Expression of Calcyclin, a Calcium-Binding Protein, in the Keratogenous Region of Growing Hair Follicles

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Changes in calcium levels control the differentiation of skin epithelial cells and thus may also affect the epithelial cells of the hair follicle. We have isolated a murine cDNA clone, pCAL-F559, for the calcium-binding protein calcyclin by differential screening of a cDNA library made from RNA isolated from hair follicles of 6-d-old mice. The identity of our cDNA clone was established by comparing the DNA sequence with the sequence of the human calcyclin gene. That the authentic calcyclin mRNA encoded by pCAL-559 is present in skin of 3-d-old mice was confirmed by S1 nuclease protection assays. As measured by RNA dot blots, calcyclin mRNA levels in the skin change in accordance with the hair cycle and reaches a peak a few days prior to the mRNA for structural hair proteins. Although we can demonstrate by in situ hybridization that mRNA for calcyclin is localized in the post-mitotic keratogenous region of the hair follicle we can only assume that this calcium binding protein is involved in the control of differentiation of these cells by regulating their Ca^{++} levels. *J Invest Dermatol 96:383–387, 1991*

The regulation of calcium levels in a cell has a decisive role in the response of the cell to many stimuli [1,2]. Most of these stimuli integrate the cell into the metabolic and biosynthetic needs of the organism. The intracellular calcium levels are controlled by several mechanisms including internal storage compartments, transport proteins, and active pumps [3,4]. Thus, the calcium fluctuations are usually limited in time and in extent. How these changes affect gene expression in detail is not known. It has been shown, however, that in epithelial cells, calcium levels regulate differentiation [1,2]. Because the differentiation of hair from epithelial cells is a very complex procedure, we started to analyze calcium-binding proteins in relation to the hair cycle in the hope of gaining insights into the regulation of hair differentiation.

Several cellular calcium-binding proteins have been reported [3,4]. Calmodulin is found in all eukaryotic cells and it is the main receptor for Ca^{++} [5]. The level of calmodulin has been shown to vary during the cell cycle, indicating that it may play an important regulatory role in the cell cycle [5,6]. It has been shown that a putative calcium-binding protein, calcyclin, is also regulated during the cell cycle [7]. Calcyclin shows elevated mRNA levels 16 h after serum stimulation in the late G_1 phase, then decreases 8 h later when cells enter the S phase. Protein synthesis is not required for the initiation of the calcyclin gene expression, because steady-state levels of mRNA increase even in the presence of cycloheximide, a known inhibitor of protein synthesis [8]. The initiation of calcyclin synthesis therefore is an immediate early response of the cell to an external or internal stimulus. Besides serum, other factors known to increase mRNA levels of calcyclin include platelet-derived growth factor and epidermal growth factor [9].

Calcyclin initially isolated as a cell-cycle-dependent cDNA clone [7] has been cloned and sequenced from humans [9,10]. The sequence shows strong similarity to two putative calcium-binding domains of the beta-subunit of the S-100 protein [11], which is a member of the calcium-binding proteins. In addition, calcyclin shows, to a lesser extent, similarities to a subunit of a protein complex [12,13] that is a substrate for tyrosine kinase [14].

The regulation of hair growth involves a cascade of events that is only partially understood [15,16]. In the murine hair cycle, there is an extended growth phase (anagen) that each time lasts for 7–10 d followed by a resting period (telogen) of different length. During anagen, a substantial cell proliferation takes place. The cells immediately differentiate to the cells of the various tissues of the hair follicle. Some of the structural genes that encode hair-specific proteins are highly expressed during anagen and are absent during telogen [17]. To isolate genes controlling the induction of these structural hair proteins in early anagen, we differentially screened cDNA libraries generated from hair tissue. In this paper, we report the isolation of a murine cDNA clone for calcyclin by differential screening and describe its accumulation during hair growth.

MATERIALS AND METHODS

Isolation of cDNA Clones For most of the molecular biology techniques, we followed standard procedures [18,19]; all enzymes were from New England Biolabs except where otherwise noted and were used following the manufacturers' instructions. In short, a cDNA library was constructed from poly(A+) RNA isolated [20] from purified [21] hair follicles of 6-d-old mice (strain TUC CF1) in the Honjo vector (Pharmacia) following the manufacturers protocol. The cDNA library was plated and screened [22] using as hybridization probes ^{32}P-dCTP (3000 Ci/mmol) -labeled single-stranded cDNA probes. The RNA used to make the cDNA probes was isolated from either the hair follicle of 3-d-old mice (TUC CF1), the skin of hairless (Hr/Hr strain SKH-1) mice or from mouse liver (TUC CF1). To label cDNA, we denatured 3 μg ran-

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Abbreviations:
bp: base pair
EDTA: ethylenediaminetetraactic acid
nt: nucleotide
SDS: sodium dodecyl sulfate
Tris: tris(hydroxymethyl)aminomethane

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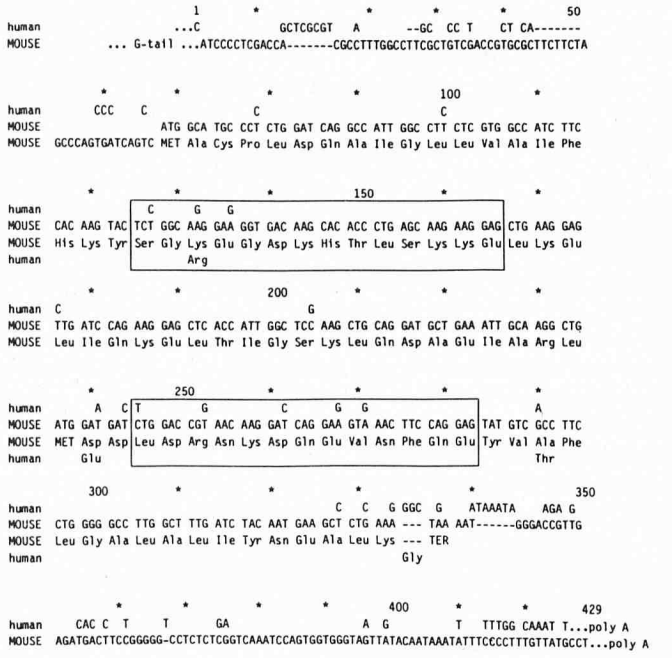

Figure 1. Nucleotide sequence of murine calcyclin. The nucleotides start numbering with nucleotide 1 of the human sequence. Only the differences between the murine and human sequences are shown. The calcium binding domains homologous to the S100 protein [9,11] are boxed.

dom oligonucleotides (P-L #2166) and 3 μg of poly A + RNA with 10 mM methyl mercury in 10 μl for 10 min at 20°C [23]. The mixture was adjusted to 105 mM 2-mercapto ethanol, 0.3 M KCl, 5 mM Tris, pH 7.5, 0.1 mM EDTA and annealed for 15 min at 31°C. Added to the mixture, final concentration, were 1 mM dATP, 1 mM dGTP, 1 mM dTTP, 1 mM Mg Acetate, 23 mM KCl, 20 mM Tris, pH 8.3 in a final volume of 100 μl. The labeling was started by the addition of 40 U RNasin, 80 U avian reverse transcriptase (Seikagaku, St. Petersburg, FL), and 150 μCi ^{32}P-dCTP (3000 Ci/mMole, Amersham). After 30 min at 37°C, 0.5 mM dCTP was added for 20 min at 37°C followed by 45 min at 42°C. An additional 20 U of reverse transcriptase was added and the incubation continued for 15 min at 50°C. The unincorporated nucleotides were removed by chromatography on NICK column (17-0855-02, Pharmacia). The cDNA library on NEN plaque screen filters was hybridized at 42°C consecutively with the three different probes (1,000,000 cpm/ml of hybridization solution) and washed under stringent conditions in 0.1 × SSC (150 mM NaCl, 15 mM trisodium citrate), 1% SDS at 51°C according to published procedure [24]. Between the different hybridizations, the old probe was removed by heating the filters for 30 min at 95°C in 0.1 × SSC, 1% SDS, 50 mM Tris-HCl, pH 7.5, and 50 mM pyrophosphate. Only clones that were positive with 3-d "cDNA" and negative with hairless "cDNA" and liver "cDNA" were further analyzed by sequencing on both strands using the dideoxy method for double stranded DNA [25] with T7 polymerase (Pharmacia).

RNA Dot Blot Longitudinal skin sections were excised from the skin of mice ranging in age from 1 to 32 d and total RNA was isolated by the RNAzol method according to manufacturers' (Cinna/Biotecx, Friendswood, TX) recommendations [20]. Ten μg of total RNA was denatured for 1 h at 50°C in 50% formamide and 6% formaldehyde, chilled on ice, spotted onto Genescreen plus (New England Nuclear), treated at 80°C for 2 h in the vacuum oven, and then hybridized as above with the 32P-dCTP (3000 Ci/mMole) random primed labeled [26] insert isolated from the calcyclin plasmid (pCAL-F559, see Fig 1). The probe was removed from the filters as above and rehybridized with an oligonucleotide for beta-actin LW299 (5′-TTTGATGTCACGCACGATTTCCCTCTCAGCTGTGGTGGTGAA)[27] and with an oligonucleotide for 18 S rRNA GV71 (5′-TGGTCACCATGGTAGGCACGGCGACTACCATCGAAAGTTGAT) [28].

In Situ Hybridization The in situ hybridization was performed according to published procedures [16,29]. In short, the skin tissue from 8-d-old mice were fixed in 4% paraformaldehyde-PBS for 4 h at 4°C and embedded in paraffin. A DNA fragment containing coding sequences for calcyclin from nucleotide (nt) 53 to nt 362 (Fig 1) was amplified by PCR [30] using 20 ng of pCAL-F559 DNA, Taq polymerase, and 50 pmol of primers LW295 (5′-GACGAATTCGCCCAGTGATCAGTCAT) and LW296 (5′-GACGGTACCGGAAGTCATCTCAACGG) containing an Eco RI restriction site and Kpn I restriction site, respectively. The amplified DNA fragment was subcloned into the Eco RI/Kpn I site of the Bluescript II vector (Stratagene) and 1 μg of the resulting plasmid (pCAL-F599p) was linearized with Sma I for synthesis of antisense cRNA using T7 polymerase (Stratagene), or linearized with Rsa I for synthesis of sense cRNA using T3 polymerase (Stratagene) [16]. Five-micron tissue sections on pretreated slides [31] were hybridized with 50,000 cpm 35S-UTP–labeled cRNA probes. The hybridized and washed slides were dipped into Kodak NTB-2 emulsion, exposed at 4°C for 21 d, and developed following Kodak's recommendations.

S1 Nuclease Protection Assay The S1 nuclease protection assay was performed according to the published procedure [32]. The plasmid pCAL-F559 (Fig 1) was digested with the endonuclease restriction enzymes PstI and Bam HI following the manufacturer's recommendations and a 280-bp fragment from the 5′ end of the cDNA (Fig 1) was isolated from the gel and end-labeled with T4 Kinase (Boehringer) using gamma-ATP (ICN). One hundred thousand cpm of this end-labeled fragment and 30 μg of total RNA isolated [20] from the skin of 3-d-old mice were resuspended in 40 μl of 75% formamide, 0.1% SDS, 20 mM Tris, pH 7.4, 0.4 M NaCl, and 1 mM EDTA. As a control we used 10 μg of tRNA. The samples were denatured at 75°C for 15 min and hybridized at 55°C overnight. Then a 350-μl solution containing 2 μg salmon sperm DNA, 0 or 20 U S_1 nuclease (Boehringer), 333 mM NaCl, 3.7 mM $ZnSO_4$, and 67 mM sodium acetate, ph 4.5, was added. The samples were digested for 1 h at 37°C, treated with phenol/chloroform, precipitated with ethanol, and electrophoresed on a 6% polyacrylamide–8 M urea gel in Tris-borate buffer [19].

RESULTS

Isolation and Characterization of a cDNA Clone for Calcyclin A murine cDNA library made from RNA isolated from 6-d-old hair follicles was differentially screened to find clones for genes involved in the early stages of hair growth. To isolate such cDNA clones we selected clones that were positive with labeled cDNA probes from 3-d-old hair follicles and that were negative with a labeled cDNA probe made from RNA isolated from the skin of Hr/Hr hairless mice and also negative with a probe derived from mouse liver RNA. A series of such clones were further analyzed and characterized. One of the clones isolated in this manner was pCAL-F559 (Fig 1), a murine equivalent of the previously published human calcyclin gene [9,10]. The murine calcyclin sequence is compared in Fig 1 with the human sequence. The murine cDNA clone is 428 nt long and ends with the poly A tail. The open reading frame encodes 88 amino acids with a 95% similarity to the amino acid sequence from human [9]. However, the mouse clone has one less amino acid than the human at the very 3′ end. The two areas that are very similar to the calcium binding domains of the S-100 protein are boxed in Fig 1. The mRNA CAP site has been determined for the human [10], making the 5′ untranslated region of the human gene one nucleotide longer than our clone.

S1 Nuclease Protection Assay and RNA Dot Blot Analysis To verify that mRNA for calcyclin is actually present in murine skin, we used the S1 nuclease protection assay [32]. Figure 2 shows that a DNA fragment with a length of 213 bp was protected from

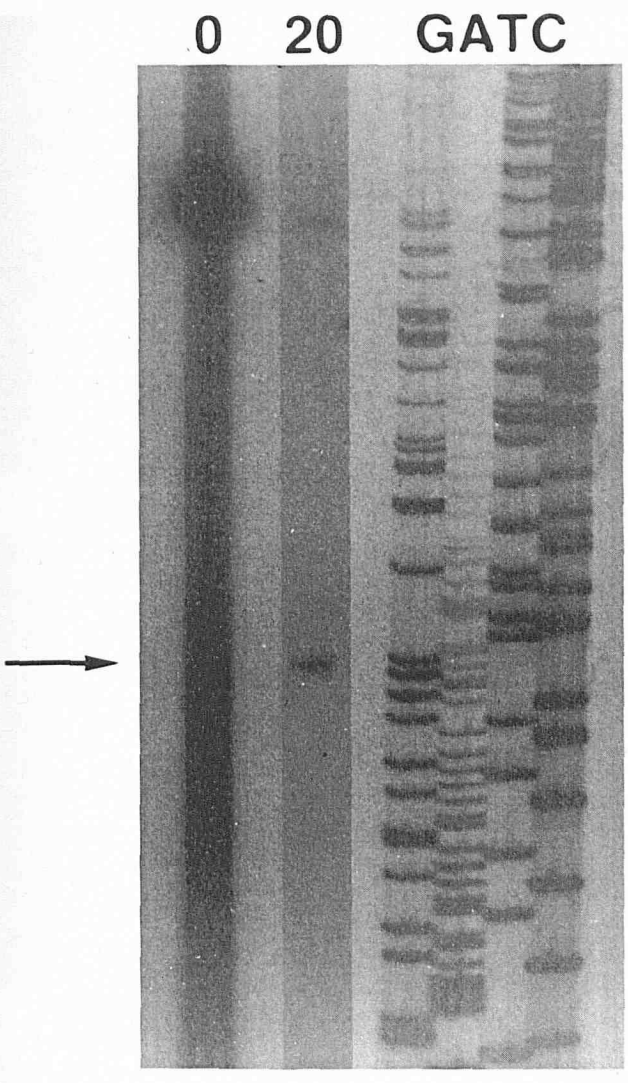

Figure 2. S1 nuclease protection assay. The protected calcyclin fragment, indicated by the *arrow*, is 206 nucleotides long as compared with a sequencing lane (GATC). The large signal at the *top* of the *lane* without nuclease S1 represents the 280-bp–labeled probe. Two different concentrations of S1 (0 and 20 units) were used to digest the hybrids.

digestion with 20 units of the single-strand specific nuclease S1 when mRNA from 3-d-old skin was hybridized to a Bam HI-Pst I restriction fragment from pCAL-F559. The large signal in the lane without nuclease S1 represents the labeled Bam HI-Pst I fragment that contained a small portion of the Honjo vector and G-tail. This protection indicates a complete sequence match between the DNA sequence shown in Fig 1 and the mRNA present in 3-d-old murine skin.

The RNA dot blot (Fig 3) shows that the level of expression of calcyclin increases during the first hair cycle, reaching its peak at day

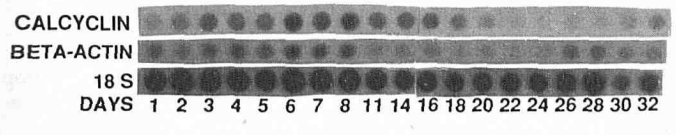

Figure 3. RNA dot blot. Each *dot* represents 10 μg of RNA isolated from excised skin of animals of different ages (day 1–day 32). The anagen phases extend from around day 3 to day 18. The anagen phase of the second postnatal hair growth cycle starts at day 30.

8 and then gradually decreases with the down cycle of hair growth during days 22 to 28. There is at least a tenfold reduction in the calcyclin levels in the telogen phase (day 22) compared to the peak of calcyclin mRNA accumulation (day 8). The level of mRNA accumulation for calcyclin increases again during the anagen phase of the second hair growth cycle (day 30). As a control, the RNA dot blot was rehybridized with a probe for beta-actin [27]. Figure 3 shows that the levels of beta-actin mRNA also varies with the hair cycle, showing increased mRNA accumulation during anagen and decreased levels during telogen. The dot blot was then rehybridized with a 18 S rRNA [28] probe to show that the same amount of RNA had been applied to each dot blot (Fig 3, bottom row).

In Situ Hybridization of Calcyclin with Hair Follicle Tissue

To determine the localization of calcyclin expression we used in situ hybridization to tissue sections of skin from 8-d-old mice. The ^{35}S-UTP–labeled riboprobe specific for the coding region of calcyclin was used as a hybridization probe. Figure 4*a,b* shows that the specific cRNA probe hybridizes to specific areas of the hair follicle and to a lesser extent to the muscle and other tissues of the skin. The control hybridization (Fig 4*c,d*) with the labeled sense strand as a cRNA probe shows that hybridization seen in Fig 4*a,b* is specific for calcyclin. The accumulation of calcyclin mRNA specific hybridization is greatest over the medulla and the inner root sheath of the hair follicle. Both are areas that are not mitotically active; rather sparse labeling is seen over the mitotically active bulb of the hair follicle (Fig 4*a,b*).

DISCUSSION

One cDNA clone, pCAL-F559, that we isolated by differential screening is homologous to the previously published human calcyclin clone [8,9]. Because it is known that calcyclin is induced early during the initiation of cell proliferation, the isolation of its cDNA by differential screening indicates that our approach is adequate to isolate cDNA clones for proteins involved in the early events of hair growth. During the isolation of calcyclin, we screened a total of around 30,000 clones; 56 were picked initially and after colony purification 26 clones were still positive with probes from 3-d-old hair follicles and negative with probes from skin of hairless (Hr/Hr) mice. We could identify four clones by comparing the DNA sequences with known sequences (data not shown). The four clones identified include calcyclin (reported here), a member of the ultra-high-sulfur keratin family [17], a clone related to cytochrome C oxidase [33], and a clone related to the lysosomal membrane glycoprotein [34]. Among the other 22 clones, we found three that had an insert size of 300 bp or more; the others contained only short stretches of DNA sequences including a poly A tail derived from the 3′ end of a mRNA. These short clones may reflect the inefficiency of the size selection during the cDNA library constructions.

Calcium levels are known to control the differentiation of skin epithelial cells [1,2], but it is unknown what role calcium levels play in hair growth. We therefore decided to investigate calcyclin's involvement during the hair cycle. First it was necessary to show that our cDNA clone, pCAL-F559, corresponded to a mRNA present in the skin. S1 nuclease digestion (Fig 2), which is a more stringent and more sensitive method for detecting RNA transcripts [32], showed that pCAL-F559 was able to hybridize to mRNA from the skin, thereby demonstrating that the clone encodes functional mRNA. The next question that needed to be addressed was whether or not the level of calcyclin expression changed during the hair cycle. A dot blot of skin RNA (Fig 3) representing one full cycle of hair growth and a small portion of a second cycle was hybridized with a probe specific for calcyclin. The dot blot demonstrated that the level of expression of calcyclin parallels the hair cycle with high levels in the anagen (growing) phase and low levels in the telogen (resting) phase. Such a hybridization pattern had been reported previously for an ultra-high-sulfur keratin, a hair structural protein [17]. The probe for beta-actin also showed varying levels of accumulation, which would be expected because it has been shown that actin levels increase during the cell cycle [35]. It has also been reported that in

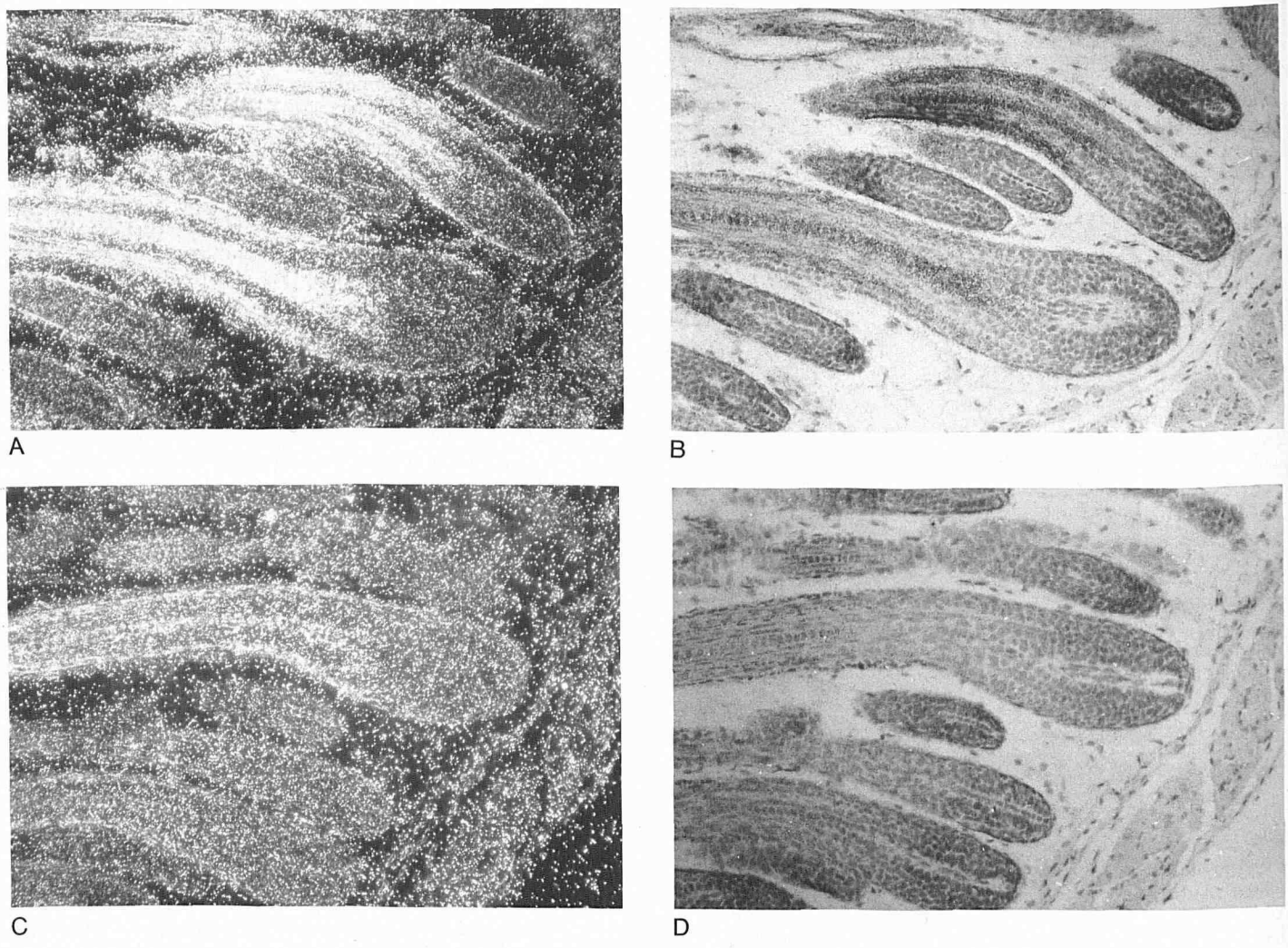

Figure 4. In situ hybridization with ^{35}S-UTP–labeled calcyclin cRNA. *A,B*) Experimental hybridization to longitudinal section through skin of 8-d-old mouse using the antisense cRNA as probe. The hybridization is prominent in the inner root sheath and the medulla of the hair follicle. *A*) Darkfield, *B*) normal image. *C,D*) Darkfield and normal image of control hybridization using the labeled sense strand as probe.

the mouse the mitotic and metabolic activity of the whole skin is in accordance with the hair cycle [15]. Thus the varying hybridization to beta-actin that we find might be related to the changes in the skin. Another cDNA probe (Type IV collagen) we used indicated changes related to the maturation of the skin (data not shown) from day 1 to day 32. Whether mRNA levels in general follow the hair cycle can not be concluded from our experiments. As a control, the hybridization to 18 S rRNA shows that the same amount of RNA had been applied to each dot (Fig 3, *bottom row*).

We expected accumulation of the calcyclin mRNA in the mitotically active cells [7] of the hair follicle bulb. However, in situ hybridization (Fig 4*a,b*) showed that calcyclin mRNA localizes to the post-mitotic keratogenous region of the hair follicle. The area of the bulb at the bottom of the hair follicle is only sparsely labeled. The control hybridization, with the "sense" cRNA probe (Fig 4*c,d*) shows no unspecific labeling to the hair follicle and its parts. The labeling seen in the surrounding, mitotically active dermal tissues of 8-d-old mice (Fig 4) agrees with studies using poly-clonal antibodies showing calcyclin staining in several tissues including smooth muscle cells [36].

It is surprising that we find calcyclin accumulating in the mitotically inactive keratogenous region of the hair follicle (Fig 4*a,b*), where the cells of the inner root sheath and the medulla are undergoing terminal differentiation. Usually calcyclin expression is coupled with mitosis and has not been reported for differentiating cells. Epithelial cells might be unique, because it has been demonstrated for the related skin epithelial cells that changes in calcium levels control terminal differentiation [1,2]. Thus, in the keratogenous region, calcyclin might also be involved in the control of differentiation by regulating Ca^{++} levels.

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