGO 5′-AAACAGGCCTGAGGCCGAGATTG-3′

B.

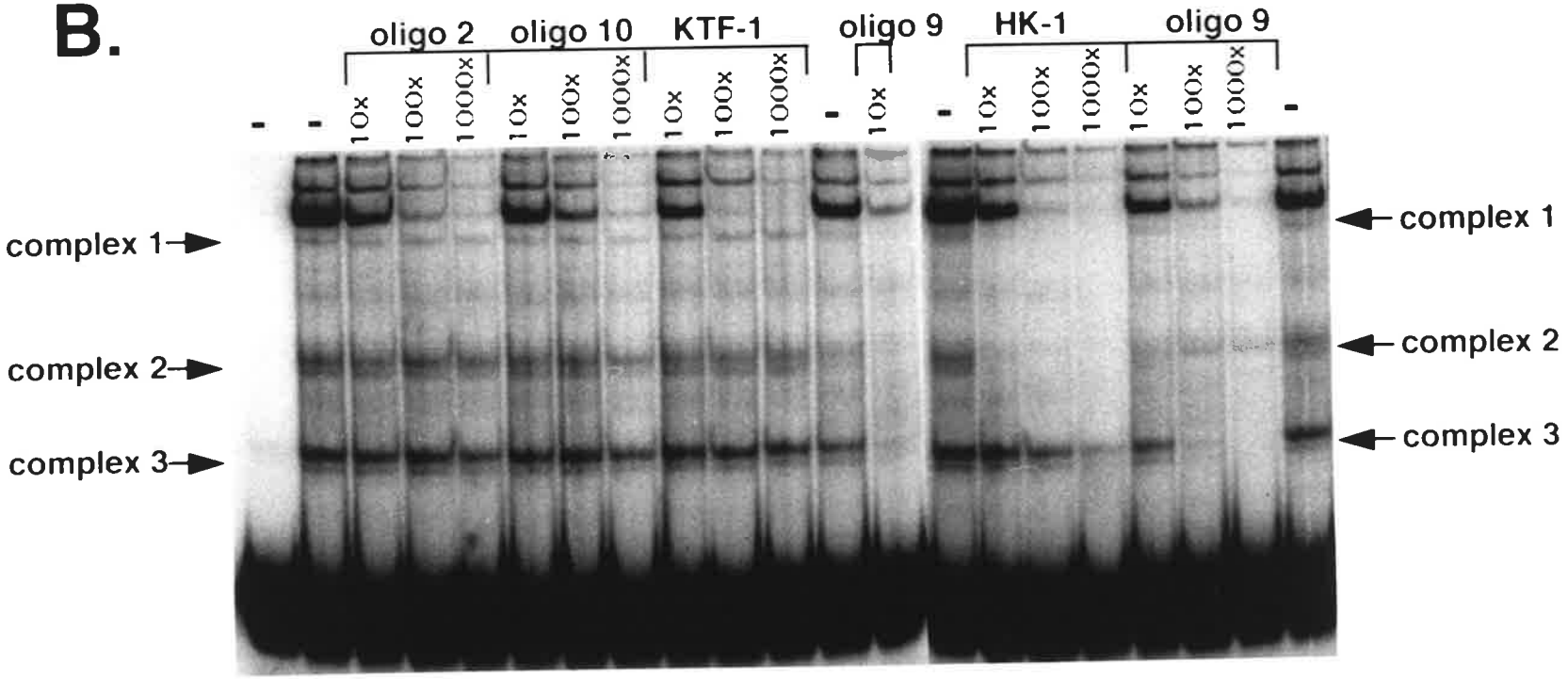


Figure 6.10 GSA using oligo 9 probe with hair follicle nuclear extracts and AP-2 extract.

(A) A diagrammatic representation of oligo 9 sequence and the overlapping sequences of oligo 8 and oligo 10. The HK-1 and KTF-1/AP-2 motifs are indicated (underlined and labelled). Oligo 8 contains the HK-1 site whilst oligo 10 overlaps the AP-2 motif.

(B) Oligo 9 forms three specific retarded complexes with hair follicle nuclear extracts (complexes 1-3; lane 2). These complexes are unable to be competed out with non-specific oligo (NS, oligo 3 [see figure 6.11]; lanes 3-5). Oligo 8, which overlaps oligo 9 at the HK-1 element, is able to compete for complex 1 and complex 2 formation (i.e. HK-1-specific complexes; lanes 6-8). The presence of excess unlabelled oligo 9 abolishes all specific complex formation (lanes 9-11). No protein binding is seen with oligo 9 and AP-2 extracts (5 μg; lane 12), i.e. oligo 9 appears unable to bind AP-2 despite spanning the KTF-1/AP-2 element. Lanes 1 and 13 contain free oligo 9 probe only. 10X, 100X, 1000X refer to molar excess amounts of unlabelled competitor oligo.

A.

OLIGO 9

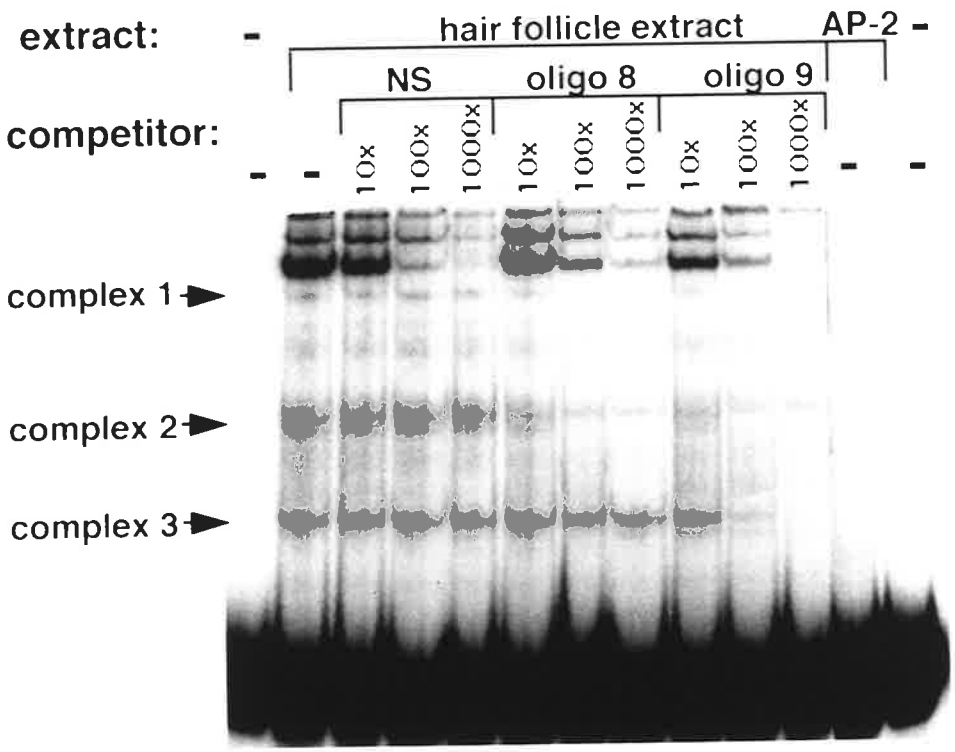
HK-1 AP-2

5′-AACGGCTTTGAAGATGAAACAGGCCTGAGGCCGAG-3′

OLIGO 8 OLIGO 10

5′-AGGGGGAGAAGAAGCTCGTAAACGGCTTTGAAGAT AGGCCTGAGGCCGAGATTGTTGACACAGCTCTACT-3′

B.



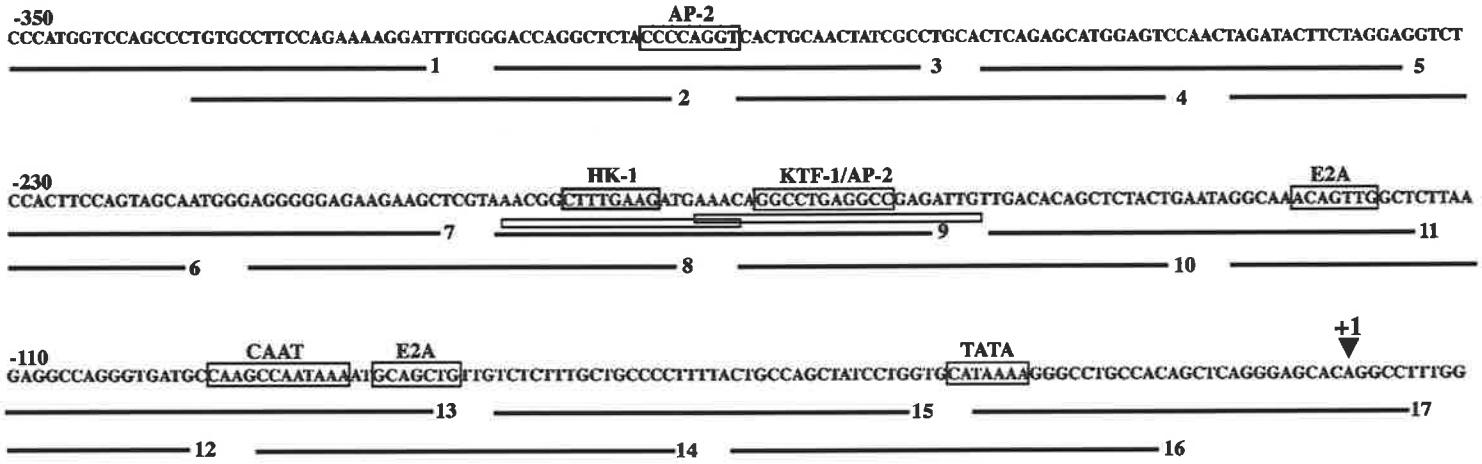


Figure 6.11 Oligonucleotide dissection of the *K2.10* 5' proximal promoter.

This is a diagrammatic representation of overlapping 35-mer oligonucleotides (oligo 1-17) spanning the *K2.10* promoter from -350 to +5. HK-1and KTF-1 oligos are representaed as hollow lines beneath their respective sequences. The CAAT and TATA boxes are labelled, and putative AP-2, HK-1, KTF-1/AP-2 and E2A transcription factor binding sequences are also marked. The arrowhead denotes the transcription start site at +1.

Figure 6.12 Analysis of *K2.10* oligos 1 and 2 for the ability to bind mouse hair follicle nuclear proteins.

(A) GSA using radiolabelled oligo 1 (which spans -350 to -315 of the *K2.10* promoter; see figure 6.11) and mouse hair follicle nuclear extracts. A major retarded complex (arrow) is formed with hair follicle nuclear proteins (lane 2) which is unable to be competed out with non-specific competitor oligo 10 (lanes 3-5), but is competed out with unlabelled cold "self" oligo (lanes 6-8). Lane 1 contains free oligo 1 probe only. Dashes (-) indicate that no competitor oligo is present.

(B) GSA using radiolabelled oligo 2 probe (which spans -330 to -295 of the *K2.10* promoter; see figure 6.11) and mouse hair follicle nuclear extracts. Several retarded complexes are formed, however, only the major band (arrow) appears to be due to specific interactions. Oligo 17 was used as a non-specific competitor (lanes 3-5). Overlapping oligo, oligo 1, is also able to compete for oligo 2 complex formation (lanes 9-11) but oligo 3 is not (lanes 12 and 13). Lane 1 contains free probe only. Arrowheads indicate non-specific (NS) complexes. Dashes (-) indicate that no competitor oligo is present. 10X, 100X and 1000X refer to molar excess amounts of competitor oligo.

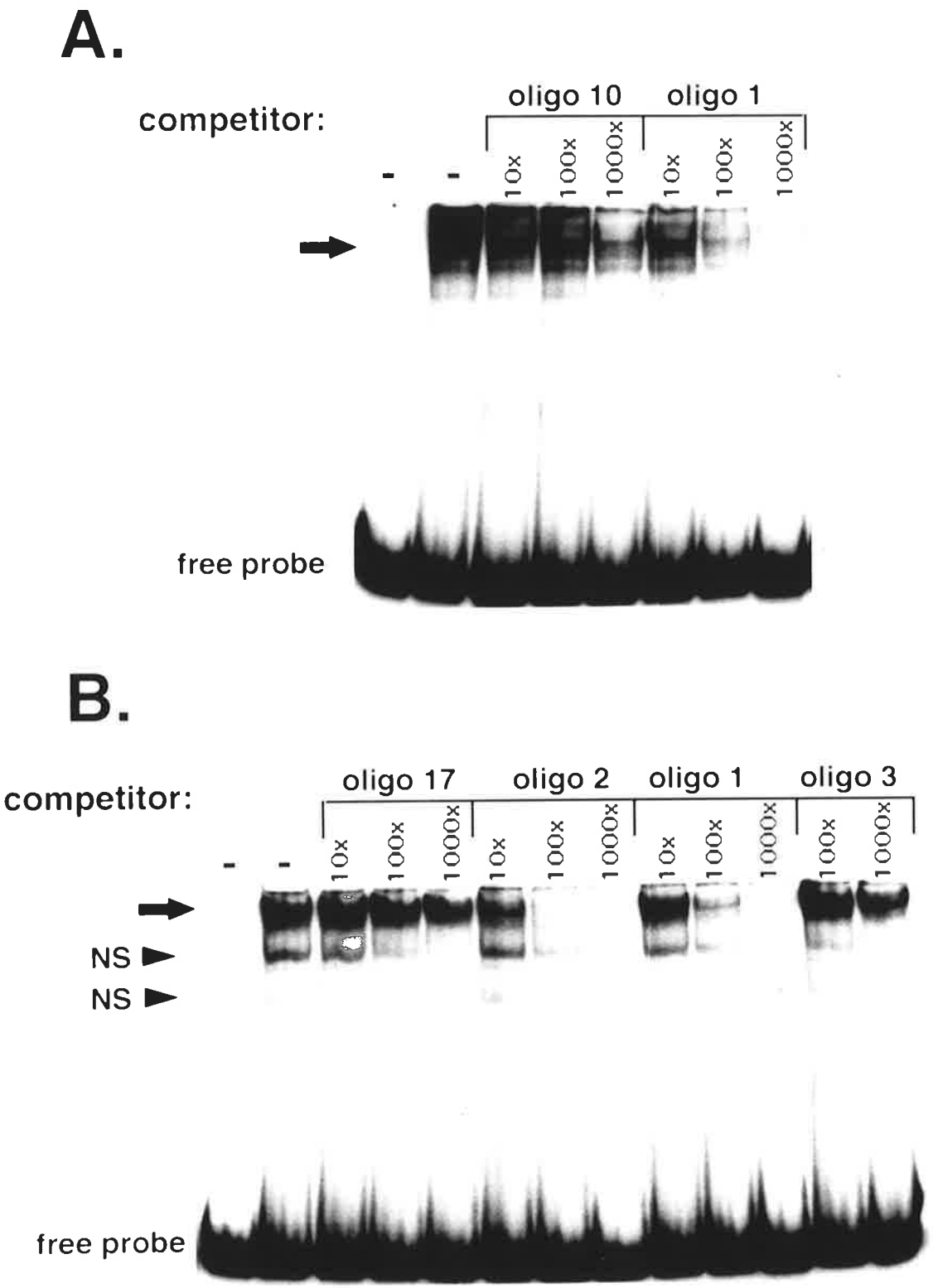

A.
competitor:
oligo 10
oligo 1
-
-
10x
100x
1000x
10x
100x
1000x
free probe
B.
competitor:
oligo 17
oligo 2
oligo 1
oligo 3
-
-
10x
100x
1000x
10x
100x
1000x
10x
100x
1000x
100x
1000x
NS
NS
free probe

Figure 6.13 Analysis of *K2.10* oligos 3 and 4 for the ability to bind mouse hair follicle nuclear proteins.

(A) GSA with radiolabelled oligo 3 (spanning -310 to -275 of the *K2.10* promoter; see figure 6.11) and hair follicle nuclear extract. A triplet of shifted bands is seen in lanes 7 and 9. (The intensity of the non-specific bands [arrows, NS] obscures the resolution of the triplet in many of the lanes). The non-specific complexes are lost or greatly reduced in intensity in the presence of 100- to 1000-fold excess of competitor oligo (lanes 4-5 and 7-9). The specific complex (bold arrow) is the central band of the triplet which is not competed out with 1000-fold excess of oligo 17 (lane 5) or oligo 2 (lane 9), but is lost upon competition with unlabelled "self" oligo (lanes 6-8). Lane 1 contains free probe only. Dashes (-) indicate that no competitor oligo is present.

(B) GSA with radiolabelled oligo 4 (spanning -290 to -255 of the *K2.10* promoter; see figure 6.11) and hair follicle nuclear extracts. At least 4 retarded complexes are formed (arrows) but these appear to be non-specific (NS) interactions as binding is lost upon competition with non-specific oligo (oligo 11; lanes 3-5). A similar pattern is observed in the presence of excess oligo 5 (lanes 9-11). Lane 1 contains free probe only. Dashes (-) indicate that no competitor oligo is present. 10X, 100X and 1000X refer to molar excess amounts of competitor oligo.

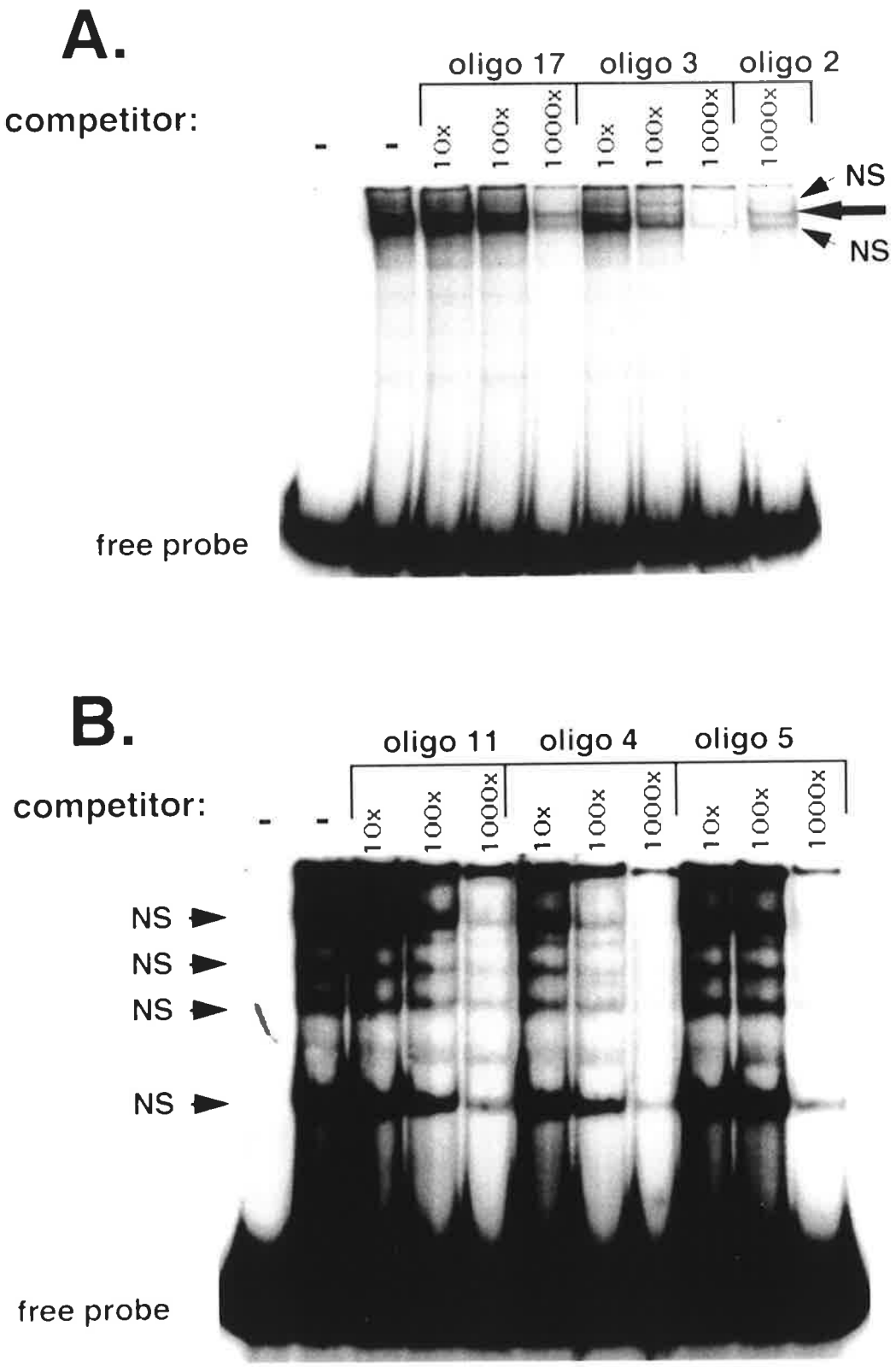
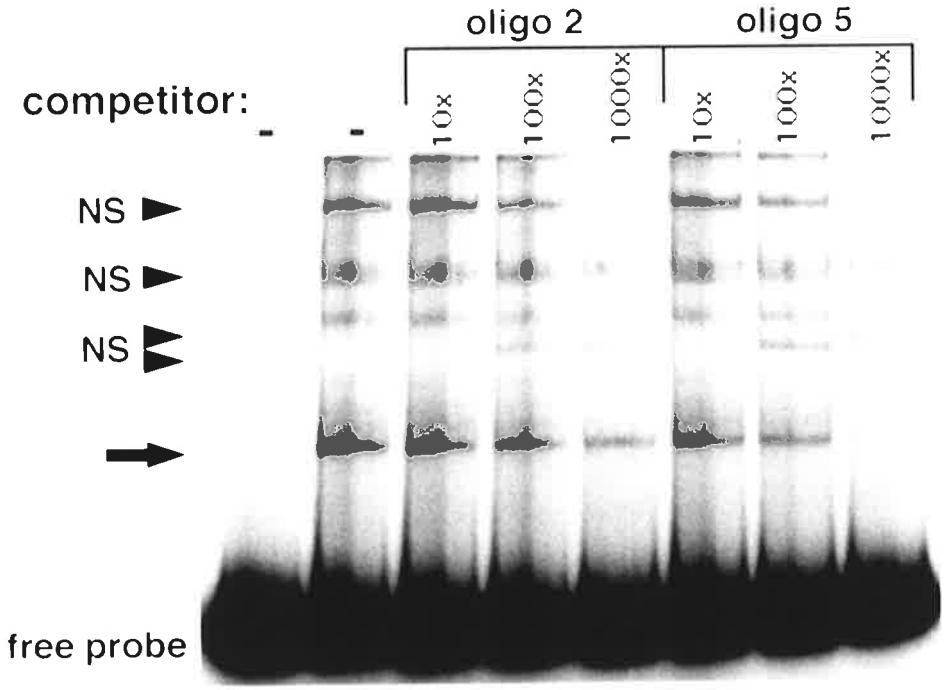

A.
competitor:
oligo 17
oligo 3
oligo 2
-
-
10x
100x
1000x
10x
100x
1000x
1000x
NS
NS
free probe
B.
competitor:
oligo 11
oligo 4
oligo 5
-
-
10x
100x
1000x
10x
100x
1000x
10x
100x
1000x
NS
NS
NS
NS
free probe

Figure 6.14 Analysis of *K2.10* oligos 5 and 6 for the ability to bind mouse hair follicle nuclear proteins.

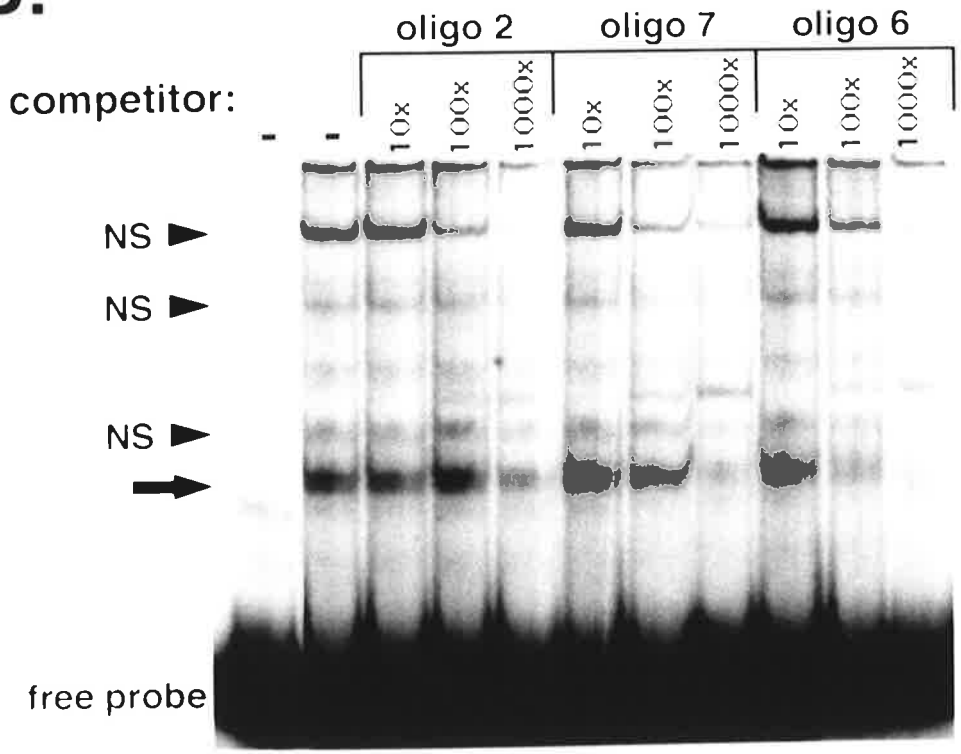
(A) GSA with radiolabelled oligo 5 (spanning -270 to -235 of the *K2.10* promoter; see figure 6.11). A series of non-specific (NS) retarded complexes (arrowheads) are produced with follicle nuclear extracts which are competed out with non-specific oligos such as oligo 2 (lanes 3-5). A specific high-mobility complex is also formed (arrow) which is only able to be competed out at high concentrations of unlabelled "self" oligo (lane 8). Lane 1 contains free probe only. Dashes (-) indicate that no competitor oligo is present.

(B) GSA with radiolabelled oligo 6 (spanning -250 to -215 of the *K2.10* promoter; see figure 6.11). A specific complex is formed (arrow) , as well as a number of non-specific retarded complexes (arrowheads) which are lost upon competition with oligo 2, a non-specific competitor. Note: the apparent loss of specific complex formation with 1000-fold molar excess of oligo 7 is an artefact of this gel. Lane 1 contains free probe only. Dashes (-) indicate that no competitor oligo is present. 10X, 100X and 1000X refer to molar excess amounts of competitor oligo.

A.


oligo 2
oligo 5
competitor:
-
-
10x
100x
1000x
10x
100x
1000x
NS
NS
NS
free probe

B.


oligo 2
oligo 7
oligo 6
competitor:
-
-
10x
100x
1000x
10x
100x
1000x
10x
100x
1000x
NS
NS
NS
free probe

appear to be able to compete with each other for specific complex formation (data not shown), suggesting that the binding site occurs in the overlapping region between these two oligos.

Oligo 7 binds specific hair follicle nuclear factors (figure 6.15A), although this high-mobility complex was often difficult to detect and appears to be present at very low levels. Oligo 8 contains the HK-1 binding site (see figure 6.11) and forms HK-1-specific complexes (figure 6.15B). Oligo 8 is able to compete for HK-1 binding and vice versa. Oligo 9 also forms HK-1-specific complexes (see figure 6.9B) as well as a third complex which is specific for oligo 9 and cannot be competed out with HK-1, KTF-1 or oligo 10 competitors (figure 6.9B).

Oligo 10 forms a specific retarded complex with hair follicle extracts which runs as a doublet in GSAs (figure 6.16A). This complex is able to be competed with oligo 11 (which overlaps at the 3' end of oligo 10), but excess KTF-1 oligo has no effect on binding indicating that this interaction does not involve the KTF-1 binding site. Oligo 11 forms three major retarded complexes which appear to be specific interactions (figure 6.16B), however, neither flanking oligo 10 nor 12 is able to compete for binding. This suggests that the specific binding site occurs in the region of oligo 11 where oligos 10 and 12 do not overlap (see figure 6.11).

Oligo 12 forms a major specific retarded complex and several minor complexes which may also be specific interactions (figure 6.17 A). Complexes formed with oligo 12 appear to be more readily competed out with oligo 13 than oligo 12, suggesting a stronger interaction with oligo 13. Oligo 13 itself forms a strong major specific retarded complex and two more minor complexes which are also able to be competed off with oligo 12, but not oligo 14 (figure 6.17B). Again, this strongly suggests that the complexes formed with oligos 12 and 13 occur in the region of overlap between the two oligos which, interestingly, does not contain the CAAT box consensus sequence.

Two major bands and one minor band appear to represent specific interactions with oligo 14 and hair follicle nuclear proteins (figure 6.18A). Complex 1 is unable to be competed out with flanking oligos 13 and 15, complex 2 appears to be competed with oligo 13 to some extent, and the minor retarded complex is competed off with oligo 15. However, oligo 15 forms a strong, specific retarded complex with hair follicle nuclear proteins which appears unaffected by competition with flanking oligos 14 or 16 (figure 6.18B).

Oligo 16 spans the TATA box in the *K2.10* promoter and forms a strong, specific retarded complex with follicle extracts (figure 6.19A). Complex formation is able to be competed with oligo 15 (which overlaps oligo 16, but not the TATA box consensus sequence) but not with oligo 17.

Figure 6.15 Analysis of *K2.10* oligos 7 and 8 for the ability to bind hair follicle nuclear proteins.

(A) GSA with radiolabelled oligo 7 (spanning -230 to -195 of the *K2.10* promoter; see figure 6.11). A specific retarded complex is formed (arrow). Oligo 1 has been used as a non-specific competitor in this experiment (lanes 3-5). Lane 1 contains free probe only. Dashes (-) indicate that no competitor oligo is present.

(B) GSA with HK-1 (lanes 1-7) and oligo 8 (lanes 8-13) radiolabelled probes. Oligo 8 spans the *K2.10* promoter from -210 to -175 (see figure 6.11). Formation of specific retarded complexes with HK-1 probe (lane 1; arrows) is lost upon competition with itself (lanes 5-7) and oligo 8 (lanes 2-4). Conversely, HK-1 oligo was able to compete for specific complex formation with oligo 8 and follicle nuclear proteins (lanes 9-11; arrows). Non-specific complexes (NS, arrowheads) were identified using non-specific competitor oligos (see figure 6.4A, 6.10B and data not shown). Lane 1 contains free probe only. Dashes (-) indicate that no competitor oligo is present. 10X, 100X and 1000X refer to molar excess amounts of competitor oligo.

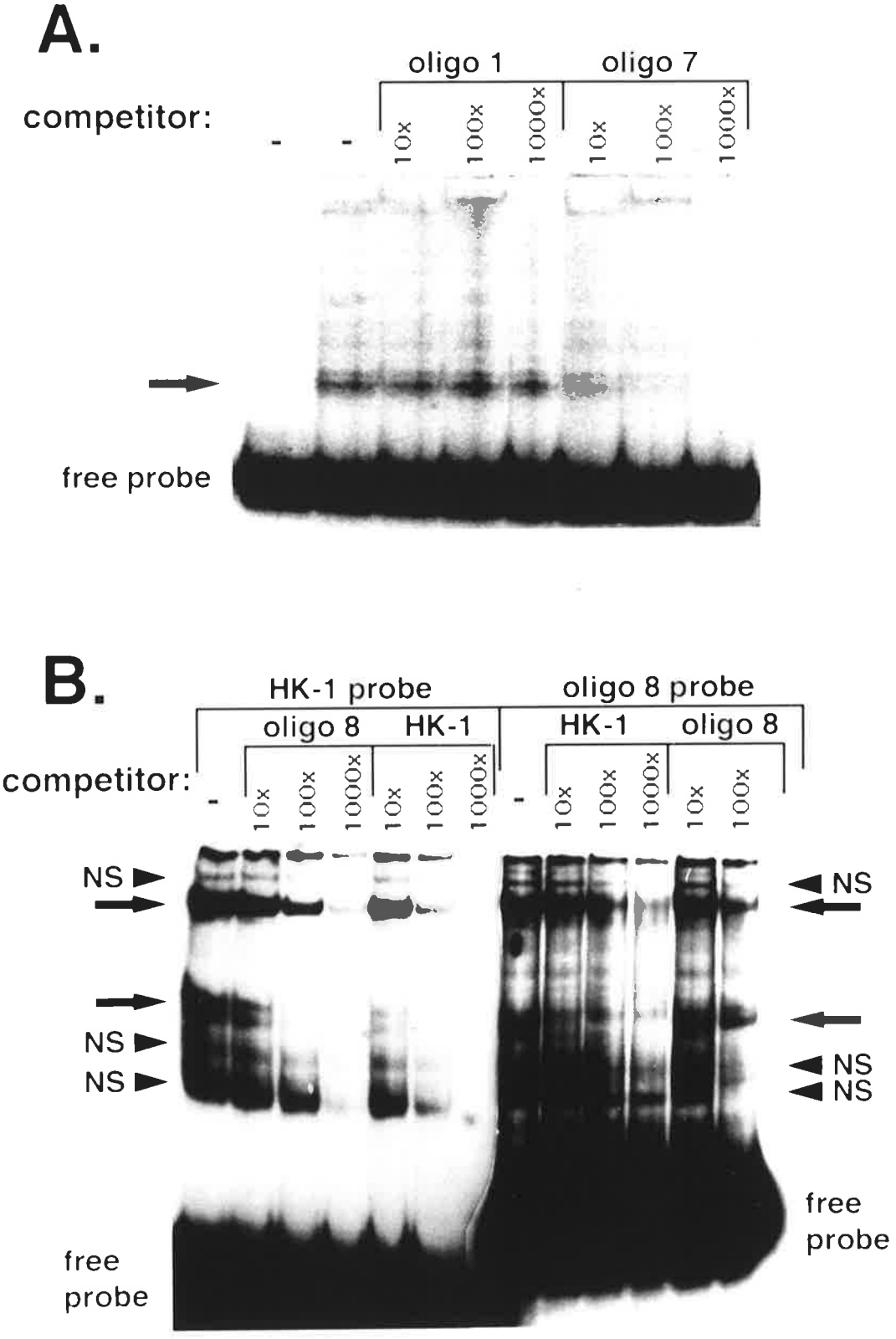

A.
competitor:
oligo 1
oligo 7
-
-
10x
100x
1000x
10x
100x
1000x
free probe
B.
HK-1 probe
oligo 8 probe
oligo 8
HK-1
HK-1
oligo 8
competitor:
-
10x
100x
1000x
10x
100x
1000x
-
10x
100x
1000x
10x
100x
NS
NS
NS
NS
NS
NS
free probe
free probe

Figure 6.16 Analysis of *K2.10* oligos 10 and 11 for the ability to bind mouse hair follicle nuclear proteins.

(A) GSA with radiolabelled oligo 10 (spanning -170 to -135 of the *K2.10* promoter; see figure 6.11). A specific retarded complex (lane 2; arrow) is formed. This complex is able to be competed out with oligo 11 (which overlaps oligo 10 at the 3' end; lanes 6-8) but not with the KTF-1 oligo (which overlaps with oligo 10 at the 5' end; lanes 12 and 13). A non-specific (NS) complex (arrowhead) is also present but is reduced upon competition with non-specific oligo (oligo 2; data not shown). Lane 1 contains free probe only. Dashes (-) indicate that no competitor oligo is present.

(B) GSA with radiolabelled oligo 11 (spanning -150 to -115 of the *K2.10* promoter; see figure 6.11). Three specific retarded complexes are formed (arrows; lane 2) with the top complex being more readily competed out with unlabelled "self" (lanes 9-11). Overlapping oligos 10 and 12 are unable to compete for binding with oligo 11 (see lanes 6-8 and 12-13 respectively). Lane 1 contains free probe only; oligo 3 is used as non-specific competitor (lanes 3-5). Dashes (-) indicate that no competitor oligo is present. 10X, 100X and 1000X refer to molar excess amounts of competitor oligo.

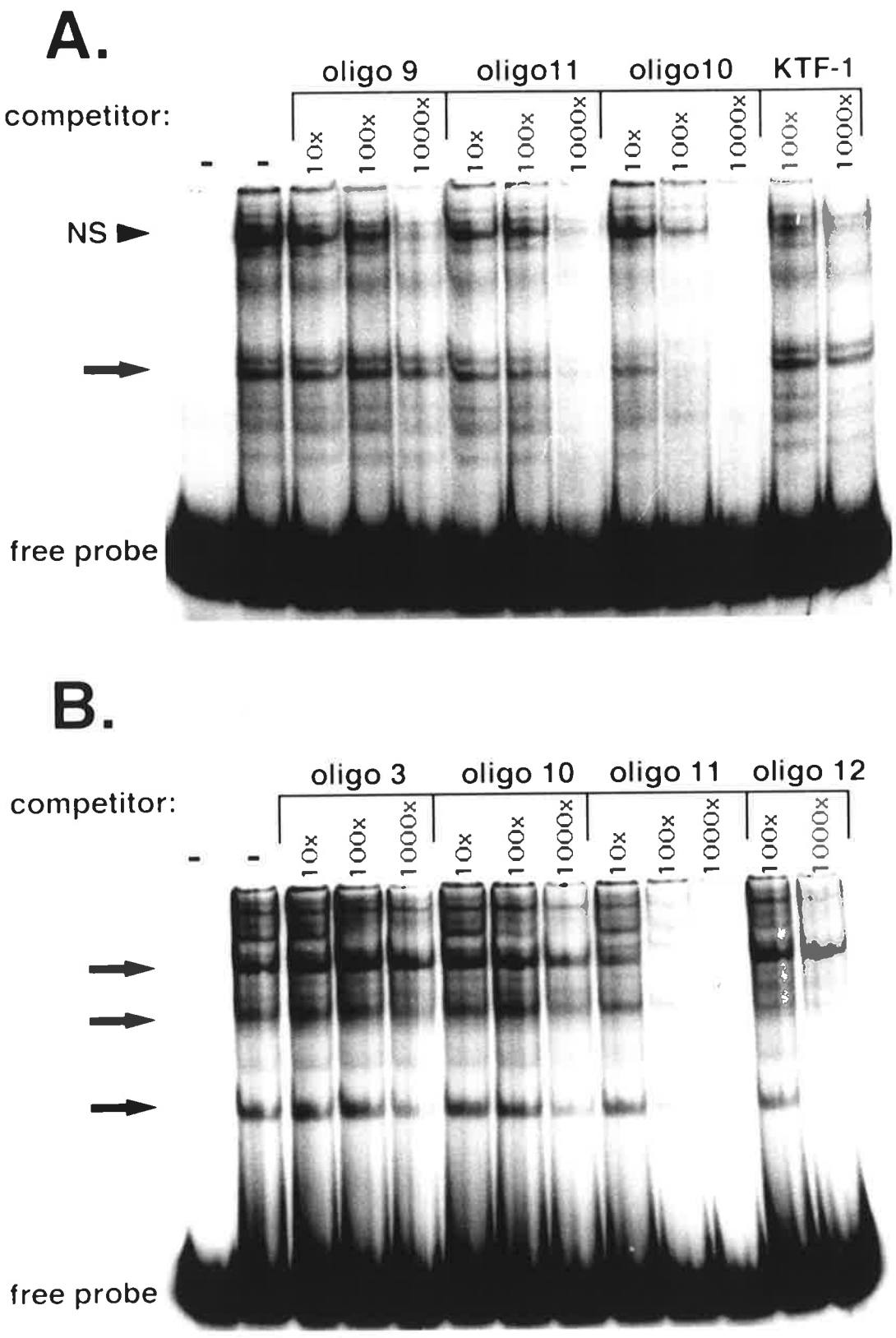

A.
oligo 9
oligo11
oligo10
KTF-1
competitor:
-
-
10x
100x
1000x
10x
100x
1000x
10x
100x
1000x
100x
1000x
NS
free probe
B.
oligo 3
oligo 10
oligo 11
oligo 12
competitor:
-
-
10x
100x
1000x
10x
100x
1000x
10x
100x
1000x
100x
1000x
free probe

Figure 6.17 Analysis of *K2.10* oligos 12 and 13 for the ability to bind hair follicle nuclear proteins.

(A) GSA with radiolabelled oligo 12 (spanning -130 to -95 of the *K2.10* promoter; see figure 6.11). A major specific retarded complex is formed (bold arrow) together with two minor complexes (arrows). These specific complexes can also be competed off with oligo 13 (which overlaps oligo 12 at the 3' end; lanes 9-11). The non-specific (NS) complex is labelled (arrowhead). Lane 1 contains free probe only; oligo 4 is used as non-specific competitor. Dashes (-) indicate that no competitor oligo is present.

(B) GSA with radiolabelled oligo 13 (spanning -110 to -75 of the *K2.10* promoter; see figure 6.11). A major specific retarded complex is formed (bold arrow) together with two minor complexes (arrows 1 and 2). Minor complex 2 is able to be competed out with oligo 12 (which overlaps at the 5' end), but the major complex and minor complex 1 are not competed as readily (lanes 6-8). Oligo 14 (which overlaps at the 3' end) is unable to compete for binding (lanes 13-15). Oligo 2 was used as a non-specific competitor oligo (lanes 3-5). Lane 1 contains free probe only. Dashes (-) indicate that no competitor oligo is present. 10X, 100X and 1000X refer to molar excess amounts of competitor oligo.

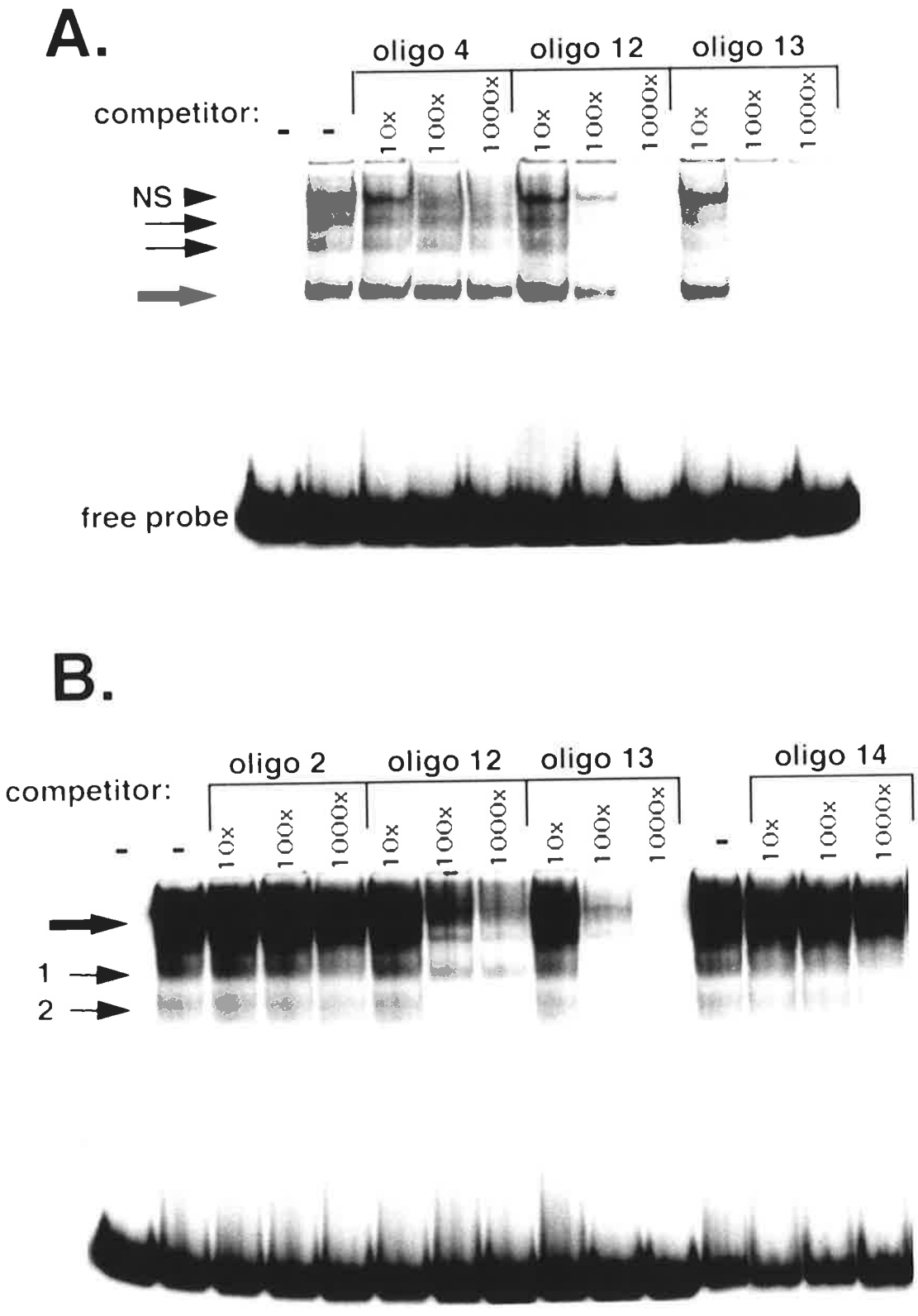

A.
oligo 4
oligo 12
oligo 13
competitor:
- -
10x
100x
1000x
10x
100x
1000x
10x
100x
1000x
NS
free probe
B.
oligo 2
oligo 12
oligo 13
oligo 14
competitor:
- -
10x
100x
1000x
10x
100x
1000x
10x
100x
1000x
-
10x
100x
1000x
1
2

Figure 6.18 Analysis of *K2.10* oligos 14 and 15 for the ability to bind hair follicle nuclear proteins.

(A) GSA with radiolabelled oligo 14 (spanning -90 to -55 of the *K2.10* promoter; see figure 6.11). Two major specific retarded complexes are formed (bold arrows 1 and 2). Note that complex 1 refers only to the upper band of the doublet which appears in some tracks. Complex 1 is unable to be competed off with non-specific oligo (oligo 2; lanes 2-4) or with overlapping oligos 13 and 15 (lanes 5-7 and 8-10, respectively). Oligo 13 appears to compete with complex 2 formation (see lane 7). A minor retarded band (arrow) appears to be a specific complex and is also able to be competed off with oligo 15 (see lane 10). Dashes (-) indicate that no competitor oligo is present. 10X, 100X and 1000X refer to molar excess amounts of competitor oligo.

(B) GSA with radiolabelled oligo 15 (spanning -70 to -35 of the *K2.10* promoter; see figure 6.11). A major retarded complex (arrow) is formed with hair follicle nuclear proteins (lane 1). Flanking oligos 14 and 16 are unable to compete with oligo 15 for binding of these proteins (see lanes 5-7 and 12-14, respectively). Oligo 2 was used as a non-specific competitor oligo (lanes 2-4). Dashes (-) indicate that no competitor oligo is present. 10X, 100X and 1000X refer to molar excess amounts of competitor oligo.

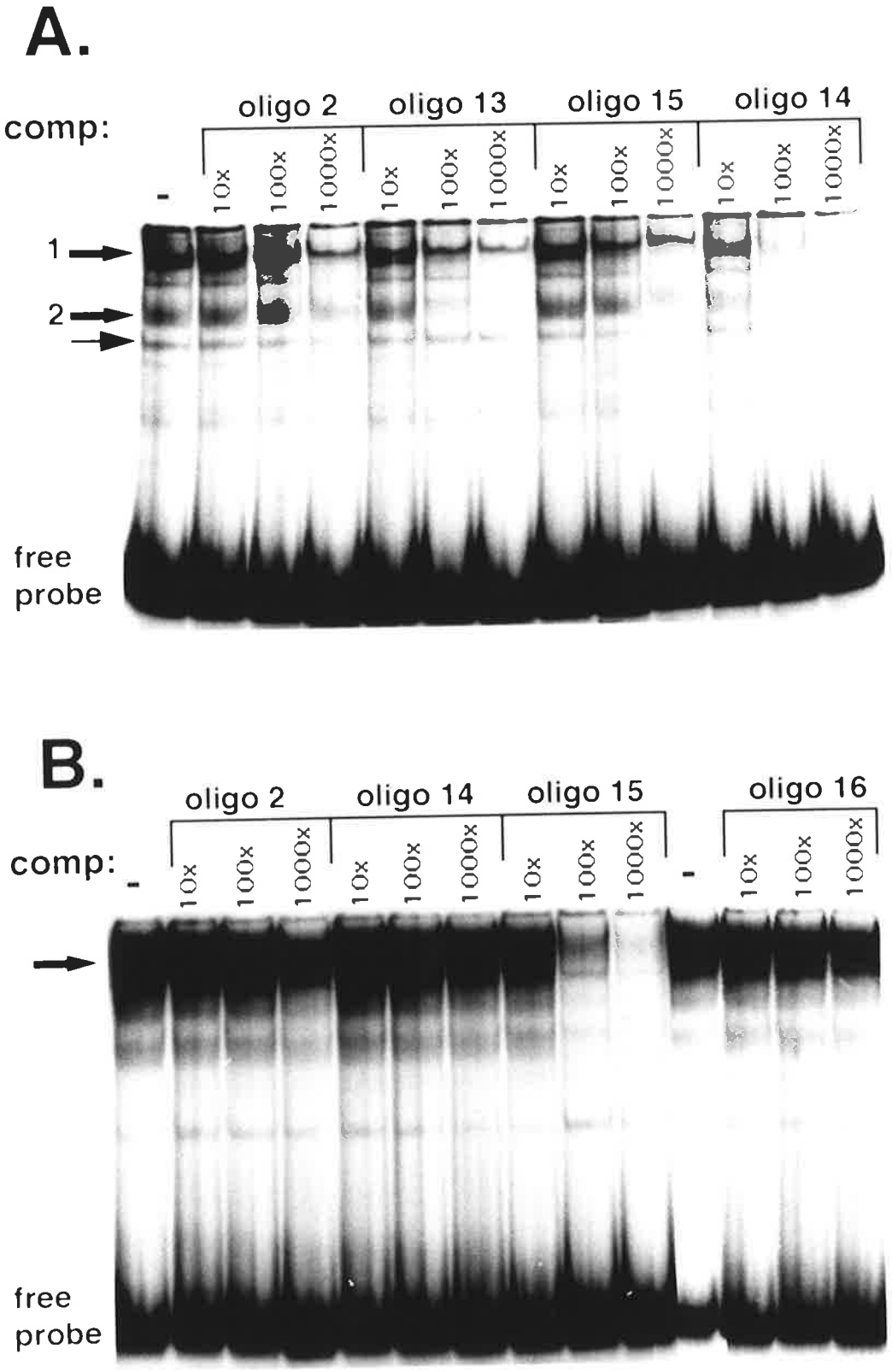
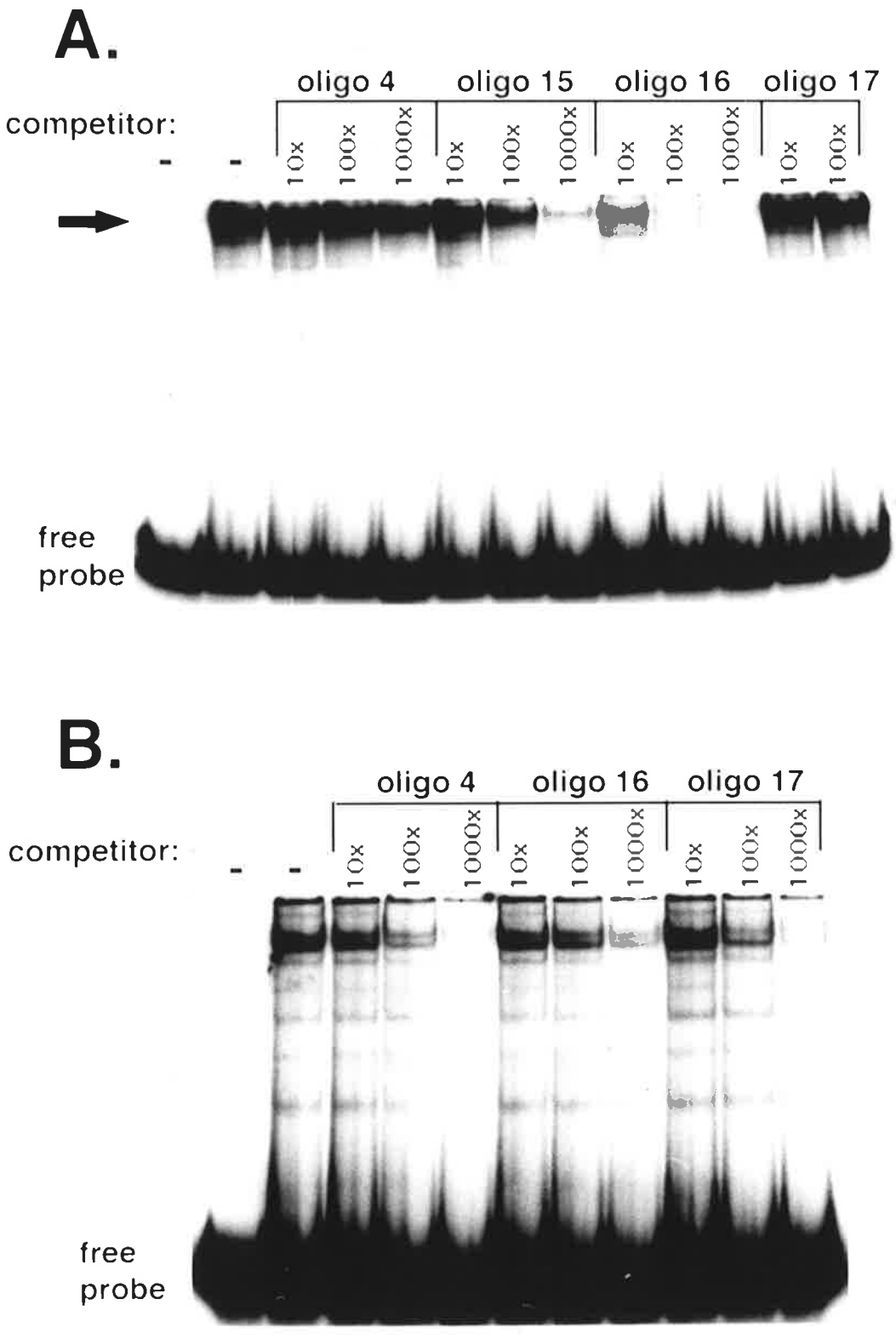

A.
comp:
oligo 2
oligo 13
oligo 15
oligo 14
-
10x
100x
1000x
1
2
free
probe
B.
comp:
oligo 2
oligo 14
oligo 15
oligo 16
-
10x
100x
1000x
free
probe

Figure 6.19 Analysis of *K2.10* oligos 16 and 17 for the ability to bind hair follicle nuclear proteins.

(A) GSA with radiolabelled oligo 16 (which spans the TATA box region of the *K2.10* promoter from -50 to -15; see figure 6.11). One major specific retarded complex is formed (arrow; lane 2). Oligo 15 is also able to compete for binding (lanes 6-8) but oligo 17 is not (lanes 12 and 13). Lane 1 contains free probe only; oligo 4 is used as a non-specific competitor (lanes 3-5). Dashes (-) indicate that no competitor oligo is present.

(B) GSA with radiolabelled oligo 17 (spanning -30 to +5 of the *K2.10* promoter; see figure 6.11). Despite a series of retarded complexes being formed, none of these interactions appear to be specific and are lost upon competition with non-specific oligo (oligo 4; lanes 3-5) and with the overlapping oligo 16 (lanes 6-8). Lane 1 contains free probe only. Dashes (-) indicate that no competitor oligo is present. 10X, 100X and 1000X refer to molar excess amounts of competitor oligo.


A.
oligo 4
oligo 15
oligo 16
oligo 17
competitor:
-
-
10x
100x
1000x
10x
100x
1000x
10x
100x
1000x
10x
100x
free
probe
B.
oligo 4
oligo 16
oligo 17
competitor:
-
-
10x
100x
1000x
10x
100x
1000x
10x
100x
1000x
free
probe

Competition with oligo 15 is not as efficient as that seen with oligo 16 itself. Oligo 17 forms a number of retarded complexes with follicle extracts, however, none of these appear to be specific interactions (figure 6.19B).

6.3 Discussion

6.3.1 Analysis of mouse hair follicle nuclear extracts

Mouse hair follicle extracts were used in gel shift assays to examine the ability of sheep *K2.10* promoter sequences to bind hair follicle nuclear proteins. The specific binding of nuclear proteins would imply the presence a possible transcription factor binding site in those sequences. As the sheep *K2.10* promoter is active in transgenic mice (see chapter 3; Powell and Rogers, 1990; Keough *et al.*, 1995) and the spatio-temporal pattern of expression correlates with the endogenous *K2.10* expression in sheep, mouse hair follicle extracts were used due to the relative ease of isolation of hair follicles from mouse compared with sheep.

Hair follicle extracts were generally prepared from normal 3-5 day old Swiss mouse pups, however, follicle extracts from *K2.10-TAg* transgenic mice were used to determine whether nuclear proteins from terminally differentiating follicle cells were present in the extracts. Western blot analysis of *K2.10-TAg* follicle nuclear extracts indicated the presence of SV-40 TAg protein (see figure 6.1) which is expressed only in the terminally differentiating cortical cells which express the *K2.10* gene (see chapter 4). The 97 kD TAg protein was detected as two bands of approximately 90 and 100 kD in *K2.10-TAg* follicle extracts. These size variations are thought to be due to post-translational protein modifications including phosphorylation, glycosylation and adenylation (see Fanning and Knippers, 1992 and references therein).

The transcription factor AP-1 which is known to be expressed in the follicle bulb region (Fisher *et al.*, 1991) also appears to be present in the follicle extracts as a specific complex was formed in gel shift assays (see figure 6.2). This indicated that the nuclear extracts contained native proteins which were capable of specific binding to DNA (i.e. "binding-competent" proteins) under the conditions used. It also confirmed the presence of an AP-1 binding activity in the extracts, as expected.

6.3.2 The HK-1 element

The 20 bp HK-1 oligonucleotide appears to bind a specific factor (or factors) in the hair follicle nuclear extracts, forming 2 retarded complexes in gel shift assays (see figure 6.3). The higher mobility complex appears to migrate as a doublet and is often less abundant than the lower mobility complex.

To examine the sequence specificity of binding in the HK-1 complex, the ΔHK-1 oligo (in which the HK-1 site has been mutated) was used as a competitor for HK-1 binding (see figure 6.4). Whilst the ΔHK-1 oligo is unable to compete for HK-1 binding when present at 100-fold molar excess, it does compete at 1000-fold excess whereas other oligos, such as NS-1, do not. ΔHK-1 has been shown to form a low-mobility HK-1-like complex in gel shift assays, although the binding activity is very low and the higher mobility HK-1 complex is undetectable. This weak binding ability, despite the disruption of the putative binding site, indicates a specific, low affinity binding with regions outside of the 8 bp HK-1 consensus sequence.

A further series of HK-1 variant oligos, HKv-1 to HKv-8, were also analysed for binding activity with follicle nuclear extracts (see figure 6.5). HKv-1, an oligo derived from the *K2.11* promoter, exhibited HK-1-like binding, although at a reduced level compared with HK-1 binding. This suggests that, similar to the K2.10 HK-1 motif, this HK-1 site may be involved in transcriptional regulation of the *K2.11* gene. Although the affinity of the binding site is somewhat reduced, it is not known whether this reflects the endogenous situation or is due to differences in the affinities of the mouse and sheep proteins.

HKv-2 contains an A to G substitution in the HK-1 consensus sequence which is identical to the 8 bp HK-1 element of the sheep *K2.11* gene but has *K2.10* HK-1 flanking sequence. This variant oligo has a slightly increased affinity for HK-1-like factors compared with HKv-1, again suggesting that flanking sequences play a role in binding. HKv-3 contains a T to C substitution in the HK-1 consensus sequence corresponding to the second, more distal HK-1 sequence of the *K2.10* promoter. HKv-3 also appeared to exhibit HK-1-like complex formation indicating that this HK-1 motif, like the more proximal HK-1 site, may be functional *in vivo*. Oligos HKv-4, HKv-5 and HKv-6 also showed HK-1-like binding activity, albeit at reduced levels. Mutations in the HK-1 consensus sequence of these oligos were random and do not correspond to known HK-1 consensus variations.

Despite the apparent reduced binding affinities of these variant oligos, binding was always stronger than with the ΔHK-1 oligo in which the consensus sequence was completely disrupted. However, the variant oligo HKv-7 contains a single A to C substiution which appears to abolish all binding activity. This same substitution is also present in the ΔHK-1 oligo, which is able to form the low-mobility complex, albeit at a very low level. This apparent anomaly is difficult to explain, given that the flanking sequences of both oligos are identical. Assuming that the low-mobility complexes formed by the wild-type, ΔHK-1 and variant oligos is due to the binding of identical proteins (this remains to be proven, although the mobilities appear identical), the following model is proposed as a possible explanation.

ΔHK-1 can only form the low-mobility complex, but it is able to compete with the HK-1 oligo for both the low-mobility and high-mobility complexes, suggesting that competition for the low-mobility complex (i.e. removal of the low-mobility proteins) results in the loss of the high-mobility complex. This implies that the low-mobility complex is required for high-mobility complex formation.

It is proposed that the initial protein interaction occurs mainly with the flanking sequences, forming the low-mobility HK-1 complex. The A to C substitution in HKv-7 abolishes this interaction, suggesting that the A residue is critical for Low-mobility complex formation. The ability of ΔHK-1 to form a low-mobility complex may be fortuitous due to other mutations which could possibly compensate for the A to C substitution. However, this is a very weak interaction and ΔHK-1 is not as efficient as HK-1 in competing for HK-1 complexes. It is further proposed that HKv-7 is unable to form the high-mobility complex due to the inability to form the initial low-mobility complex, whilst ΔHK-1 is unable to form the high-mobility complex because the required factor(s) is unable to bind to the mutated HK-1 site. Confirmation of this model of protein binding to the HK-1 oligo awaits further experimentation. It should be mentioned that gel shift assays use non-denaturing gels , therefore, it may be possible for a complex of a higher molecular weight to exhibit a higher mobility than a lower molecular weight complex (depending on factors such as protein conformation and charge).

A further variant oligo, HKv-8, maintained an intact HK-1 element but an A to G substitution was introduced just outside of the consensus sequence. Whilst not as highly conserved as the 8 bp sequence, an A nucleotide is often found in this position. It was of interest to see whether this nucleotide change would affect binding, perhaps increasing binding affinity since it would more

closely resemble the HK-1 element in the mouse *HKA1* gene. HKv-8 exhibited strong HK-1-like binding activity which was only slightly reduced compared to the binding activity of the HK-1 oligo.

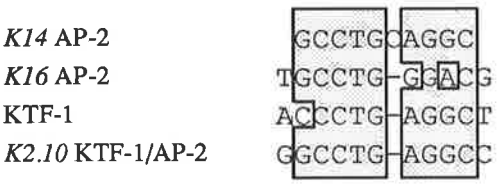
Thus, it appears that sequences outside of the 8 bp HK-1 consensus element play a role in HK-1 complex formation. Changes within the consensus sequence appear to reduce binding affinity, but may not abolish binding, depending on the surrounding sequence. Although use of further variant oligos could be used to extend the mutational analysis of the HK-1 site, these experiments did show that nucleotides within the HK-1 element are involved in the binding of specific factors. However, the exact binding site remains to be defined. An important experiment to determine the role of the flanking sequences would be a gel shift assay using an oligo containing the HK-1 consensus motif, CTTTGAAG, with mutated flanking sequences. DNA footprinting and methylation interference studies of this region should enable exact protein-DNA contact points to be determined.

The presence of proteins which bind specifically to the HK-1 oligo in CHO cell nuclear extracts (see figure 6.6), rat brain , testis and spleen extracts (see figure 6.7) suggest that this factor (or factors) may not be exclusive to the hair follicle. It is interesting to note that whilst CHO cells and HeLa cells express a factor (or factors) which binds to the HK-1 oligo, other epithelial-like cell types such as HepG2 and Ptk2 cells do not. Except with HeLa cell extracts, complex formation in these non-follicular extracts is poorer than with hair follicle extracts. This may be due to a lower abundance of factor(s), or because different factors with a lower affinity are binding. Despite the strong binding and high level of complex formation with HeLa cell extract, the second, higher-mobility complex characteristic of hair follicle extract was not observed. Also, complexes formed with extracts from testis migrated differently from the hair follicle extract complex in gel shift assays, again suggesting that these factors may not be identical or may be interacting with other sites within the HK-1 oligo which differ from the hair follicle protein binding site. Methylation interference studies or DNA footprinting would enable this to be determined.

6.3.3 The KTF-1/AP-2 element

A second putative transcription factor binding site adjacent to the HK-1 sequence is an AP-2-like binding site, termed KTF-1 in this thesis due to the high similarity with the Xenopus KTF-1 site (figure 6.20A and Snape *et al.*, 1991). KTF-1 shares a strong sequence similarity with AP-2 sites identified in other epidermal genes such as *K14* (Leask *et al.*, 1990, 1991) and *K16* (Magnaldo *et al.*, 1993). KTF-1 is thought to bind an AP-2-like factor, if not AP-2 itself (Snape *et al.*, 1991).

A.



B.

K16 E site	TTCTTCTGAAGGGCTGAC
K2.10 HK-1	CTT-TGAAG

Figure 6.20

(A) Comparison of the *K2.10* KTF-1/AP-2 motif with Xenopus KTF-1 and epidermal AP-2 sequences. The KTF-1 sequence was identified in the promoter of the Xenopus keratin gene, *XK81A1* (Snape *et al.*, 1990). AP-2 binding sites have been found in the human *K14* (Leask *et al.*, 1990,1991) and *K16* (Magnaldo *et al.*, 1993) genes. The stippled boxes represent nucleotides common to all sequences. Dashes indicate insertions which are made to enable alignment of the sequences.

(B) Comparison of *K2.10* HK-1 and *K16* E-site (Magnaldo *et al.*, 1993) promoter elements. The dash represents an insertion included to assist in sequence alignment.

The 23 bp KTF-1 oligo was shown to bind proteins from hair follicle nuclear extracts and form specific retarded complexes in gel shift assays (see figure 6.8A). This oligo was also shown to form a similar specific complex with AP-2 extract, indicating that this sequence contains a functional AP-2 binding site (see figure 6.8B). The slightly different mobilities of the KTF-1 complexes formed with hair follicle and AP-2 extracts may be due to differences between mouse and human AP-2 proteins, or due to the fact that the AP-2 extract was produced by over-expression of the human AP-2 protein in a bacterial system in which protein modifications may differ from eukaryotic cells, or simply that the commercially produced extract contained different salt or glycerol concentrations affecting migration of the complex through the gel. In order to show conclusively that the KTF-1 binding activity found in hair follicle extracts is due to AP-2, supershift gel assays could be performed using AP-2 antibodies.

The close proximity of the HK-1 and AP-2 binding sites suggested a functional interaction between the proteins involved. It was therefore of interest to examine the binding activity of an oligo containing both sites, such as oligo 9. It was surprising to find that oligo 9 demonstrated no AP-2 binding (see figure 6.10) and that the KTF-1 oligo was unable to compete for any specific complexes formed with oligo 9 in gel shift assays (see figure 6.9B. Conversely, oligo 9 was also unable to compete for KTF-1 complexes (see figure 6.8A). As oligo 9 is 4 bp shorter than the KTF-1 oligo at the 3' end, this must be preventing AP-2 binding. However, it was interesting to note a new binding activity for oligo 9 in addition to the HK-1 complexes. This activity, referred to as complex 3, is unable to be competed with excess HK-1 oligo or KTF-1 oligo and appears to be unique for oligo 9 (see figure 6.9B), suggesting that the binding site occurs in the central region of oligo 9, between the flanking oligos 8 and 10.

The occurrence of 3 possible protein binding sites, HK-1, AP-2 and complex 3, in this region suggest that these sequences may play an important role in expression of the *K2.10* gene. A similar element has been identified in the human *K16* gene which consists of an enhancer, termed the E site, and an adjacent AP-2 binding site (Magnaldo *et al.*, 1993). Interestingly, the 18 bp E site bears some similarity to the HK-1 motif (figure 6.20B). An oligonucleotide containing both the E and AP-2 sites also exhibited a new binding activity, producing a higher mobility complex that was unable to be competed out with excess AP-2 oligo or E site oligo, somewhat analogous to complex 3 formed with oligo 9. The E site alone was shown to act as an enhancer in human foreskin keratinocytes, however, both the E and AP-2 binding sites appear to act as a single transcriptional activation unit in the *K16* promoter, with both sites being required for activity. Like the HK-1

element, the E site does not appear to confer cell type specificity. The E site-binding protein(s) is abundant in HeLa cells. Interestingly, strong, specific complex formation is also observed with the HK-1 oligo and HeLa cell extracts. Whether or not the E/AP-2 element and the HK-1/KTF-1 site are functionally analogous remains to be shown.

6.3.4 Gel shift analysis of the 400 bp *K2.10* proximal promoter

AP-2, HK-1 and KTF-1/AP-2 motifs had previously been identified in the *K2.10* proximal promoter (Powell *et al.*, 1991; see figure 5.1). A Signal Scan (Prestridge, 1991) analysis also identified potential E2A transcription factor binding sites in this sequence (figure 6.11). No other known protein binding motifs were found in the 400 bp *K2.10* promoter. Thus, in order to identify other binding sites which may be important in the regulation of *K2.10* gene expression, a series of overlapping oligos spanning the promoter were generated for use in gel shift binding studies (see figure 6.11).

GSAs identified possible nuclear protein binding sites in the sequence spanning the overlap between oligos 1 and 2 (i.e. between approximately -330 and -315 of the *K2.10* promoter). A specific complex was formed with oligo 3 (see figure 6.13A) which spans a putative AP-2 site (see figure 6.11). This complex appeared to be formed at a very low abundance, especially when compared with the binding of hair follicle extracts to the KTF-1 oligo (see figure 6.8). Further experiments are required to determine whether this complex is due to AP-2 binding. The region of overlap between oligos 5 and 6 (i.e. approximately -250 to -235) also binds a specific protein(s) and a low abundance complex was observed with oligo 7 and hair follicle nuclear extracts, suggesting further potential binding sites in this region.

Oligo 8, containing the HK-1 sequence, formed HK-1-specific complexes and was able to compete for proteins binding to the HK-1 oligo. Similar HK-1-specific complexes were formed with oligo 9, as well as a third complex unique to oligo 9. Neither oligo 9 nor oligo 10 were capable of forming KTF-1-like complexes, possibly because neither oligo contained enough flanking sequence to enable binding. Oligo 10 did, however, form a specific retarded complex which was also competed out with oligo 11. Whilst oligo 11 could compete with oligo 10 for protein binding, oligo 10 was unable to affect protein binding to oligo 11. This suggests that oligo 11 binds a set of nuclear proteins distinct from those bound by oligo 10. The ability of oligo 11 to compete for oligo 10 complexes is therefore difficult to interpret. It is possible that proteins binding strongly to oligo

11 may interact with those binding to oligo 10 such that these protein-protein interactions are stronger than the DNA-protein interactions between oligo 10 and its binding factor(s).

Another putative protein binding site is located at the 5' end of oligo 13 around the region of overlap between oligos 12 and 13 (approximately -110 to -90) as an abundant, low-mobility complex is observed in GSAs. Oligo 13 spans the CAAT box region of the promoter, however, the competition studies with oligos 12 and 14 suggest that this site does not bind any proteins present in hair follicle nuclear extracts. It is possible that oligo 13 does not contain enough flanking sequence to allow CAAT factors to bind. However, DNA footprinting of CAAT factors present in liver nuclear extracts indicates an asymmetrical binding around the CAAT motif, with approximately 18 bp upstream and 7 bp downstream of the CCAAT pentanucleotide motif being protected (Graves *et al.*, 1986). Such a region is amply covered by oligo 13.

Further possible protein binding sites are found in sequences spanned by oligos 14, 15 and 16. Oligo 14 forms 2 major specific complexes in GSAs with hair follicle proteins, one which is specific to oligo 14 and one which is able to be competed out with oligo 13 (possibly corresponding to E2A binding, although further experiments are required to show this). A minor complex is also observed which is competed out with oligo 15. These low-abundance oligo 14 complexes are not observed in GSAs with oligo 13 and 15 probes, however, oligo 13 and oligo 15 complexes are quite abundant and oligo 14 complexes may be undetectable. As mentioned, oligo 15 contains a protein binding site, forming a strong retarded complex with follicle extracts which is unaffected by competition with oligos 14 and 16. Oligo 16 contains the TATA element and forms a specific low-mobility protein complex in GSAs with follicle extracts. This complex is also competed out with oligo 15, albeit less efficiently. At least seven different factors (not including RNA polymerase II) operate via the TATA element: TFIID, TFIIA, TFIIB, TFIIF, TFIIE, THIIH and TFIIJ (for review see Zawel and Reinberg, 1993). The TATA-binding protein (TBP), TFIID, is the first protein bound and the only factor with specific DNA binding activity. The binding of the associated factors appears to be mediated by protein-protein interactions. Thus, the ability of oligo 15 to compete for oligo 16 protein complexes is puzzling because oligo 15 does not contain the TATA motif. Also, excess oligo 16 has no effect on protein complex formation with oligo 15. It is possible that the oligo 15 protein complex, rather than oligo 15 itself, is able to compete for proteins binding to oligo 16. The lack of specific protein binding to oligo 17, which spans the transcription start site, can be explained by the fact that oligo 17 does not contain the TATA-binding site and, without TFIID being able to bind, the other proteins of the transcription initiation complex cannot bind.

From the GSA data it is possible that the putative AP-2 site at -298 (see figure 6.11) is functional as specific protein binding was detected in this region (although further experiments are required to show this, such as GSAs using AP-2 and supershift assays). The sequence spanning the HK-1 and KTF-1/AP-2 motifs appears to bind at least three specific factors, including AP-2, suggesting that this region plays a regulatory role in gene expression. Oligos which spanned the two putative E2A sites (at -125 and -80) also showed specific protein interactions, indicating possible binding sites for E2A or other factors. GSA data also suggests that the CAAT box site may not be functional in this promoter. Whilst the CAAT box is considered to be a common transcriptional element and CAAT binding proteins are ubiquitous, not all genes contain this motif (for example, the SV-40 early promoter; McKnight and Tjian, 1986).

Thus, this preliminary gel shift study has identified a number of regions in the sheep *K2.10* proximal promoter with the ability to bind specific nuclear proteins in mouse hair follicle extract. This data can be used to generate a model of the *K2.10* promoter showing the minimum number of protein binding sites identified by this work (figure 6.21). These binding sites are based purely on specific DNA-protein interactions *in vitro* using oligos and crude follicle nuclear extracts derived from the different cell types of the follicle. Whether these binding sites are functional in the context of the entire intact 400 bp promoter is unknown. Evidence suggests that the HK-1 element is likely to play important roles in hair keratin gene expression given the high conservation of the HK-1 sequence in hair keratin IF genes, the ability to bind hair follicle nuclear proteins and the reduction in gene expression upon mutation of the element (see 5.2.3). The KTF-1/AP-2 site adjacent to the HK-1 motif is also of interest. This site is able to bind AP-2 protein *in vitro* and binds specific nuclear proteins in hair follicle extracts. The expression of AP-2 in the hair follicle (C. Byrne, personal communication) and its known role in the expression of epidermal keratin genes implies a possible function in hair keratin gene expression.

The identification of putative E2A binding sites in the *K2.10* promoter are also of interest. E2A helix-loop-helix proteins are generally involved in the control of various developmental pathways, but have also been found in a number of tissue-specific complexes that regulate tissue-specific enhancers involved in muscle-specific (Weintraub *et al.*, 1991), pancreatic-specific (Cordle *et al.*, 1991) and B cell immunoglobulin (Murre *et al.*, 1991) gene expression. E2A transcripts are present in most tissues, and high levels of expression are seen in rapidly proliferating and differentiating cells, including those of the hair follicle (Roberts *et al.*, 1993). Putative E2A binding sites have also been identified in the epidermally expressed *L-CAM* gene (Sorkin *et al.*, 1993). Although there is

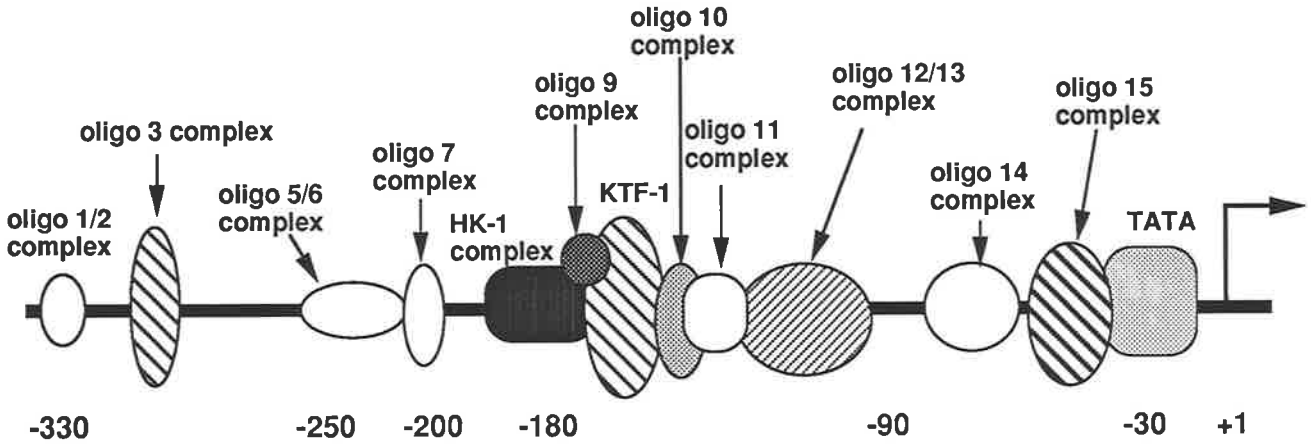


Figure 6.21

A diagrammatic representation of putative protein binding sites in the *K2.10* proximal promoter identified by gel shift analysis with hair follicle nuclear extracts. Note, "oligo 9 complex" refers to the oligo 9-specific complex, termed complex 3 in figure 6.9B.

no evidence that the E2A sites in the *K2.10* promoter are functional, oligos spanning these sites do bind specific nuclear proteins in follicle extracts.

It is interesting to note that apart from AP-2 and E2A binding sites, no other known transcription factor binding motifs have been identified in this promoter. However, these studies have localised regions which now warrant further analysis as putative regulatory elements.

Chapter Seven

Conclusions and final discussion

7.1 Introduction

The work presented in this thesis forms part of an on-going investigation into the regulation of hair keratin gene expression. A major aim of this work was to produce an immortalised hair keratinocyte cell line capable of supporting the expression of hair-specific keratin genes. Such a cell line would enable the rapid analysis of gene promoter constructs and subsequent identification of possible regulatory elements. In the absence of a suitable cell, mouse transgenesis and *in vitro* gel mobility shift assays were employed in order to identify sequences in the sheep hair keratin *K2.10* gene promoter which may be important in controlling the expression of this gene.

7.2 Development of a hair follicle cell line

In an attempt to produce an immortalised cell line from hair follicle cortical cells SV-40 TAg expression was targeted to these cells in transgenic mice using the sheep hair keratin *K2.10* promoter. The expression of TAg in the follicle produced a marked phenotypic effect. TAg expression disrupted the cellular ultrastructure of the cortex and altered the normal abundance of keratin proteins, producing crooked and weakened fibres. *K2.10-TAg* mice exhibited various other abnormalities such as growth retardation, bladder, intestinal and stomach tumours and spinal defects, resulting in premature death. However, despite cortical cell expression of TAg, no neoplasia or hyperplasia was observed in the skin or follicles. Prolonged culturing of follicles isolated from transgenic mice was performed in an attempt to allow cell immortalisation to occur *in vitro*. Unfortunately, no follicle cell immortalisation was achieved and cells expressing TAg were found to be non-viable after 5-6 days in culture.

The inability of TAg to transform the cortical keratinocytes appears to be due to the differentiation state of the cells at the time the promoter is active. Cortical keratinocytes are derived from rapidly dividing follicle bulb cells which cease proliferation and undergo terminal differentiation accompanied by the activation of hair-specific keratin gene expression. The expression of *K2.10* appears to occur just after cell division has ceased. Despite the potent oncogenic effect of TAg and

its ability to stimulate and maintain cell proliferation, TAg seems incapable of inducing these cells back into the cell cycle. Thus, the use of the *K2.10* promoter to target TAg expression was unsuccessful in the production of an immortalised cell line. Oncogene expression earlier in cortical cell or follicle bulb cell differentiation would be desirable.

The use of the sheep *K1.15* promoter to direct TAg expression to the follicle should be more successful in producing a useful follicle-derived cell line. Cells expressing K1.15 are localised around the dermal papilla in the follicle bulb (as well as the ORS and other epithelial tissues) in the zone of bulb cell proliferation. It is hoped that immortalisation of this unique population will not only provide cell lines capable of supporting hair keratin gene expression, but will also provide an insight into the role of these cells in the follicle and their interaction with the dermal papilla.

The effect of TAg expression on the keratin composition of *K2.10-TAg* hair is also interesting. It is presumed that the changes in the protein profile are due to changes in keratin gene expression, perhaps via the interaction of TAg with transcription factors. Given that a cell line would be used for the analysis of keratin gene expression, it may be important to understand how normal gene expression is altered in such a cell line. However, the use of a temperature-sensitive TAg protein (as is planned for the *K1.15-TAg* construct) may overcome this problem by inactivating TAg at the restrictive temperature, although this would depend on whether the temperature-sensitive mutation affects the ability of TAg to alter gene expression.

Another possibility for the production of hair follicle cell lines is to use follicles from H-2K^b-tsA58 transgenic mice (Jat *et al.*, 1991). The major histocompatibility complex H-2K^b class I promoter is active in most cell types at various levels but can be induced to high levels of expression in almost all tissues in the presence of interferon-γ. The inducibility of the promoter, together with the temperature-sensitive regulation of TAg function, would allow tight control over TAg expression. A number of different cell lines from various tissues have been successfully produced from these transgenic mice (Jat *et al.*, 1991; Whitehead *et al.*, 1993).

The use of nail matrix cell cultures should also be considered as an alternative culture system for the analysis of hair keratin gene expression. Both hair- and epithelial-specific keratins are expressed in nail (Lynch *et al.*, 1986; Heid *et al.*, 1988b; Kitahara and Ogawa, 1993). Hair keratins are expressed mainly in the ventral nail matrix and partially in the dorsal and apical matrices, although some cells of the dorsal and apical matrices co-express both epithelial- and hair-specific keratins (Kitahara and Ogawa, 1993). *In situ* hybridisation studies to foetal mouse nail demonstrate the expression of *K2.10* in the nail bed (Powell and Rogers, 1994), suggesting that analysis of *K2.10*

gene expression should be possible in cultured nail bed cells. Recently, a method to culture nail matrix cells has been established and characterisation of cultured human nail matrix cells performed (Picardo *et al.*, 1994). These cells exhibited characteristic markers of nail differentiation, including hair keratin synthesis. Thus, it appears that nail keratinocytes may prove to be a suitable culture system for studying hair keratin gene regulation. It is not known, however, whether the control of keratin expression in the nail differs from that in the hair follicle or other epithelia, especially given the ability of some nail cells to co-express both hair- and epithelial-specific genes. Other tissues which express hair keratin genes such as tongue (Winter *et al.*, 1994) and intestine (see 3.3.1; B. Powell, unpublished results) could also be considered as a source of cells for the production of cell lines capable of supporting hair keratin gene expression.

7.3 Regulation of *K2.10* gene expression

Expression of the sheep *K2.10* gene in transgenic mice was shown to mimic the pattern of endogenous expression in the sheep wool follicle (Powell and Rogers, 1990), indicating that keratin gene regulatory mechanisms are conserved between mouse and sheep species. The 5' flanking region from this transgene construct containing 3 kb of 5' proximal promoter was used to target TAg expression to the follicle cortex in *K2.10-TAg* transgenic mice and correct timing and localisation of expression was observed. Shortening of the promoter to 400 bp of 5' proximal sequence produced a similar expression pattern in *K2.10(400)-lacZ* mice. Thus, as little as 400 bp of *K2.10* promoter sequence contains all of the necessary regulatory elements required for correct *K2.10* expression in the follicle cortex.

No expression was detectable in *K2.10(200)-lacZ* mice where only 200 bp of promoter was used. This construct lacked approximately 200 bp of *K2.10* exon 1 sequence that was present at the 3' end of the *K2.10-TAg* and *K2.10(400)-lacZ* constructs. Sequence analysis of this DNA revealed the presence of AP-2 and Sp1 binding motifs and a γ-interferon responsive element. It is not known whether these elements are functional, nor whether they affected *K2.10-TAg* and *K2.10(400)-lacZ* transgene expression. The complete lack of detectable expression from the 200 bp promoter was unexpected as widespread low level expression from the minimal promoter was predicted if tissue-specific elements were lost. This lack of expression may indicate the presence of a silencing element within this region of the promoter, although the inactivation (or silencing) of weak promoters by the *lacZ* gene has also been reported (Paldi *et al.*, 1993). Therefore, it would be desirable to use a different reporter gene in future constructs to avoid this possibility. Analysis of the 200 bp *K2.10*

exon 1 sequence for its possible effect on gene expression should also be investigated. It is interesting to note that 90 bp of *K5* promoter was sufficient to direct *lacZ* expression in transgenic mice and that expression was virtually epidermal-specific, although the differentiation-specificity was altered in this construct (Byrne and Fuchs, 1993).

Closer inspection of the 400 bp promoter sequence identified putative binding motifs for AP-2 and E2A transcription factors, as well as the HK-1 element. No other known protein binding sites, apart from CAAT and TATA motifs, were identified in this sequence. The high conservation of the HK-1 element in hair keratin genes, particularly IF genes, suggested a likely role in gene expression. *In vitro* gel mobility shift assays using oligos spanning the *K2.10* HK-1 site showed that hair follicle nuclear proteins can bind specifically in this region. The HK-1 element was mutated in the 400 bp promoter and expression was analysed in *K2.10(400ΔHK-1)-lacZ* transgenic mice. Mutation of the HK-1 site did not alter the cortical-specific expression of the *K2.10* promoter. However, low level, patchy expression was observed in a small percentage of follicles. Expression was only seen in two out of thirteen transgenic mice compared with four out of twelve *K2.10(400)-lacZ* transgenic mice. Expression was significantly reduced in mice with the mutated HK-1 element compared with those carrying the wild-type 400 bp promoter. Whilst transgene integration site effects cannot be ruled out given the low number of expressing transgenics in each case, two independent *K2.10(400ΔHK-1)-lacZ* Fo mice showed similar expression characteristics that suggest the HK-1 element may act as an enhancer.

An AP-2 binding site with strong homology to the Xenopus KTF-1 element is located adjacent to the HK-1 motif in the *K2.10* promoter. *In vitro* gel shift assays showed that a partially purified AP-2 extract was able to bind in this region and similar binding was seen using hair follicle nuclear extracts suggesting a possible role in *K2.10* gene expression.

The close proximity of the HK-1 and AP-2 sites in the promoter implies a possible functional interaction. Further analysis of these elements is required to determine their role in *K2.10* gene expression and how they interact. A strikingly similar element is found in the human *K16* gene promoter (Magnaldo *et al.*, 1993) in which the so-called E site (which bears some similarity to the HK-1 site) and AP-2 site are adjacent and exhibit a functional dependence on each other. Whether or not the HK-1/AP-2 and E-site/AP-2 elements are functionally analogous, or even bind the same factors, remains to be determined. Given the similarity between the E-site and HK-1 sequences, and that HK-1 binding activity is seen in a number of different tissues and cell lines, this may represent a

new family of transcription factors. Isolation and characterisation of the HK-1 factor (or factors) would be an obvious avenue to pursue.

Preliminary analysis of the 400 bp *K2.10* proximal promoter for other potential regulatory elements was undertaken using gel shift binding assays. Overlapping oligos spanning the promoter were tested for the ability to bind mouse hair follicle nuclear proteins. A number of regions for further investigation have been defined by this study. At least twelve different protein binding regions were identified, excluding TATA box binding proteins. However, given the *in vitro* nature of the assay and the use of crude nuclear extracts prepared from whole follicles (composed of a number of cell types including cortical cells), some of these potential binding sites may not play a role in *K2.10* gene expression *in vivo*. DNA footprinting and methylation interference studies would be useful in defining the sequence of the specific protein binding sites more closely. Further investigation into the function of putative regulatory elements would have to be performed in transgenic mice given the present lack of a suitable cell line. It would also be of interest to more closely map the expression patterns of transcription factors such as AP-2 and E2A (which are known to be expressed in the follicle) in order to determine the timing of their expression as well as the cell types involved.

7.4 Final remarks

The lack of suitable cell lines for the rapid analysis of hair keratin gene expression is a major hindrance to understanding the regulation of these genes. Cell lines would greatly facilitate gene promoter analysis and the isolation and characterisation of transcription factors. Given the successful production of eye lens cell lines which express terminal differentiation markers, it is likely that oncogene expression in the hair follicle will also be successful in generating useful cell lines provided that the expression of the oncogene is targeted to an "immortalisation-competent" cell population.

Understanding the regulation of keratin gene expression may provide an insight into the mechanisms controlling follicle cell differentiation. It may also have important implications in the study of disease states and the effects of drugs, nutrition and other environmental factors on hair and wool growth.

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