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Hair-specific expression of chloramphenicol acetyltransferase in transgenic mice under the control of an ultra-high-sulfur keratin promoter

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ABSTRACT **We have generated a transgenic mouse line by microinjection of a chimeric DNA fragment (KER-CAT) containing a hair-specific, murine ultra-high-sulfur keratin promoter (KER) fused to the coding region of the bacterial chloramphenicol acetyltransferase (CAT) gene. A 671-base pair (bp) stretch of the 5′ promoter region was used to direct the expression of the CAT gene in this construct. Of the tissues tested for CAT activity in these transgenic animals only skin with growing hair, isolated hair follicles, and microdissected vibrissae showed substantial levels of activity. These are the same tissues where the endogenous ultra-high-sulfur keratin gene is expressed as shown by *in situ* hybridization. Furthermore, analysis of the CAT activity during the developmental stages of the hair growth cycle shows that the chimeric gene is expressed during the anagen phase of the hair growth cycle; this is the expected time during development for its expression. From these results we conclude that 671 bp of the promoter sequence from the ultra-high-sulfur keratin gene is sufficient to direct the correct development-specific and tissue-specific expression of the reporter gene construct in transgenic mice. The appropriate expression of the KER-CAT construct in transgenic mice is an important step in understanding the regulation of this gene during hair organogenesis.**

Differentiation of epithelial cells in murine hair follicles is a tightly regulated cyclic process (1). Cycles of hair growth are controlled by intimate interactions between the mesenchymal dermal papilla and the epithelial components of the hair follicle (1–3). Hair follicles dramatically change their histological appearance, metabolic activity, and synthetic patterns during their cyclic growth (4). These changes are best documented in the epithelial components of the hair. As the epithelial cells move distally in the hair follicle, they differentiate to form the diverse tissues of the hair. During this process the cells express in a tightly regulated manner specific proteins, including hair-specific cytokeratins (5, 6), trichohyalin (7), and high-sulfur (8, 9) and ultra-high-sulfur keratins (10). Immunofluorescence (5, 11, 12) and *in situ* hybridization (13) evidence shows that expression of specific cytokeratins and matrix proteins is localized to the keratogenous zone of the hair follicle. These hair-specific proteins provide a set of markers for differentiation stages of epithelial cells during hair development. Thus analysis of the regulation of one of these genes promises an efficient way to begin to elucidate molecular mechanisms that control hair growth.

We have previously reported the isolation and characterization of a hair-specific gene coding for an ultra-high-sulfur keratin protein (10). Ultra-high-sulfur keratins belong to a family of highly insoluble proteins that have a high cysteine content; these proteins do not have any structural similarities with cytokeratins (8–10). The ultra-high-sulfur keratin gene is expressed during the hair cycle in the same pattern as hair growth proceeds along the body of the mouse (10). To use the expression of this specific gene as an indicator of hair differentiation, we have generated a transgenic mouse line containing regulatory elements from the ultra-high-sulfur keratin gene controlling the expression of the bacterial enzyme chloramphenicol acetyltransferase (CAT) (14). Transgenic mice carrying one or several copies of a recombinant plasmid have provided useful and powerful tools for the study of gene regulation in a number of developmental systems. Examples include the human K14 cytokeratin gene (15), the rat pancreatic elastase gene (16, 17), the chicken A-crystallin gene (18), the mouse vimentin gene (19), and rearranged light and heavy chain immunoglobulin genes (20, 21). For the human K14 cytokeratin gene it has also been shown that the promoter controls tissue-specific and differentiation-specific expression in skin of transgenic animals (15) and in transfected epithelial cells (22). In this paper, we report that our chimeric ultra-high-sulfur keratin–chloramphenicol acetyltransferase (KER-CAT) construct expresses CAT activity in the appropriate epithelial tissues and at the correct time during the hair growth cycle.

MATERIALS AND METHODS

Subcloning and Characterization of DNA Constructs. The recombinant DNA work was done following standard texts (23, 24) and published procedures (25–27) using the manufacturers' recommendations for all enzymatic reactions. Except where noted enzymes were from New England Biolabs. pKER-CAT was constructed in two steps (Fig. 1). First, we isolated the CAT-SV40 DNA fragment from pSV0CAT DNA (ref. 14; a gift from B. Howard, National Cancer Institute) by complete digestion with the restriction endonuclease *Hin*dIII and partial digestion with *Eco*RI. Then, we ligated these partially digested DNA fragments into the *Hin*dIII/*Eco*RI site of the pBluescript plus cloning vector (Stratagene) and isolated plasmids that contained the internal *Eco*RI site of the CAT-SV40 DNA fragment by hybridization (26) with radioactively labeled oligonucleotide GV-92 (5′-GTCTTTCATTGCCATACGGAATTCCGGATGAGCATTCATCAG). Positive clones were sequenced (29, 30) by using oligonucleotides GV-1 (5′-GTAAAACGACGGCCAGT), GV-2 (5′-AACAGCTATGACCATG), and GV-99 (5′-TTTCTCCATTTTAGCTTC) to select the vector, pBlue-CAT.

Second, the promoter region from the ultra-high-sulfur keratin gene was isolated (25) from phage gUHSK-701 DNA (10) after digestion of the DNA with the restriction endonuclease *Fok* I. The DNA fragment was ligated into the *Hin*dIII

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Abbreviation: CAT, chloramphenicol acetyltransferase.

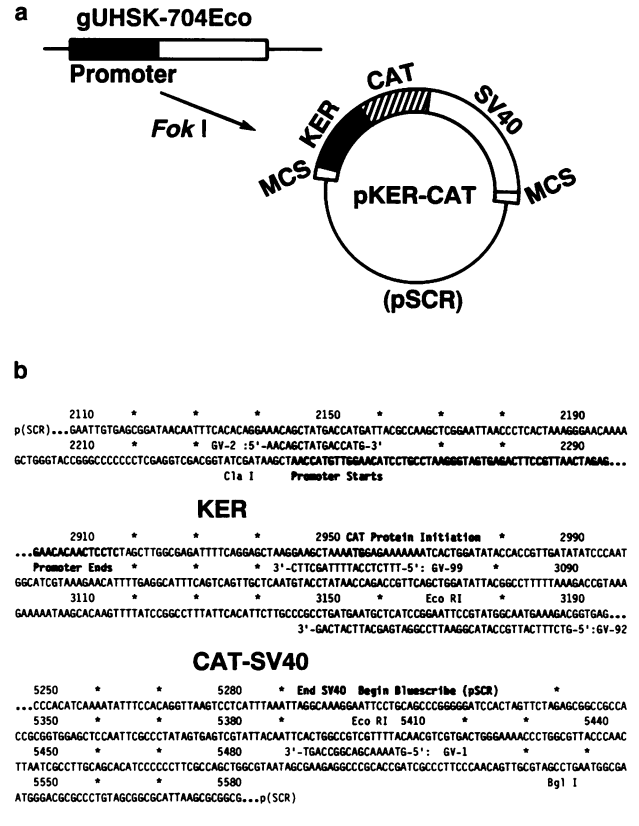

FIG. 1. (*a*) Diagram of the plasmid pKER-CAT used for the generation of transgenic mice. CAT-SV40 sequences were excised from pSV0CAT by digestion with *Hin*dIII and partial digestion with *Eco*RI and then subcloned into the multiple cloning site (MCS) of pSCR (Bluescript plus cloning vector). The promoter region (KER) of the ultra-high-sulfur keratin gene was isolated from gUHSK-701 by digestion with *Fok* I and then inserted ahead of the reporter gene sequences (CAT-SV40). (*b*) DNA sequences at the junctions between the promoter (KER), the CAT and SV-40 sequences, and the rest of the pSCR vector. The *Cla* I and *Bgl* I restriction sites used to excise the transgene KER-CAT are shown. Oligonucleotides GV-2, GV-99, GV-92, and GV-1 were used to characterize pKER-CAT. The remainder of the DNA sequences have been published: Bluescript plus vector (Promega; ref. 28), the ultra-high-sulfur keratin gene promoter (10), and the CAT-SV40 fragment (14).

site of pBlue-CAT after treatment of both the DNA fragment and the vector with the Klenow fragment of *Escherichia coli* DNA polymerase. Transformants that hybridized to an oligonucleotide from the promoter region (AMC 102: 5′-AACATCCTGCCTAAGGGTAGTGAGTCTTCCGT-TAACTAGAGT; Fig. 1*b*) were further analyzed by sequencing with oligonucleotides GV-2 and GV-99 to ensure the correct orientation of the promoter compared to the coding sequence of the CAT-SV40 DNA fragment. Because all the DNA sequences have been published, we show in Fig. 1*b* only the sequences at the junctions of the different fragments of the recombinant plasmid pKER-CAT.

Transgenic Animals. Details of standard procedures to generate transgenic mice have been published (refs. 31–34, and references cited therein). In brief, the 3.3-kilobase *Cla* I–*Bgl* I restriction fragment from plasmid pKER-CAT (Fig. 1*b*, nucleotides 2237–5540) containing the ultra-high-sulfur keratin promoter (KER) ahead of the CAT-SV40 (CAT) DNA fragment was isolated from an agarose gel (35) and was microinjected into the pronuclei of fertilized mouse eggs (200–500 copies of DNA per egg) from B6SJL/F1J mice (The Jackson Laboratory). Then, 311 injected eggs were transplanted into the oviducts of 15 pseudopregnant mice. DNA was isolated from surgically removed tails of 49 offspring and was probed using the labeled (27) CAT insert (*Cla* I–*Bgl* I restriction fragment) of plasmid pBlue-CAT to determine whether the mice were transgenic. Three mice transmitted the recombinant gene in a Mendelian fashion but the pups from one line (28–4) only expressed substantial amounts of CAT; only offspring from this line were analyzed further.

Preparation of Tissue Samples for Analysis of CAT Activity. Hair follicles from 3-day-old mice were isolated from skin by homogenization (three times, 10 sec each) in phosphate-buffered saline (GIBCO). Large debris was removed from the homogenate with a stainless steel filter and the follicles were cleaned by centrifugation (three times, 5 min each at 400 rpm; Sorvall RT6000 table top centrifuge; ref. 36 and U. Lichti, personal communication). Shaved skin and internal organs were isolated from 4-day-old mice. A sample of 50 hairs in anagen was plucked from 10-day-old mice with forceps and determined by light microscopy to contain no exogenous tissue. Twenty vibrissa were isolated by microdissection from 3-day-old mice and were separated by dissection into the outer connective tissue sheath and the vibrissae shafts. The plucked hair shafts and the dissected vibrissa follicles were suspended in 50 μl of lysate buffer (0.25 M Tris·HCl, pH 7.8/10 mM EDTA) and assayed for CAT activity. Protein was not determined for follicle samples. The other tissue samples were cut into small pieces and suspended in 0.25 M Tris·HCl, pH 7.8/10 mM EDTA at 1 mg of tissue per ml and sonicated with a vibra cell (Sonics and Materials, Danbury, CT). All samples were lysed by repeated freeze-thaw cycles. Samples containing 150 μg of protein from each of these internal organ lysates were assayed for CAT activity as described previously (37). Briefly, samples were incubated with [^{14}C]chloramphenicol (Amersham; 57 mCi/mmol; 1 Ci = 37 GBq), the products of the enzymatic reaction were separated using thin layer chromatography, and the plates were air dried and exposed to x-ray film (Kodak).

***In Situ* Hybridization.** The procedure used for *in situ* hybridization was that described by Fuchs (ref. 3, personal communication). Vibrissa follicles from 3-day-old CF1 mice (Charles River Breeding Laboratories) were isolated by microdissection. Tissues were fixed in 4% paraformaldehyde for 4 hr at 4°C and 5-μm paraffin sections were hybridized with the [^{35}S]cRNA probe. After washing, the sections were coated with NTB2 emulsion (Kodak), exposed at 4°C and then developed according to the manufacturers' recommendations.

The cRNA hybridization probe was transcribed using SP6 RNA polymerase (Promega) and uridine 5′-[α-[^{35}S]thio]triphosphate (Amersham; 650 mCi/mmol) from a plasmid containing the 2-kilobase *Eco*RI fragment of the ultra-high-sulfur keratin gene (10) in the pGEM-4Z vector (Promega). To ensure that the cRNA transcript contained unique sequences from the 3′ untranslated region, the plasmid was linearized with the restriction endonuclease *Stu* I. This DNA template gave a radioactive 1.3-kilobase cRNA terminating at nucleotide 430 of the 3′ sequence of the ultra-high-sulfur keratin gene (10). A control hybridization probe containing a λ phage sequence was transcribed from the control plasmid (Promega).

RESULTS

Generation and Characterization of Transgenic Mice. Three pups born to founder mouse 28-4 showed substantial CAT activity in skin lysates (Fig. 2), indicating that the founder mouse was heterozygous for the transgene. This founder mouse was used to establish the transgenic line. Southern analysis of DNA from CAT-positive mice from this founder showed that the DNA sequences of the *Cla* I–*Bgl* I fragment had been integrated into the mouse genome without apparent rearrangement (data not shown).

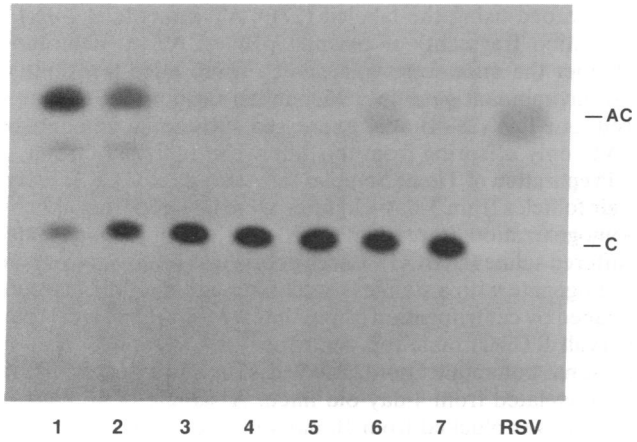

FIG. 2. CAT activity in the skin of one litter derived from founder mouse 28-4. An autoradiogram of the thin-layer chromatographic analysis of skin samples from offspring 1, 2, and 6 shows high CAT activity as conversion of chloramphenicol (C) to the acetylated form (AC). A lysate from bacteria containing plasmid pRSVCAT was used as a control (lane RSV).

Tissue-Specific Expression of KER-CAT and *in Situ* Hybridization. To determine whether the KER-CAT construct is expressed in the correct tissue, CAT activity in whole skin samples, in purified hair follicles, and in internal organs were compared within the same animal (Fig. 3). In this 6-day-old mouse the only detectible CAT activity was in the skin and the partially purified hair follicles. No CAT activity could be detected in any other tissue. These data demonstrate the correct tissue localization of CAT expression. Attempts to demonstrate CAT enzyme or CAT mRNA within histological sections of hair tissues were not successful. Neither an antibody against CAT (3'prime-5'prime) nor northern analysis showed any positive signals for the CAT enzyme (data not shown). In comparison, our *in situ* hybridization data using a probe specific for the 3' end of the endogenous ultra-high-sulfur keratin gene showed that the expression of the endogenous ultra-high-sulfur keratin gene is in the anagen vibrissae of normal CF1 mice (Fig. 4). The radiolabeled cRNA localizes specifically to the keratogenous zone of the cuticle and the cortex region of the hair shaft (Fig. 4). No hybridization was detected in either the inner or the outer root sheath or in the mesenchymal cells in the adjacent skin tissue. Furthermore, the ultra-high-sulfur keratin probe did not hybridize to murine spleen sections. The control λ cRNA probe did not hybridize with vibrissae sections (data not shown).

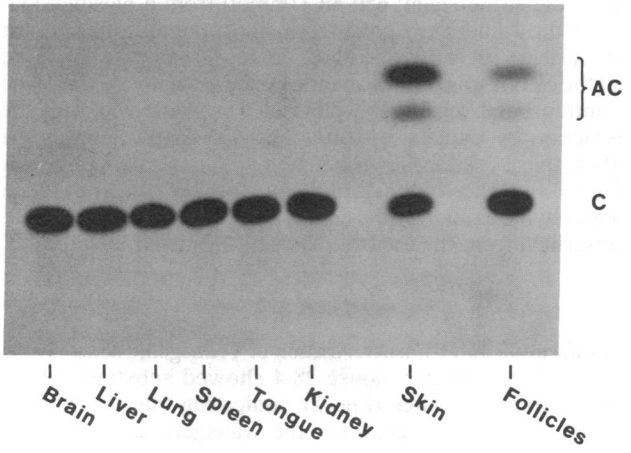

FIG. 3. CAT expression in different tissues from a mouse in the first anagen phase of the murine hair cycle (6-day-old mouse). An autoradiogram of the CAT assay is shown. C, chloramphenicol; AC, acetylated chloramphenicol.

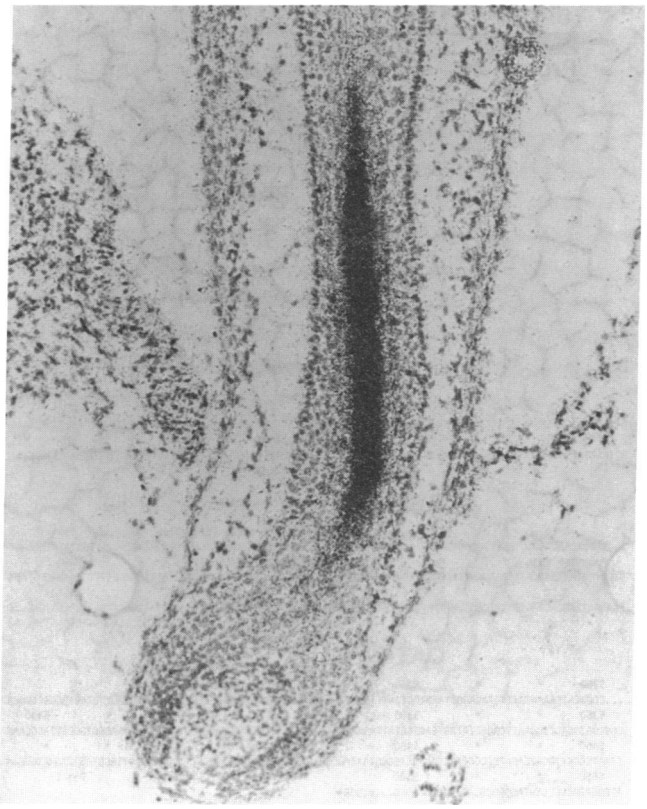

FIG. 4. *In situ* hybridization to vibrissa from a 3-day-old mouse. A ^{35}S-labeled cRNA probe corresponding to the negative strand of the 3' end of the ultra-high-sulfur keratin gene hybridizes to ultra-high-sulfur keratin mRNA in the cortex and the cuticle of the hair shaft. (×155.)

To corroborate that the CAT activity is expressed from the transgene in the same compartment of the integument as the endogenous ultra-high-sulfur keratin protein, we analyzed CAT activity in skin, plucked hair follicles, and microdissected vibrissae (Fig. 5). CAT activity was detected in the skin sample and plucked hair in the growth phase of the hair

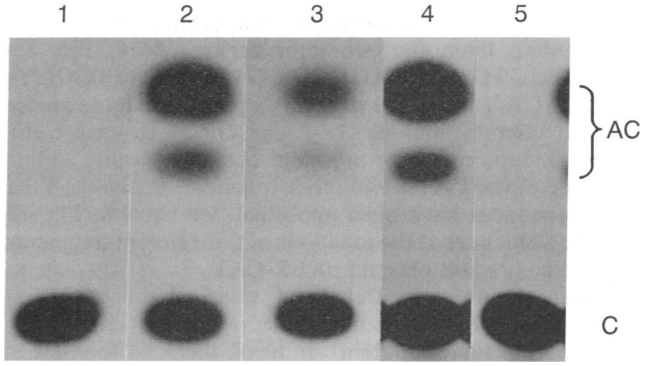

FIG. 5. CAT expression is localized to the hair follicle during the growth phase of the hair cycle. An autoradiogram of CAT assays of lysates from different tissues is shown. Lanes: 1, skin lysate from the telogen phase (resting phase) of the hair cycle; 2, skin lysate from the anagen phase (active growth); 3, lysate from plucked hairs in the anagen phase of hair growth; 4, lysate from the epithelial portion of 20 dissected anagen vibrissae; 5, lysate from mesenchymal portion of the dissected vibrissae. C, chloramphenicol; AC, acetylated chloramphenicol.

cycle (anagen, lanes 2 and 3) but not in skin samples from the resting phase (telogen, lane 1) even after prolonged exposure of the film. This result was further supported by demonstrating that the CAT activity in microdissected vibrissae was restricted to the epithelial cells of the vibrissae follicle (lane 4) and that no CAT activity was detected in the surrounding mesenchymal cells (lane 5). Thus, the CAT activity is localized exclusively to the hair follicle. This localization of the enzymatic activity is consistent with our *in situ* hybridization data for the endogenous ultra-high-sulfur keratin gene (Fig. 4).

Hair Cycle-Specific Expression of the KER-CAT Transgene. To determine whether our transgene is regulated in a developmentally specific manner, transgenic mice were assayed through two consecutive hair cycles (Fig. 6). CAT activity was found in lysates of plucked hairs isolated from the first anagen (lane 1) and from skin during the second anagen (lane 3). No CAT activity was found in telogen skin (lane 2). This pattern of CAT expression demonstrates the correct timing for the promoter activity of the KER-CAT transgene during the murine hair cycle.

DISCUSSION

Only one founder animal (28-4) expressed CAT at high levels and was thus analyzed further. As we did not map the precise integration sites of the transgenes, we do not know whether all the 5–10 copies of the transgene that integrated into the haploid genome are active or whether the CAT activity is due to only one functional copy of the transgene. Furthermore, we cannot rule out the possibility that flanking sequences near the integration site influence the expression of the transgene in a hair-specific manner. Nevertheless, for the transgenic mouse line 28-4 we can conclude that some of the integrations occurred without any deleterious rearrangements, thus allowing expression of the CAT gene in a manner similar to that of the endogenous gene. We have followed the CAT activity in this transgenic mouse line through six generations and we do not find substantial variations in either the level or the pattern of CAT expression (data not shown). The active copies of the transgene do not appear to be subject to gross alterations during consecutive breedings.

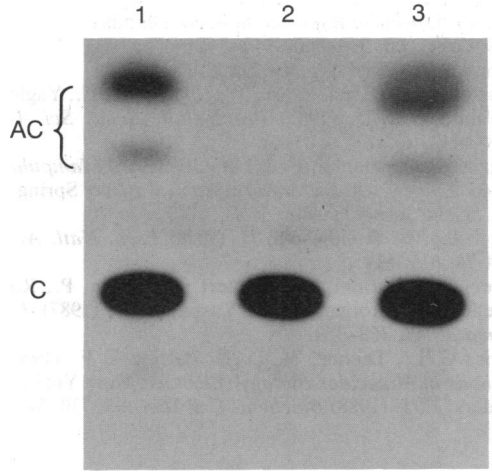

FIG. 6. Developmental expression of KER-CAT in transgenic mice. An autoradiogram of CAT assays of lysates isolated from tissues during the first and second hair cycles of one transgenic mouse is shown. Lanes: 1, lysate prepared from hair plucked during the first hair cycle (anagen phase, 6-day-old mouse); 2, lysate from skin removed surgically from the same mouse at day 22 (telogen phase); 3, lysate from skin removed surgically from the same mouse at day 38 (anagen phase).

The advantages of CAT as a reporter gene are that it provides a highly sensitive enzyme assay and that it is relatively easy to assay. However, in our hands it has proven difficult to localize histologically the CAT expression to a specific cell type. This problem has been overcome by Vassar *et al.* (15), who have used an immunological marker to find the transgenic product in epithelial cells. Using a cytokeratin promoter to express a human cytokeratin in transgenic mice they showed that the recombinant cytokeratin is stably integrated into the cytoskeleton and that immunologically it can be easily detected. Using antibodies against the CAT enzyme, we have not been able to localize it histologically to specific cells of the hair shaft. Since we were unable to detect CAT mRNA in hair follicles by northern analysis, we did not attempt to use *in situ* hybridization with the CAT cRNA probes. Thus, we have relied on two different experiments to ascertain that CAT is expressed in the same tissue in which ultra-high-sulfur keratin is expressed.

In the first set of experiments we determined the exact tissue localization of the endogenous mRNA for ultra-high-sulfur keratin by *in situ* hybridization using a highly specific probe. To avoid cross-hybridization with other members of the ultra-high-sulfur keratin multigene family, we used a cRNA probe for the divergent 3′ end of the specific gene that contains mostly untranslated sequences. Spleen sections were negative when hybridized with this 3′ ultra-high-sulfur keratin-specific probe, demonstrating tissue specificity of the probe. Since the ultra-high-sulfur keratins have a completely different structure compared to the cytokeratins we did not expect cross-hybridization with mRNAs encoding cytokeratins. Heterologous cRNA transcripts made from a λ DNA fragment did not hybridize to vibrissae, also demonstrating that there was no unspecific binding of labeled probes. It is clear from our results that mRNA for the ultra-high-sulfur keratin gene is restricted to the keratogenous zone of the hair shaft, since no mRNA was detected in cells outside of this zone. Thus, the *in situ* hybridization results demonstrate that the pattern of hybridization obtained with the 3′ probe is specific for ultra-high-sulfur keratin expression. This localization of the endogenous ultra-high-sulfur keratin to the hair shaft is in agreement with the synthesis of other hair-specific proteins in the same region (5, 11–13).

Our second set of experiments show that CAT expression is localized to hair, since there was no CAT expression in other tissues of transgenic mice (Fig. 3). Plucked hair yields relatively clean preparations and we found that CAT activity is high in this material (Fig. 5, lane 3, and Fig. 6, lane 1). By microdissecting the vibrissae follicle, we could show that the CAT activity is found exclusively in the hair shaft and that no activity could be found in the outer hair shaft, the adjacent mesenchymal portions, or the connective tissue of the vibrissae. Since we did not find CAT activity in skin samples containing hair in the resting phase of the hair cycle (Fig. 5, lane 1, and Fig. 6, lane 2), we assume that the CAT enzyme becomes inactivated when the cells terminally differentiate. This inactivation may result from crosslinking with the endogenous ultra-high-sulfur proteins that are expressed concomitantly with the CAT enzyme or by proteolytic activities within the terminally differentiated cell. Together these data demonstrate that the transgene is expressed in the hair shaft of both pelage hair and vibrissae and thus that it is likely that the CAT enzyme is expressed in the same cells that show mRNA accumulation for the endogenous ultra-high-sulfur keratin gene.

We conclude that the main regulatory elements of the ultra-high-sulfur keratin gene are contained within 671 base pairs (bp) of the 5′ untranslated region. It is this 671 bp of the 5′ region of the ultra-high-sulfur keratin gene that is sufficient to program CAT expression in the same tissue at the same time as the endogenous ultra-high-sulfur keratin gene (10).

Since the ultra-high-sulfur keratin genes do not contain intervening sequences and our KER-CAT construct does not have the 3′ untranslated region, our results indicate that these regions do not play an essential role in control of the expression of the transgene. In a skin epithelial cell-specific transgenic construct, Vassar *et al.* (15) have shown that 2500 bp of 5′ upstream sequences and 700 bp of 3′ downstream sequences of the authentic human K14 gene are sufficient to express the K14 cDNA. In addition Jiang *et al.* (22) have shown by deletion experiments and transfections into cell lines that a 300-bp element isolated from a pseudogene of human K14 is sufficient for epithelial cell-specific expression of CAT. The strict control of CAT expression by the ultra-high-sulfur keratin promoter of 671 bp may reflect an evolutionary selection mechanism that established tight control on the ultra-high-sulfur-gene product. This gene product is expressed only in terminally differentiated epithelial cells of the hair follicle (10).

Finally, the ability of the ultra-high-sulfur keratin regulatory region to target the expression of our reporter gene to the correct tissue with appropriate timing provides a potentially useful gene expression system to study the effects of other gene products on the development of the hair follicle. Perturbation of either cell growth or differentiation within the hair follicle will give insight into specific cell–cell interactions during the formation of hair.

CONCLUSION

Collectively our results show that the KER-CAT construct and the endogenous ultra-high-sulfur keratin gene respond in similar ways to the mesenchymal/epithelial inductive events that trigger the hair cycle. Thus, the expression of the CAT enzyme in epithelial cells can be used as a marker for hair organogenesis. As the hair cycle has commonality with the mesenchymal/epidermal interactions shared by a number of developmental systems in vertebrate organogenesis (38), this transgenic mouse line should provide a tool to analyze these processes further.

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