ISSN 0370-2731/2000 Proceedings of the New Zealand Society of Animal Production **60:** 147-154

Genetic manipulation to modify wool properties and fibre growth rates

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ABSTRACT

Over many centuries, genetic selection of sheep for particular meat and wool traits has made steady progress and given producers the opportunity to choose from a broad spectrum of well defined breeds. Selection for specific features of body frame or fleece has resulted in the emergence of true "meat" breeds (e.g. Poll Dorset, Suffolk, Texel) and other breeds highly valued for their fleece characteristics (e.g. Merino, Romney). Whilst such selection tends toward the outer limits of genetic variation among sheep, the sheep genome itself and natural mutation rates provide an inbuilt restriction to genetic change and the appearence of new phenotypic varieties. In comparison, the use of transgenesis to add specific functional sheep or non-sheep genes to the sheep genome has the potential to greatly expand the phenotypic range possible among sheep. For example, in a preliminary sheep transgenesis experiment using a cortical type II keratin intermediate filament transgene, we have shown it is possible to alter fibre structure and properties. The fact genes from any species may be used in the transgenesis process provides many experimental options simply not possible using the sheep genome alone. This paper is a discussion of some of the recent attempts to modify the genotype / phenotype of wool producing breeds using transgenesis. In particular, results of the earliest programmes designed to modify wool fibre properties and fibre growth rates are reviewed.

Keywords: Sheep transgenesis; Wool fibre properties; fibre growth rates.

INTRODUCTION

Wool, though a remarkable natural fibre in its own right, is disadvantaged in the textile fibre marketplace because of the non-uniformity in its composition and diameter. The inherent difficulty lies in the fact that it is produced by a biological system, itself subject to environmental stresses which affect fibre production rates and regulation of structural protein synthesis. Indeed, in comparison to other natural and synthetic fibres, wool generally has a higher and more variable diameter (fineness is highly sought after in the manufacture of apparel garments and is the main determinant of raw wool price) and less strength per unit cross-sectional area (Table 1). Ideally, transgenesis might be used to reduce fibre diameter and attempt control of diameter fluctuations. However, the precise regulatory mechanisms involved in controlling follicle diameter are yet to be determined and likely to involve a complex interplay between genotype and environment, including the processes regulating fibre elongation rates (Hynd, 1994a, b). If one is restricted to working with fibre diameters achievable on farm, modification of wool properties by transgenesis provides a logical alternative means to fibre improvement. This has been the primary aim of preliminary experiments in the sheep transgenesis programme of the Cooperative Research Centre for Premium Quality. We have attempted to overcome fibre flaws introduced by seasonal nutritional shifts and more random environmental stresses by manipulating fibre protein composition in the cortex for improved strength and elasticity (see below).

Another structural feature of the wool fibre which reduces its value and increases production costs is its uneven surface. Ridges occur at the point of cuticle "scale" cell overlap. These require smoothing during wool processing; a combination of harsh chemical etching then coating of the surface via a polymer treatment is normally used. These treatments act to lower the surface profile of the fibres such that apparel garments are less prone to shrinkage. Modification of the fibre surface by manipulating the protein composition of cuticle cells, to lessen the need for such processing, is another entry point for the transgenesis technology.

Genes encoding regulatory proteins such as growth factors and enzymes have also been used as transgenes in sheep and some examples will be discussed here.

FIBRE STRUCTURE MODIFICATION

The nature of fine, crimped wool fibres indicates an underlying unevenness in strength and elasticity within the cortex, itself bilaterally segmented into the orthocortical (soft) and paracortical (hard) compartments. In this case, fibre form manifests the compositional differences between cells of these two compartments (see below) and interactions between keratin intermediate filaments (KIF) and keratin-associated proteins (KAPs) at the molecular level. In broader, medullated fibres, orthocortical and paracortical cells are also present but are distributed randomly within the cortex (Orwin *et al.*, 1984). So it is reasonable to expect that manipulation of either the KIF or KAP content of wool cortical cells would cause changes in the macromolecular properties of wool fibres. Variation in fibre diameter, seen in fine and broad fibres, is a product of mitotic rates and KIF / KAP synthesis in the follicle and also impinges upon fibre properties. The present sheep transgenesis study has investigated the consequences of the separate introduction of either (i) additional KIF protein or (ii) additional KAP protein (cysteine-rich and glycine/tyrosine-rich KAPs) into the cortex of the wool fibre. In comparison to selective breeding, this approach has allowed single gene changes to be tested successively for their ability to enhance fibre properties. Such transgenesis has the advantage that genetic modification is not restricted to genes of the sheep genome, but may include specifically

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engineered KIF, KAP or KIF / KAP-related protein coding regions or coding regions not normally associated with wool.

Wool follicle gene expression

Previous studies of wool follicle structure, follicle development and the spatio-temporal patterns of synthesis of specific keratin intermediate filament (KIF) and keratin-associated proteins (KAP) in the sheep wool follicle have defined candidate genes most likely useful in wool fibre structure modification by transgenesis (Powell *et al.*, 1991; Powell and Rogers, 1994a, 1997). Merino wool fibres are comprised of a central cortical region encased within a single layer of cuticle cells. The orthocortical and paracortical cells differ in the proportion of glycine / tyrosine-rich and cysteine-rich KAPs they contain. In the paracortex, a greater proportion of the KAPs are cysteine-rich KAPs, hence more disulphide bonding occurs within this compartment. Overall, some 50 - 100 proteins are synthesised to form the fibre. Isolation and characterisation of genes encoding most of the structurally important KIF and KAP molecules expressed within the wool follicle cortex and cuticle cells has been followed by mapping of the patterns of expression of wool KIF and KAP genes during terminal differentiation in the follicle (Powell *et al.*, 1991; **Figure 1**). cRNA *in situ* hybridisation analysis of gene expression in the follicle cortex has shown that four type I and type II KIF gene pairs are expressed first, followed by the glycine / tyrosine-rich KAP genes (KAP6 family, KAPs 7 and 8, predominantly in the orthocortex) then the cysteine-rich KAP gene families (KAP1, 2 and 3 throughout the cortex; KAP4 and 12 in the paracortex). Similarly, KIF genes are those first expressed in the cuticle cells, beginning at the widest point of the follicle bulb (zone of Auber), followed by cysteine-rich KAP gene families (KAP5 and 10). Synthesis of all of these proteins in the follicle results in the production of fibre comprised of approximately 50% KIF and 50% KAP (proportions differ somewhat in the ortho- and paracortex, the latter with less KIF). Not surprisingly, each KIF gene promoter is more transcriptionally active than the individual promoters that drive expression of the multiplicity of KAP genes.

Design of transgenesis experiments

Transgenesis using genes active in the wool follicle has taken into account the complexity of endogenous gene expression in the follicle. A gene promoter able to function specifically in the follicle cortex (or cuticle) is central to fibre modification. For temporal specificity of transgene function within the follicle, the promoters of characterised KIF and KAP genes allow transgene expression to be timed early, mid or late in cortical differentiation. The results of our work suggest that the temporal specificity of transgene function within the follicle has a large bearing on the resultant fibre properties. An equally important consideration is the level of transgene expression which might be required to effect measurable changes to protein composition and fibre properties. In our experience, a wide range of expression levels (very low to very high) is possible using a KIF gene promoter, but that low efficiency of the KAP gene promoters limits them to applications where only

FIGURE 1. Keratin intermediate filament (KIF) and keratin-associated protein (KAP) gene expression patterns in the wool follicle cortex and cuticle (adapted from Powell *et al.*, 1991). Shown are diagrammatic representations of the distribution of KIF and KAP gene expression in both transverse (left) and longitudinal (right) aspects. The spatial distribution of gene expression throughout the orthocortex (O) and paracortex (P) in the transverse representation is is depicted in the longitudinal representation, which also demonstrates temporal distribution of the KIF and KAP gene expression. The position of the inner root sheath (IRS) and outer root sheath (ORS) which encase the developing fibre are shown.

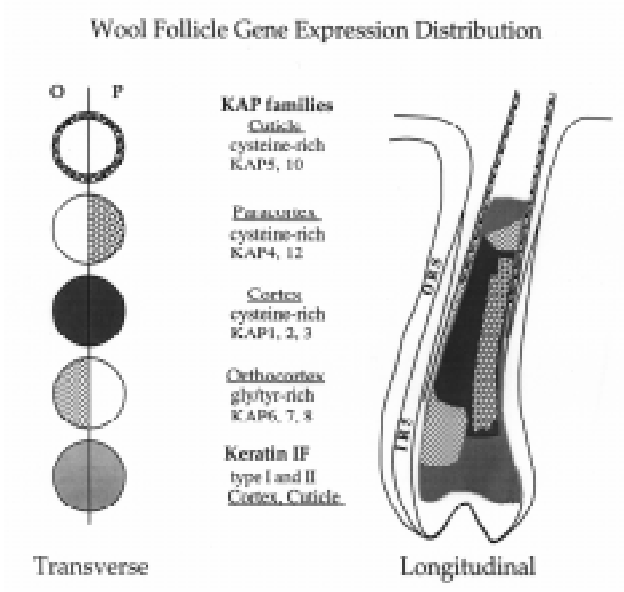
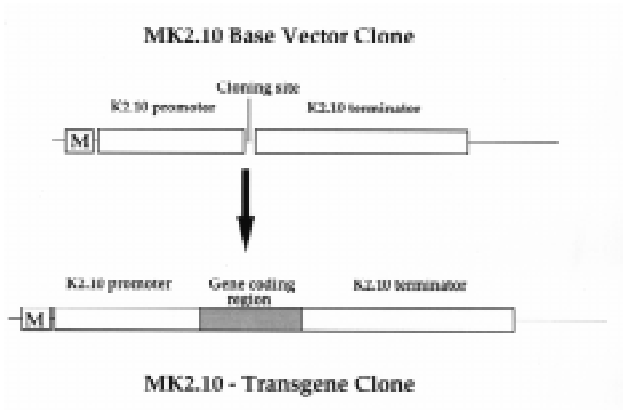


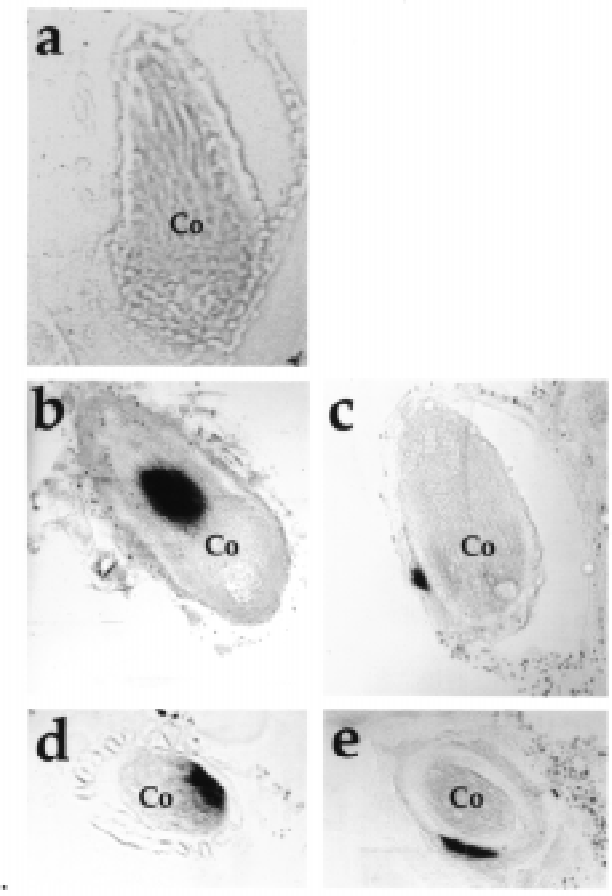
FIGURE 2. MK2.10 transgene base vector clone. The K2.10 promoter (proximal promoter and 5' flanking region; 2.8 kb), K2.10 terminator (polyadenylation and 3' flanking region; 5.2 kb) and M (matrix attachment region from the mouse kappa immunoglobulin gene; 0.9 kb) are shown. Not drawn to scale; see Bawden *et al.*, 1998).



minimal transgene expression is sought. It is possible that a hybrid KIF-KAP gene promoter could be used to achieve high-level, temporally late transgene expression. In any case, it is difficult to estimate the level of transgene product required to facilitate change, so founder transgenic sheep expressing transgenes over a broad range were routinely produced for analysis.

To facilitate transgene expression in the wool follicle cortex, we have used a transgene vector cassette which includes the promoter region of the wool cortical type II KIF gene, K2.10 and a cloning site able to accept DNA

FIGURE 3. β-galactosidase assay of sectioned wool follicles in Merino skin. 1 mm thick skin strips placed onto 1% agar (made with William's E cell culture medium) were biolistically transfected (from the side of the strips, through one of the cut surfaces) under vacuum (27 inches Hg) using the Bio-Rad Biolistic PDS-1000/He transfection apparatus with 2000 p.s.i. rupture caps and 1 mm diameter gold particles. After transfection, skin was incubated for 48 hours under William's E medium to allow follicle growth (previously proven possible for vacuum-treated individual follicles;data not shown) then the b-galactosidase (LacZ) assay performed on whole skin strips. Following this assay, an aqueous eosin counterstain was used to visualize follicles then frozen skin sections were prepared by cryostat sectioning. Slightly oblique sections through follicles show the result of transfection with gold particles either (a) uncoated or coated with (b, d) K2.10-LacZ or (c, e) K1.15-LacZ plasmid. Longitudinal sections (a,b,c); transverse sections (d,e). Gold particles used in the transfections are visible as small black dots within the sections and LacZ-staining as large dark areas in the cortex (b, d) and outer root sheath (c, e) of transfected follicles. In similarly transfected skin sections, control CMV-LacZ transfections gave LacZ activity in all follicle cell types (data not shown). Position of the cortex (Co) is indicated in each follicle section.



encoding a heterologous protein of choice **(Figure 2)**. When DNA encoding such proteins is inserted into the transgene vector, it is surrounded by the K2.10 regulatory sequences which restrict transgene expression to the follicle cortex and allow a wide range of transgene expression levels to be achieved. A functional matrix attachment region sequence (MAR; Cockerill and Garrard, 1986) was inserted upstream of the K2.10 5'-flanking sequence, so that in multi-copy transgenics, transgene functionality would be protected from inhibitory positional effects often mediated by chromatin surrounding transgene loci. Using the MK2.10 base vector, we have produced KIF and KAP transgene proteins early in cortical cell differentiation in the wool follicle, at the time when endogenous KIF genes are being expressed. Prior to commencement of the sheep transgenesis, the regulatory elements of the K2.10 gene used here had been proven to function in a follicle-specific manner in mice (Powell and Rogers, 1990; Keough *et al.*, 1995). More recently, in the absence of a follicle cell line and to reduce transgenesis costs, we have developed the use of biolistic transfection (Birch and Franks, 1991; Klein *et al.*, 1992; Sanford *et al.*, 1993) of transgene constructs into follicles in order to pretest transgene promoter function (**Figure 3**). One mm thick midside skin strips removed from Merino sheep were transfected with various promoter-LacZ reporter constructs; 1 mm diameter gold particles coated with supercoiled plasmids were used as the biolistic material. Using this approach, we have found expression of a β-galactosidase (LacZ) reporter to be tightly regulated by the follicle-specific promoters tested to date. As per characteristic K2.10 gene expression (Powell *et al.*, 1992), expression of the K2.10-LacZ construct was restricted to the cortex (**Figure 3 b, d**; see Dunn *et al.*, 1998) and K1.15-LacZ expression to the outer root sheath (**Figure 3c, e**; see Whitbread and Powell, 1998).

To predict the effect of transgenesis using specific KIF or KAP coding regions is difficult. However, considering what is known of protein composition in the orthocortex and paracortex and the physico-chemical properties of individual KIFs and KAPs, we have performed sheep transgenesis experiments aimed to (i) improve intrinsic fibre strength by increasing levels of the cysteine-rich KAP proteins which contribute to disulphide cross-linking of cortical proteins, or by increasing the level of isopeptide bonds between cortical proteins by introduction of an exogenous transglutaminase enzyme, and to (ii) raise elasticity of the fibres by converting the fibre cortex to a predominantly orthocortical nature, or by increasing the proportion of intermediate filaments in the cortex. Although conceived with minimal knowledge of the direct interactions between KIF and KAP proteins, these experiments formed a logical platform for the transgenesis programme. In each case, fibre structure modification was sought by mis-expression of the chosen coding region. Equally important would be testing of similar transgenes designed to either over-express or under-express the products but at their spatio-temporally correct location in the wool follicle.

Overexpression of cysteine-rich proteins in the cortex

Expression of the cuticle-specific KAP5.1 protein (36 mol % cysteine; MacKinnon *et al.*, 1990) and the paracortical-specific KAP4.2 protein (29 mol % cysteine; Powell and Rogers, 1997) throughout the cortex as transgene products was demonstrated by both cRNA *in situ* hybridisation analysis (data not shown; for examples, see Powell *et al.*, 1991, 1992) and 2-D gel electrophoresis (**Figure 4a,b** for K2.10-KAP5.1 wool fibres). Along with increased levels of KAP5.1, a decrease in the amount of the other KAP proteins is evident. Despite the presence of greater quantities of this cysteine-rich protein in the cortical KAP protein pool, intrinsic strength of the fibres produced by the G1 animals of transgenic lines was lower than that of non-transgenic G1 sibling control fibres. Extension of KAP5.1 fibres in water was equivalent to that seen in control fibres but occurred under considerably lower load (**Figure**

FIGURE 4. 2-D gel fractionation of S-carboxy-methylated wool proteins. Protein extracts from KAP5.1 G1 ewe sibs (a, b) and G0 KAP6.1 transgenic (d) and non-transgenic (c) ewes were S-carboxy-methylated using 14C-Iodoacetic acid (Powell and Rogers, 1994b). Approximately equal amounts of radioactively labelled products were fractionated and gels were electrophoresed in pairs, as shown. Arrows indicate the position of migration of the transgene products. Autoradiography was carried out at -80 0C for 24 (a,b) and 48 (c, d) hours respectively, using the Kodak BIOMAX signal intensification system.

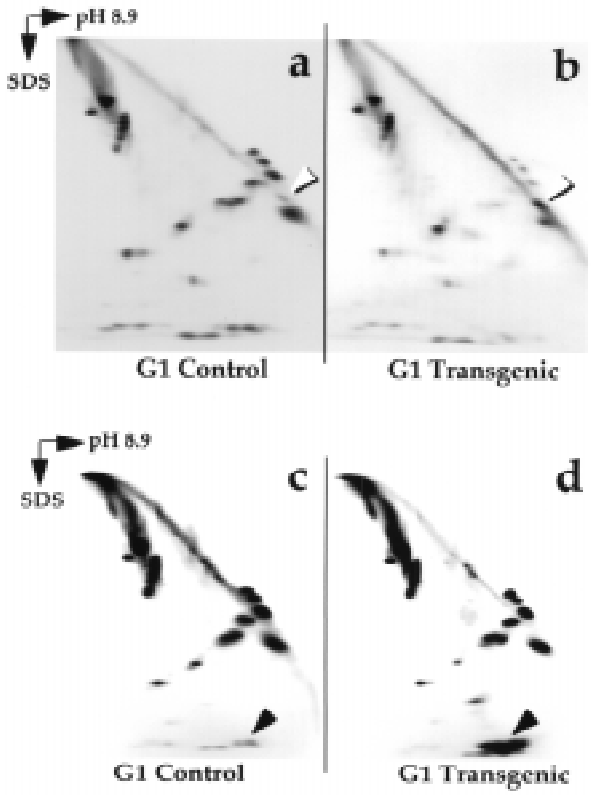
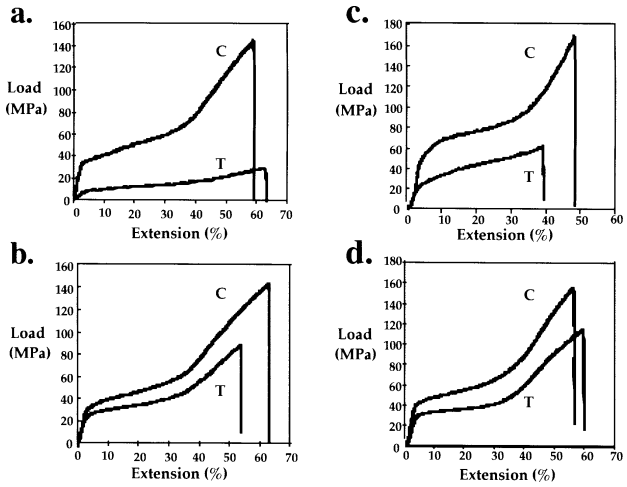


FIGURE 5. Load (MPa) / Extension (% gauge length) curves prepared for individual wool fibres from transgenic (T) and control (C) animals. For each midside wool sample, 30 single fibres were broken. One single fibre breakage curve, representing a typical result from each 30-fibre group is shown. Gauge length was 20 mm prior to extension and fibres were extended in water at 21 0C and at a rate of 5mm per minute in an Instron Tensile Tester (model 4501). Data was recorded and analysed using the Instron Series IX Automated Materials Testing System software, version 5.0. Midside fibres tested were from (a) KAP5.1, (b) KAP4.2, (c) KAP6.1 and (d) transglutaminase transgenic and control non-transgenic sheep. At the point of fibre breakage, the degree of fibre extension was significantly lower for KAP4.2 and KAP6.1 transgenics versus non-transgenic controls (for KAP4.2 fibres, mean extension = 55.3 % +/- 5.7 % for transgenic, 59.3 % +/- 5.6 % for control, $p < 0.01$; for KAP6.1 fibres, mean extension = 40.9 % +/- 5.5 % for transgenic, 49.6 % +/- 6.3 % for control, $p << 0.001$) but not significantly different for fibres from KAP5.1 or transglutaminase transgenic sheep compared with those from non-transgenic controls. The load bearing capacity of broken fibres from transgenic KAP5.1 (mean = 31.3 MPa +/- 9.6 MPa), KAP4.2 (mean = 115.3 MPa +/- 25.8 MPa), KAP6.1 (mean = 68.8 MPa +/- 13.8 MPa) and transglutaminase sheep (mean = 129.6 MPa +/- 15.6 MPa) are each significantly lower than fibres from non-transgenic controls (means ~ 150 MPa +/- 25 MPa; $p << 0.001$ in each case).



5a). For KAP4.2 fibres, both extension and load-bearing capacity were decreased (**Figure 5b**).

Increasing isopeptide crosslinks in the cortex

Expression of the human keratinocyte transglutaminase enzyme within the cortex increased isopeptide crosslinks; shown by reduced solubility of fibres under strongly denaturing conditions. Fibre extension was the same for control and transglutaminase-modified fibres but extension and breakage of the latter required only ~ 75 % of the load force required to achieve breakage of control fibres (**Figure 5d**).

Overexpression of a glycine/tyrosine-rich protein in the cortex: 2-D gel analysis of KAP6.1 G1 wool fibre extracts (**Figure 4c,d**) indicated the production of large quantities of the type II glycine/tyrosine-rich KAP6.1 protein (Fratini *et al.*, 1993) throughout the cortex of fibres (cf. a predominance of glycine/tyrosine-rich KAPs in the orthocortex in control fibres). When expressed throughout the cortex early in cortical cell differentiation, it appears that additional KAP6.1 protein reduced both the load-bearing capacity and extensibility of wool fibres (**Figure 5c**).

Increasing the proportion of intermediate filaments in the cortex

With the aim of increasing the elasticity of the wool fibre, we have attempted to increase the proportion of intermediate filaments within the cortex by the simultaneous introduction of type I and type II KIF transgenes. Expression of each is driven by the K2.10 promoter. Two different type I IF coding sequences, K1.1 and K1.2 (C. McLaughlan, unpublished and Wilson *et al.*, 1988) have been used to partner the K2.10 coding region. Preliminary electron microscopic examination of fibres from the G0 sheep shows no detectable alteration of the fibre ultrastructure. Tensile testing of the fibres is awaiting sufficient fibre growth and production of G1 animals. A parallel experiment is being conducted in mice. Currently, mouse lines are being created from founder transgenics and analysis of fibres at the EM level will follow.

Cortex transgenesis summary

In each transgenesis experiment described above, a significant change to the normal wool follicle gene expression profile was made using a promoter known to be very active in the follicle early in cortical cell differentiation. Addition of gene function through the introduction of transgenes was achieved and transgene products invariably

altered fibre protein composition and properties. In these experiments, neither the intrinsic strength nor elasticity of fibres was improved but the data presented here relate largely to analyses of transgenic sheep expressing large quantities of the respective transgene products. In those transgenic lines where differences in physical properties of the fibres were measurable, interference to endogenous KIF and KAP gene expression was also detected (Bawden *et al.*, 1998). Considering the dual effects in such high-expressor transgenic lines, transgenic sheep expressing low to moderate levels of the transgene products are more likely to provide wools with subtly altered properties. Lines from these founders have been produced but fibres are yet to be fully analysed.

With respect to the structural effects seen in high-expressors, one must consider the two main structural phases present within the cortex; the filament bundles and matrix of KAPs which surround and cement them in place. It is possible that synthesis of large quantities of KAP products at a time when they are not normally found within the developing structure, may disrupt formation of the filament phase. Hence, addition of larger quantities of specific matrix KAP components at the correct time would likely have different structural effects to those we have observed.

Whilst we have made no direct measure of wool production levels in the transgenic lines, results from RNA, amino acid and 2-D gel analyses have indicated that overall protein synthesis in the follicle is not increased but that a redistribution of amino acids occurs. This redistribution certainly favours synthesis of the transgene product. However, when the K2.10 filament gene promoter is linked to transgenes, wool fibre amino acid levels reflect an overall increase in the proportion of KIF proteins, probably due to depression of endogenous KAP synthesis.

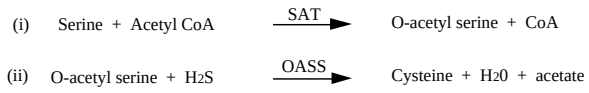
FIBRE GROWTH RATES

The plethora of regulatory factors and processes involved in the development of wool and hair follicles in the skin and the growth required to produce fibres (for reviews, see Stenn *et al.*, 1994; Nixon and Moore, 1998) seem to defy the transgenic route to modification of fibre growth. Key factors have been investigated with respect to their ability to induce follicle formation and follicle branching in skin (eg. see Wilson *et al.*, 1999) and experiments have been performed to supply growth factors directly to the follicle (ovine IGF-1; Damak *et al.*, 1996, Su *et al.*, 1998) and to the whole animal for general growth enhancement and improved wool growth (ovine GH; Nancarrow *et al.*, 1991; Ward, 1999). However, the results of these experiments, discussed in detail elsewhere, illustrate problems which may be encountered when seeking to modify complex growth control pathways. Other experiments have attempted to increase the supply of raw materials for fibre production (cysteine synthesis pathway) and nutrients for follicle growth (glyoxylate synthesis pathway). Below is a discussion of the cysteine and glyoxylate synthesis experiments and results to date.

Cysteine biosynthesis in the rumen epithelium

It is possible to improve wool growth rates through increasing the supply of cysteine available for protein synthesis and cell division in the wool follicle. Mammals can only synthesise cysteine indirectly from methionine via trans-sulphuration. Hence, expression of transgenes encoding microbial cysteine biosynthesis enzymes could provide an alternative pathway to cysteine synthesis in sheep. Metabolism and degradation of the sulphur-containing amino acids, cysteine and methionine, by microorganisms of the sheep rumen leads to loss of dietary sulphur. In turn, this limits the growth of wool which is composed of many cysteine-rich proteins (Gillespie, 1983). Infusion of methionine or cysteine either directly into the bloodstream or the abomasum of Merino sheep at a rate of 1.0 - 3.0 g per sheep per day leads to an increase in the rate of cell division in the follicle bulb (Hynd, 1989) and wool growth (Reis and Schinkel, 1963, 1964; Reis *et al.*, 1990). Infusion of methionine at a rate of 1.0 g per day has a similar effect upon mohair yield in Angora goats (Sahlu and Fernandez, 1992).

To overcome the apparent loss of sulphur from sheep, we and others (Rogers, 1990; Bawden, 1991; Ward and Nancarrow, 1991; Sivaprasad *et al.*, 1992; Ward *et al.*, 1994; Bawden *et al.*, 1995) aimed to express the microbial cysteine biosynthesis pathway in epithelial cells of the rumen, to capture the abundant sulphide produced by ruminal microorganisms, for conversion to cysteine. In bacteria and lower eukaryotes, the cysteine biosynthesis pathway has been well characterised (Kredich, 1987; Cherest *et al.*, 1993). The two-step pathway of conversion of serine to cysteine via the intermediate, O-acetyl serine (OAS) is catalysed by the enzymes (i) Serine acetyltransferase (SAT; encoded by the *cysE* gene) and (ii) O-acetyl serine sulphydrylase (OASS; OASS-A encoded by the cysK gene or OASS-B by the *cysM* gene) as shown below:



Using the cysteine biosynthesis genes of *Salmonella typhimurium*, *cysE*, *cysM* and *cysK*, and linked *cysEM*, *cysME* and *cysKE* genes, we first demonstrated a functional pathway in mammalian cell culture (Sivaprasad *et al.*, 1992). Initial experiments performed *in vitro*, involving coexpression of genes encoding the bacterial cysteine biosynthesis pathway (Bawden, 1991; Sivaprasad *et al.*, 1992) indicated that it is possible to coexpress linked *cysE* and *cysM* genes in mammalian cells to produce cysteine. Gene expression in cell culture produced up to 17.5 µmol cysteine per minute per gram of protein extract. This suggested that similar coexpression of the linked gene combinations in transgenic sheep could supply 1.5 g per sheep per day (17.2 µmol per minute), enough *de novo* synthesised cysteine to overcome the sulphur deficit.

The same linked gene constructs were then used as transgenes in mice and sheep, to demonstrate the feasibility of adding the new biosynthetic pathway to mammals (Bawden *et al.*, 1995). Linked transgenes were constructed with each gene driven by a separate promoter, either with the Rous sarcoma virus long terminal repeat (RSVLTR)

promoter or the mouse Phosphoglycerate kinase-1 (mPgk-1) promoter. The human Growth Hormone gene 3' non-coding / flanking region provided polyadenylation sequences. Transgenesis of mice with such constructs produced heritable expression of the transgenes. Results of a concurrent sheep transgenesis experiment using the RSVLTR-*cysEM* and -*cysME* linked transgenes revealed that the RSVLTR promoter was inadequate for expression in the rumen. Instability of transgenes containing the RSVLTR sequence was also observed, in mice and sheep. For mPgk-1-promoted genes, expression of mPgk-*cysME* and -*cysKE* linked transgenes in the stomach and small intestine of mice (**Figure 6**) suggested that this promoter would be a better candidate for expression in the analogous tissues of sheep. However, sheep transgenesis indicated that the mPgk-1 promoter, active ubiquitously and early in development, is also inappropriate for expression of the cysteine biosynthesis transgenes. In mouse experiments with the corresponding *Escherichia coli* cysteine biosynthesis genes driven by the sheep metallothionein Ia promoter, transgenesis with linked *cysE-cysK* genes was reported to rescue hair loss in mice placed on a sulphur-deficient diet (Ward *et al.*, 1994).

The transgenesis results presented here and other results obtained to date have shown that (i) cultured mammalian cells are able to endure constitutive cysteine synthesis mediated by an introduced pathway (Sivaprasad *et al.*, 1992), (ii) coexpression of the cysteine biosynthesis genes is possible in mice and sheep (iii) the bacterial *cysE, cysM* and *cysK* gene products are each stable and active *in vivo*, and that (iv) cysteine synthesis rates measured in cells and assayed in tissue extracts from transgenic mice are sufficient, relative to the cysteine required, for improved wool growth. In future, to avoid problems arising from synthesis of cysteine in remote locations, sheep transgenesis experiments in this laboratory will test *cysE - cysM* and *cysE - cysK* gene combinations constructed with sheep rumen epithelium-specific gene promoters which only function in the adult tissue.

A glyoxylate cycle in the wool follicle

Wool follicles have a high demand for glucose (Chapman and Ward, 1979). In this case, as for use of sulphur in the sheep, ruminant nutrition has a bearing upon the levels of glucose available to the follicle. During metabolism of carbohydrates in ruminal fermentation, the resident microbes produce a range of products, including volatile fatty acids, some of which can be used directly in gluconeogenesis. Depending upon the pasture, sheep may produce large quantities of acetate, a volatile fatty acid which cannot enter this pathway. As a consequence, ketonuria and sub-optimal wool growth may occur. It is possible that such excess acetate could be used for glucose production with an overall increase in available energy to the follicle (Ward and Nancarrow, 1991). Acetate usually enters the citric acid cycle and is converted to isocitrate (**Figure 7**). Normally, this is converted through a-ketoglutarate and succinyl CoA to succinate, itself a gluconeogenic substrate, with the loss of two carbon dioxide molecules. However, a pathway for conversion of isocitrate through glyoxylate to malate, with co-generation of succinate, known as the glyoxylate cycle, exists in plants and bacteria. The second step of the pathway, conversion of glyoxylate to malate, uses another molecule of acetate. In this reaction mechanism, citric acid cycling is maintained but an extra substrate molecule is made available for gluconeogenesis. If introduced to function in the wool follicle, the glyoxylate cycle may help ovecome the problems associated with acetone build-up and allow an increase in the rate of fibre synthesis.

FIGURE 6. Assay of cysteine synthesis activity in mouse tissue protein extracts (adapted from Sivaprasad *et al.*, 1992). Ascending thin layer chromatographic fractionation of the products of cysteine synthesis assays using protein extracts prepared from the tissues of an mPgk-cysE / mPgk-cysM transgenic mouse. 14C-L-serine was used as the substrate and hydrogen sulphide provided to the reactions via sodium sulphide. 4-vinyl-pyridine (4VP) was used to capture and stabilise cysteine for detection. The positions of the loading origin and of migration of reference compounds serine (ser), O-acetyl serine (OAS), N-acetyl serine (NAS) and cysteine-4VP (cys) are indicated on the left and the tissue extract assayed is given below the figure. A positive control extract assayed (+) was from a CHO cell line transfected with the same linked-gene construct (see Sivaprasad *et al.*, 1992 for details). Different to all other protein extracts, no cysteine synthesis activity was detected in heart extract. Autoradiography was for 3 days at room temperature.

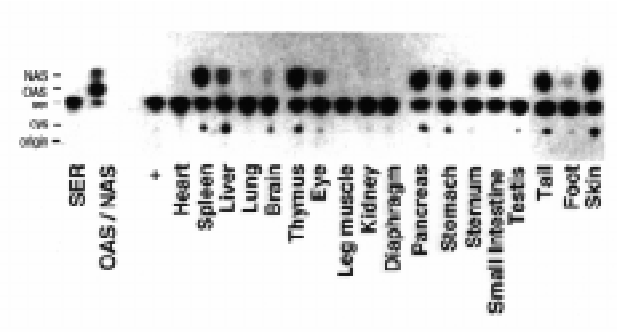
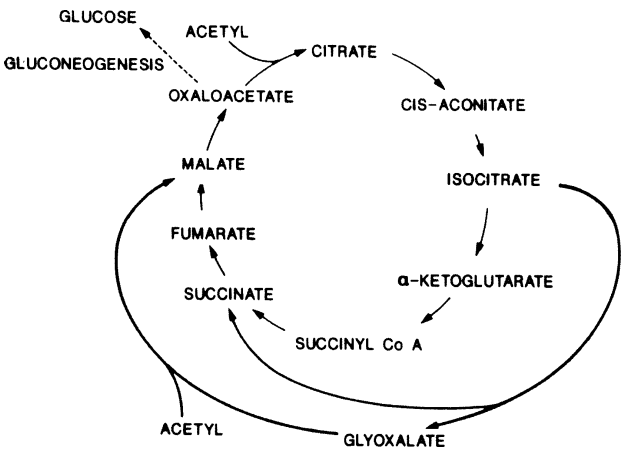


FIGURE 7. The citric acid cycle and glyoxylate pathway bypass from isocitrate through glyoxylate to malate (from Rogers, 1990). Enzymes which catalyse the conversion of isocitrate to glyoxylate (isocitrate lyase) then glyoxylate to malate (malate synthase) are encoded by the bacterial genes *aceA* and *aceB*, respectively.



Toward this goal, *E. coli* genes encoding the two required enzymes of the glyoxylate cycle, isocitrate lyase (*aceA*) and malate synthase (*aceB*), have been cloned and used as transgenes in mice (Saini *et al.*, 1996). With transgene expression driven by the sheep MT-Ia promoter, coexpression produced glyoxylate cycle activity in both the liver and intestine. The long term effect of this activity upon cellular biochemistry and physiology of the mice has not yet been reported. Testing of the effect of an added glyoxylate cycle directly on the wool follicle awaits linkage of these genes to a follicle-specific promoter.

SUMMARY

In order to effect phenotypic changes to existing breeds, the possibilities for alteration of the sheep genotype through transgenesis are broad. Included is genetic manipulation of known hormone-, growth factor- and enzyme-mediated metabolic pathways and also of the complement of structural proteins in terminally differentiating tissues. Though subject to normal physiological constraints, in sheep, genetic manipulation for different meat and wool present perfect opportunities for use of the transgenesis technology, in alteration of either quality or quantity. In relation to wool production, a future challenge lies in the manipulation of developmental regimes which determine parameters such as follicle size and follicle density in the skin, though one suspects much more is to be learned before transgenesis will find application there.

ACKNOWLEDGEMENTS

We wish to acknowledge the skill of Dr Paul Verma, Ms. Cathy Brand and Ms. Jen Kelly, of Turretfield Research Centre (South Australian Research and Development Institute) in microinjection to produce transgenic sheep in the KIF / KAP gene and cysteine biosynthesis gene work and dedication of Mr. John Evans, Ms Toni Heard and Dr. Dyane AuClair in tending the sheep flocks. We thank Dr. Peter Turner, Dr. Mickey Huson, Dr. Kerry Hansford, Mr. Gary Corino, Ms. Kerryn Saltmarsh and Ms. Nicole Phair, members of the CSIRO Textile and Fibre Technology Division (Belmont, Geelong, Victoria) for measurement of fibre properties and 2-D gel analysis of fibre proteins and Dr. Kevin Ward, CSIRO Animal Production Division (Prospect, NSW) for providing updates on the transgenesis conducted in that laboratory. We also acknowledge the funding support of the Australian Wool Research and Promotion Organisation and the CRC for Premium Quality Wool.

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