Targeting Gene Expression to the Wool Follicle in Transgenic Sheep

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To establish the feasibility of overexpressing foreign genes in the wool follicle, transgenic sheep were produced by pronuclear microinjection of a DNA construct consisting of a mouse ultrahigh-sulfur keratin promoter linked to the bacterial chloramphenicol acetyl transferase (CAT) gene. Four of 31 lambs born were transgenic. The overall efficiency of transgenesis was 1.1% of zygotes injected and transferred. Two transgenic rams were mated to nontransgenic ewes, and both transmitted the gene to their offspring in Mendelian fashion. CAT expression was found in the skin of one G0 ram and in 9 out of 26 transgenic G1 progeny. Two G1 lambs were sacrificed to study tissue specificity. Both had high levels of expression in skin but One had high expression in spleen and kidney with lower levels of expression in lung; the other had low expression in spleen, lung, and muscle. *In situ* hybridization demonstrated that transgene expression in the skin was confined to the keratogenous zone of the wool follicle cortex. Expression of CAT activity in skin was correlated with diet-induced or seasonal changes in the rate of wool growth. This keratin promoter appears useful for overexpressing factors in the wool follicle that might influence wool production or properties.

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Since the first demonstration of the production of a transgenic sheep[1], the ability to manipulate the sheep genome has been amply documented[2-4]. The greatest success has been achieved in targeting transgene expression to the gland[5,6] notably in the production of factor IX and α_1-antitrypsin[8] in milk. Targeting of other genes, such as a viral envelope gene[9] or immunoglobulin genes[10], has given more variable results. Despite considerable effort to replicate the effects of growth hormone overexpression in transgenic mice[11] by introducing growth hormone genes into transgenic sheep[12-14], the resulting phenotype has been generally detrimental[15,16].

Manipulation of the germ line to improve a production trait in sheep remains an elusive goal. One approach to enhance wool production has been to transfer bacterial genes encoding enzymes for the biosynthesis of cysteine[3], in the expectation that availability of dietary cysteine may be rate-limiting for wool growth. While expression of these enzymes prevents alopecia in transgenic mice placed on a diet deficient in sulfur-containing amino-acids[17], to date only one transgenic sheep has been reported[18] that failed to give consistent expression.

With the goal of affecting wool production or fiber characteristics, we elected to target transgene expression directly to the wool follicle. In order to develop the technology for producing transgenic sheep and to demonstrate ability to obtain expression in the wool follicle, we started with a follicle-specific promoter linked to a marker gene. The chosen construct was a bacterial chloramphenicol acetyl transferase (CAT) cDNA driven by an ultra-high-sulfur keratin promoter that had been used to target the hair follicle in transgenic mice[19]. We report the successful targeting of the marker gene to the wool follicle in transgenic sheep with stable, heritable expression that reflects the pattern of fiber growth.

Results

Transgenic animals. A DNA construct made of a mouse ultra-high-sulfur keratin gene promoter ligated to the CAT

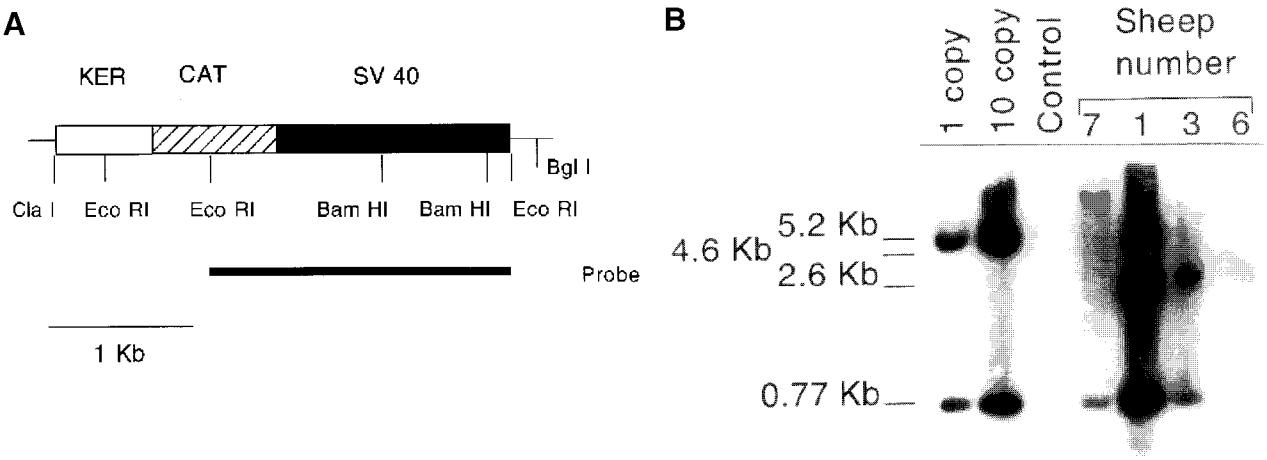

FIGURE 1. (A) The KERCAT construct used to generate transgenic sheep. (B) Southern blot analysis of BamHI digested transgenic sheep DNA probed with a CAT-SV40 probe. The probe detected a 0.77 Kb band corresponding to the BamHI internal fragment as well as bands corresponding to tandem array integration and integration fragments. One copy and 10 copy—standard are pKERCAT mixed with non-transgenic sheep DNA in a ratio equivalent to 1 copy and 10 copies of pKERCAT per haploid genome, respectively. Control is non-transgenic sheep DNA.

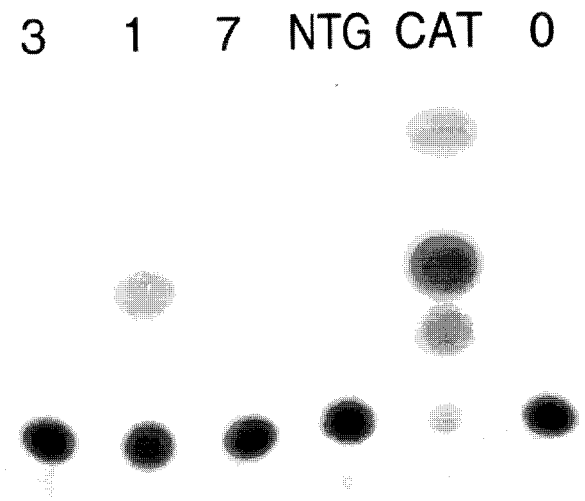

FIGURE 2. Detection of CAT activity in the skin of transgenic sheep by CAT assay. The numbers above the lanes represent the sheep number. NTG is non-transgenic sheep skin protein extract. CAT is purified chloramphenicol acetyl transferase. 0 is buffer alone.

coding sequence (Fig. 1A) was microinjected into one-cell sheep zygotes.

From 371 injected zygotes transferred to 76 recipient ewes, 31 lambs were born. Screening by Southern blotting revealed that four lambs, two male and two female, were transgenic. Transgene copy number varied from less than one per cell, in Line six, to more than 20 in Line one (Fig. 1B). The blot from one ram (Line one), subsequently selected for mating, displayed a band migrating between the 0.77-Kb internal fragment and the 2.6-Kb tandem array fragment obtained with BamHI digestion. We interpret this as indicating a single site of integration, resulting from 0.22 Kb of 3′ CAT sequence plus genomic DNA. The screening was confirmed by digestion with EcoRI (not shown). This ram was mated to 42 random-bred nontransgenic ewes, resulting in 80 lambs born of which 36 (45%) were transgenic. Mating the second ram (Line three) to 29 ewes produced 57 lambs of which 23 (40%) were transgenic.

Gene expression. CAT assay, on skin biopsies taken at 1 month of age from the four founder animals (Fig. 2), showed expression of enzyme activity in one ram (Line one) and no expression in the second ram (Line three) or either of the two ewes. From the G1 progeny of both rams, 26 Line one transgenic lambs were tested at three month intervals and nine showed CAT activity in the skin (see below). None of 17 transgenic progeny tested in Line three demonstrated expression of the transgene.

TABLE 1. Transgene expression in different tissues.

Lamb No.	118	57
Skin	9.0	12.4
Liver	0.3	0.2
Spleen	3.4	18.3
Kidney	0.1	25.8
Thymus	0.2	0.5
Hoof	0.6	0.3
Heart	0.1	0.5
Lung	2.8	2.5
Uterus	0.1	N.D.
Ovary	0.1	N.D.
Muscle	2.5	0.2
Intestine	0.1	0.9
Pancreas	N.D.	0.2
Blank	0.2	0.1

Expressed as percent conversion of substrate. N.D.: not done.

Tissue specificity. Two G1 progeny from Line one were sacrificed and CAT assays were carried out on a variety of tissues. Strong expression was found in the skin but activity was also detected in spleen, lung, and kidney in one of the two animals; and in spleen, lung, and muscle of the other (Table 1).

The cell type in which skin expression occurred was investigated by *in situ* hybridization, using a ^{35}S-labeled antisense riboprobe on frozen skin sections. Figure 3 shows that CAT expression was localized to the keratogenous zone of the cortex in the wool follicle.

Regulation of gene expression. Since the ultrahigh-sulfur keratin gene from which the KER promoter is derived is expressed only during active hair growth[19,20], we investigated whether CAT activity under the control of the mouse KER promoter would be correlated with wool growth in transgenic sheep.

Wool growth was assessed by weighing the wool clipped from a 10×10-cm midside patch. At each sampling, a skin biopsy was taken for CAT assay. When the rate of wool growth was manipulated by altering the plane of nutrition for ram Number one, a linear relationship was found between CAT expression and wool growth (Fig. 4A). Sampling was carried out at 3 month intervals in the nine expressing G1 progeny of this ram. Comparison of winter and spring growth (Fig. 4B) shows that expression of the transgene paralleled the expected seasonal changes in wool growth.

Discussion

The KER promoter was derived from a mouse ultrahigh-sulfur keratin gene that is expressed during anagen (growing phase) in the anterior-posterior temporal pattern characteristic of mouse hair growth[20]. Our data indicate that this mouse promoter functions in a similar way in the sheep, driving expression of a heterologous coding sequence in concert with the pattern of wool growth.

The KER-CAT construct had been previously used to generate transgenic mice, in which one out of three independent lines expressed CAT activity in skin[19]. This result is consistent with our finding of expression in 25% of founder transgenic sheep. Lack of expression, despite integration of the transgene, is a common occurrence that can be attributed to several factors including positional effects[21]. In the mouse line, CAT activity was developmentally regulated, occurring in anagen but not telogen (resting phase) of the hair cycle[20]. Likewise, our results show that the activity of the promoter correlates with variations in wool growth, produced either by dietary manipulation or seasonal changes. The highly synchronized pattern characteristic of hair growth in mice is not found in sheep where most follicles are in anagen[22]. Thus CAT activity can be detected in expressing sheep, regardless of the time of the year when the biopsy is taken.

Studies of tissue specificity for KER-CAT in transgenic mice showed expression was mainly in the skin[20,23] however weak expression was also found in the small intestine, which was attributed to bacterial contamination[23]. No data were given for the proportion of offspring expressing the transgene. In transgenic sheep, CAT activity was expressed in the skin of 9 out of 26 animals examined. Skin expression was strong in two G1 lambs in which 13 different tissues were assayed (about 70 times higher than background). Expression also occurred in lung, spleen and kidney in one animal and in muscle in the other. Overall, skin expression was found in 35% of G1 lambs. Tissue specificity can thus vary between animals and not all offspring of the same line express the transgene in the target tissue. We have no expla-

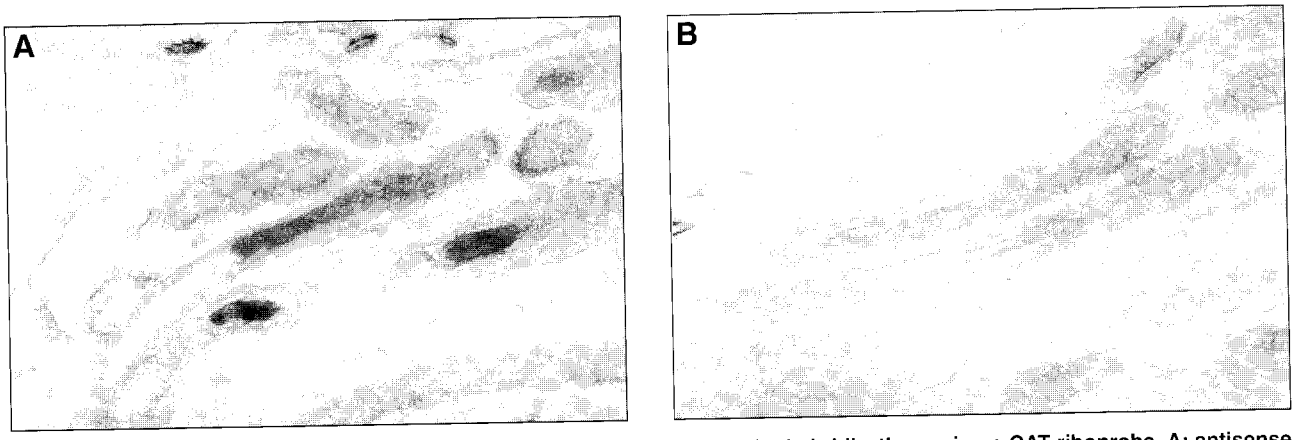

FIGURE 3. Detection of CAT mRNA in the skin of transgenic sheep by in situ hybridization, using a CAT riboprobe. A: antisense probe. B: sense probe. Sections were viewed by bright field illumination.

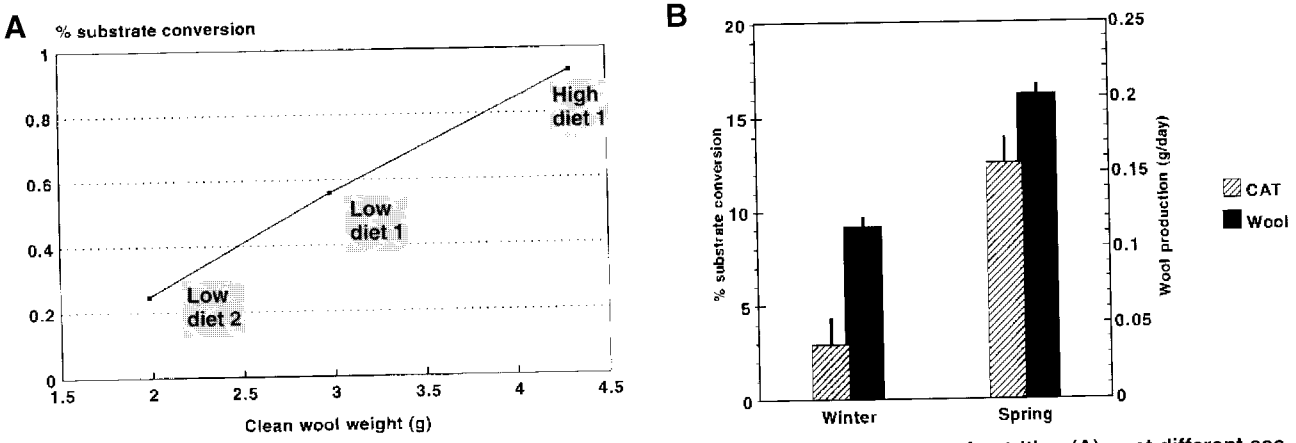

FIGURE 4. Relationship between CAT expression and rate of wool growth on different planes of nutrition (A) or at different seasons (B; mean±s.d., n=9).

nation for the incomplete penetrance or the variation in tissue specificity between animals within the same transgenic line. Southern blotting of DNA from G1 lambs did not reveal any rearrangements of the transgene between animals (not shown). The pattern of expression was not sex-related (5 females and 4 males from Line one expressed the transgene) nor was it related to twinning (in three sets of twins only one of each set showed expression). It is important to demonstrate the penetrance of expression among transgenic progeny and to examine more than one animal in order to assess the tissue specificity of expression. Few authors have commented on these aspects, but similar inconsistencies have been seen previously[24-27].

Our results show a high rate of success in generating transgenic sheep and demonstrate the use of a mouse keratin promoter to target expression of a heterologous gene to the wool follicle. The promoter activity corresponded to its behavior in the mouse. Expression of the transgene occurred in the expected follicle cells and was correlated with their metabolic activity in terms of wool growth. The mouse promoter functions normally in the skin of a different species and appears useful for overexpressing factors in the wool follicle that might influence wool production or properties.

Experimental Protocol

Superovulation program. Donor animals were selected at random from a flock of 400 mixed-age Coopworth ewes maintained outdoors on rye grass/white clover pasture. Six ewes underwent synchronization of ovulation every week. Synchronization was achieved by insertion of an intravaginal device (CIDR: controlled internal drug release device, type-G, New Zealand Dairy Board) containing 300 mg progesteron. On Day -12 the CIDR was removed and replaced with another on Day -3. The second CIDR was removed on Day0 (i.e., 12 days of CIDR treatment). Superovulation involved twice-daily i.m. injection of follicle stimulating hormone (FSHp, Schering Corp.): 5 mg and 4 mg on Day-2, 4 mg and 3 mg on Day1, and 3 mg and 2 mg on Day0, followed by a single i.m. injection of 80 μg gonadotropin-releasing hormone (GnRH, Fertagyl, Intervet) 28 h after removal of the second CIDR. Ewes remained on pasture during this period, but were mustered into yards for each step in the procedure. From Day0, a vasectomised ram with a crayon harness was run with the ewes. Ewes that were marked by the vasectomised ram were inseminated 48 h after CIDR removal with 250 μl of fresh semen placed in the lumen of the uterus by a laparoscopic technique. Surgical recovery of the zygotes was performed 64 to 67 h after CIDR removal. Ewes were anesthetised with i.v. sodium pentobarbitone and the uterine horns and oviducts exteriorized via a mid-ventral laparotomy using sterile technique. A polythene tube (5 cm×1.8 mm o.d.) was inserted into the oviduct via the fimbria and the oviduct was flushed with 10 ml Dulbecco's phosphate-buffered saline solution containing 5% (w/v) BSA (Sigma) from a syringe with a blunt 20G needle inserted into the uterus near the utero-tubal junction. Flushing was performed on both oviducts and the numbers of corpora lutea and eggs were recorded for each ewe.

Microinjection of DNA into one-cell sheep embryos. Plasmid pKERCAT[20] contains the promoter region of the ultra-high-sulfur keratin gene UHSK-704-Eco, the chloramphenicol acetyl transferase (CAT) coding sequence, and the SV40 poly A region (Fig. 1A). pKERCAT was prepared essentially as described[28], except that two rounds of cesium chloride purification were performed. The recombinant fragment was removed from the plasmid pKERCAT by restriction digestion with ClaI and BglI then purified from an agarose gel by the glass milk binding method using a GeneClean kit (Bio 101, La Jolla, California), according to the manufacturer's instructions. The purified DNA was microinjected at a concen-

tration of 4 ng/μl into the pronucleus of one-cell sheep embryos as described[29]. The microinjected embryos were transferred into the oviduct of recipient ewes which were synchronized with the donors by inserting a CIDR and removing it on the same day as for the donors. During the non-breeding season, recipients were also given a single i.m. injection of 500 IU pregnant mare serum gonadotropin (Sigma) on Day-2 at the same time as the first FSHp injection of the donors.

Screening of transgenic sheep. Lambs born from microinjected embryos (G0) were tested for integration of the transgene by Southern blotting analysis of DNA extracted from an ear biopsy taken shortly after birth[28]. The probe used was a 2.1-Kb EcoRI fragment of pKERCAT containing part of the CAT gene and the SV40 region (Fig. 1A). Progeny of the founder animals (G1) were screened with the polymerase chain reaction using primers 5′-CAGTTGCTCAATGTACCTAT-3′ and 5′-ATGATGAACCTGAATCGCCA-3′ which bind to the CAT gene.

CAT assays. A 0.5-cm^2 skin biopsy was taken from the back of transgenic sheep midway between the head and the tail. The tissue was homogenized in 0.25 M Tris pH 7.8, submitted to 3 cycles of freezing and thawing and centrifuged at 13,000g for 15 min. To neutralize possible inhibitory factors in the extract, the supernatant was incubated at 65°C for 15 minutes[30] and used in a CAT assay[31].

***In situ* hybridization.** *In situ* hybridization was performed essentially as described[32]. Briefly, skin biopsies were fixed for 6 hours in 4% (v/v) paraformaldehyde, 0.25% (v/v) glutaraldehyde in phosphate-buffered saline, pH 7.0, and 10-μm frozen sections were made. RNA probes for hybridization were prepared from a 240-bp EcoRI fragment corresponding to the 5′ end of the CAT gene subcloned into pBluescript. Transcription was carried out with T7 (antisense probe) or T3 (sense probe) RNA polymerase in the presence of ^{35}S-UTP (400 Ci/mmole), according to the manufacturer's instructions (Boehringer-Mannheim). The sections were incubated briefly in 0.1% (v/v) triethanolamine then in 0.25% (v/v) acetic anhydride, 0.1 M triethanolamine for 10 min. at room temperature, washed briefly in 2 X SSC (1 X SSC is 0.15 M NaCl, 0.015 M sodium citrate), and dehydrated by passing through 30%, 50%, 70%, 85%, 99% (v/v) ethanol solutions. Hybridization to antisense or sense probes was carried out in a buffer containing 50% (v/v) formamide, 0.3 M NaCl, 10 mM EDTA, 10 mM Tris, pH 8.0, 1 X Denhart's solution[33], 500 μg yeast tRNA/ml, 10% (w/v) dextran sulfate, and 100 mM dithiothreitol (DTT) for 48 hours at 42°C.

After hybridization the slides were treated with RNase A (20 μg/ml) in RNase buffer (0.5 M NaCl, 10 mM Tris, pH 8.0, 0.1 mM EDTA), then washed with RNase buffer for 30 min. at 37°C; 2 X SSC for 30 min. at room temperature; and 0.1 X SSC, 10 mM DTT for 60 min. at 45°C. The slides were then dried, coated with NTB2 photographic emulsion (Kodak, Rochester, NY), exposed for 7 days, developed and stained with haematoxylin-eosin.

Manipulation of wool growth. In the first experiment wool growth was manipulated by altering the diet. Ram number one was fed a high-caloric diet (1200 g nuts and 400 g hay) for 4 weeks then a maintenance diet (475 g nuts and 100 g hay) for 2 consecutive 4-week periods. Two 10×10-cm mid-side skin patches were defined prior to the first 4-week interval and the wool was clipped, cleaned and weighed at the end of each 4-week period. Two skin biopsies were taken in the middle of each interval and used to perform a CAT assay as described above.

In the second experiment, mid-side skin patches were defined on nine transgenic offspring of ram number one in April and the wool was clipped, cleaned and weighed at 3-month intervals, when skin biopsies for CAT assay were also taken. Data are presented for July (winter growth) and October (spring growth). The animals were kept on pasture during the trial.

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