Transgenic mice carrying the guinea-pig α -lactalbumin gene transcribe milk protein genes in their sebaceous glands during lactation

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We have generated transgenic mice carrying the entire guinea-pig α -lactalbumin gene. Lactating transgenic mice expressed high levels of correctly initiated and processed guinea-pig α -lactalbumin mRNA in the secretory epithelium of their mammary glands, and secreted guinea-pig α -lactalbumin in their milk. Transcripts were detectable after 7 days of pregnancy, indicating that the transgene was under correct hormonal control. Whereas no or negligible transcription was detectable in all other tissues tested, high levels of transcripts were found in the skin of lactating transgenic mice. Guinea-pig α -lactalbumin protein was undetectable in the skin, however. *In situ* hybridization analysis showed that expression was localized to the undifferentiated cells in the basal layer of the sebaceous glands. Further studies revealed high levels of endogenous β -casein mRNA in normal lactating mouse skin, demonstrating that the transcription of milk protein genes in lactating mouse skin is a normal event, and is not peculiar to the transgene. This surprising finding highlights the developmental relationship of the mammary gland to other specialized structures of the skin, supports a role for epithelial–extracellular matrix interactions in the regulation of milk protein gene expression *in vivo*, and identifies the skin as a particularly accessible model system in which to study the regulation of milk protein gene expression. In addition, the guinea-pig α -lactalbumin gene will be a source of regulatory sequences with which to direct heterologous gene expression to the sebaceous glands of transgenic mice.

INTRODUCTION

During pregnancy, the inactive mammary epithelium of the female mammal undergoes extensive development and begins to secrete large quantities of milk. The proteins in milk are either synthesized by mammary epithelial cells or, in the case of proteins such as serum albumin and immunoglobulins, directed into milk secretions from the blood. Milk protein synthesis by mammary epithelium requires the activation of milk protein gene expression to high levels, in response to a combination of peptide and steroid hormones (Banerjee, 1976) and cell–cell and cell–substratum interactions (Wiens *et al.*, 1987; Chen & Bissell, 1989). In order to understand how these factors regulate milk protein gene expression during pregnancy, we have studied the gene which encodes α -lactalbumin. This is a whey protein with an M_r of 14500 which is abundant in human and bovine milk and is the major whey protein in guinea pigs (Craig *et al.*, 1976). We have previously isolated and characterized the human (Hall *et al.*, 1987) and guinea-pig (Laird *et al.*, 1988) α -lactalbumin genes, and have identified consensus sequences which might be involved in the regulation of α -lactalbumin gene expression.

One direct test of the function of these putative regulatory sequences would be to transfect the cloned α -lactalbumin gene into cells in culture. Although this approach has worked for other hormonally responsive genes (Kelly & Darlington, 1985), we (Laird *et al.*, 1988) and others (David-Inouye, 1986; Lee *et al.*, 1988) have been unable to obtain correct hormonal regulation of milk protein gene expression in tissue culture. An alternative approach to this problem is to generate transgenic mice (Palmiter & Brinster, 1986) carrying foreign milk protein

genes. Here we report the generation and characterization of transgenic mice carrying the entire guinea-pig α -lactalbumin gene, and show that these animals regulate the transgene correctly in the mammary gland. We also describe how studies of the expression of this gene suggest that normal lactating mice transcribe milk protein genes in their sebaceous glands.

MATERIALS AND METHODS

Preparation of DNA for microinjection

A 3627 bp *HindIII*–*EcoRI* fragment of clone pgpH68 (Laird *et al.*, 1988), containing all four exons of the guinea-pig α -lactalbumin gene, along with 1195 bp of 5' flanking sequence and 398 bp of 3' flanking sequence, was used for microinjection (Fig. 1). This fragment included 675 bp of a guinea-pig LINE sequence at its 5' end, and did not contain vector sequences. The fragment was separated on a 0.8% low-melting-point agarose gel, extracted using a GeneClean kit (Stratagene Scientific) and dissolved in 10 mM-Tris/HCl (pH 7.4)/0.25 mM-EDTA.

Generation of transgenic mice

Transgenic mice were generated as described by Murphy & Hanson (1987). Superovulated (B10 $\times$ CBA/Ca/NIMR)F1 female mice were mated with CBA/Ca/NIMR male mice and single-cell embryos were flushed from their oviducts. The male pronuclei of the embryos were microinjected with 2 μ l of a 1 ng/ μ l solution of DNA, and surviving cells were transferred to the infundibulum at the top of the uterus in pseudopregnant (B10 $\times$ CBA/Ca/NIMR)F1 female mice which had been mated with vasectomized CBA/Ca/NIMR male mice. The resulting

Abbreviations used: TBS, Tris-buffered saline; WAP, whey acidic protein.

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Fig. 1. Map of the 3627 bp *HindIII*-*EcoRI* fragment used for microinjection

The shaded box is a LINE repetitive element. Exons 1-4 of the guinea-pig α -lactalbumin gene are indicated, with white boxes representing coding sequences and black boxes representing 5' and 3' untranslated regions. The *RsaI*-*HpaII* fragment used as an S1 mapping probe is also indicated.

Fig. 2. Generation of transgenic mice

A Southern blot is shown of *EcoRV*-digested genomic DNA (10 μ g) isolated from guinea-pig liver (track o) and from tails of a normal (B10 $\times$ CBA/Ca/NIMR)F1 mouse (track p), founder mouse A1 (track n) and thirteen offspring of mouse A1 (tracks a-m). Filters were hybridized with the guinea-pig α -lactalbumin gene-specific probe R205. Sizes of selected size markers (λ DNA cut with *HindIII*) are indicated in kilobase pairs.

pregnancies were allowed to develop to term, and the pups were tested for the presence of the transgene by tail blotting.

Tail blotting

The end 10 mm of the tail was removed from each pup at 10 days of age, under CO₂ anaesthesia, and placed into 700 μ l of 50 mM-Tris/HCl (pH 8.0)/100 mM-EDTA/100 mM-NaCl/1% (w/v) SDS. After addition of 25 μ l of proteinase K (10 mg/ml) (BCL), the mixture was shaken overnight at 55 $^{\circ}$ C and gently extracted twice with phenol (equilibrated in Tris/HCl, pH 7.4)/chloroform/3-methylbutan-1-ol (25:24:1, by vol.). After a final chloroform extraction, genomic DNA was precipitated by the addition of 0.6 vol. of propan-2-ol. The DNA was spooled out, washed 4 times in 70% (v/v) ethanol and once in absolute ethanol, air-dried and resuspended in 10 mM-Tris/HCl (pH 7.4)/1 mM-EDTA. A 10 μ g portion of genomic DNA was digested with *EcoRV*, electrophoresed on 0.8% agarose gels and transferred to Hybond-N filters (Amersham International) as described previously (Williams *et al.*, 1988). Filters were probed with a 205 bp *RsaI* fragment of the guinea-pig α -lactalbumin cDNA clone pgpK9 (Hall *et al.*, 1982), radiolabelled with [α -³²P]dCTP (800 Ci/mmol; New England Nuclear) by the random primer method (Feinberg & Vogelstein, 1984). This probe, designated R205, contains sequences from exons 2, 3 and 4 of the guinea-pig α -lactalbumin gene. Hybridization was

performed at 65 $^{\circ}$ C in 6 $\times$ SSC/5 $\times$ Denhardt's solution/10% (w/v) dextran sulphate/0.5% (w/v) SDS/denatured sonicated herring testis DNA (250 μ g/ml), containing 10⁶ c.p.m. of probe/ml (1 $\times$ SSC = 0.15 M-NaCl/0.015 M-sodium citrate; 1 $\times$ Denhardt's = 0.02% Ficoll/0.02% polyvinylpyrrolidone/0.02% BSA). Final washes were in 0.1% SSC/0.1% (w/v) SDS at 65 $^{\circ}$ C.

Isolation of total RNA and Northern blotting

Total RNA was isolated from fresh or frozen tissues by the acid guanidinium thiocyanate/phenol/chloroform method (Chomczynski & Sacchi, 1987). Samples (20 μ g) of total RNA were electrophoresed on 1% agarose/Mops/formaldehyde gels, and blotted on to Hybond-N filters (Amersham International) as previously described (Brickell & Patel, 1988). Guinea-pig α -lactalbumin transcripts were detected using the radiolabelled R205 probe described above. Mouse β -casein transcripts were detected using the radiolabelled β -casein cDNA probe pCM β 13 (Gupta *et al.*, 1982). Hybridization was performed at 65 $^{\circ}$ C in 5 $\times$ SSC/10 $\times$ Denhardt's solution/10% (w/v) dextran sulphate/7% (w/v) SDS/denatured sonicated herring testis DNA (250 μ g/ml) containing 10⁶ c.p.m. of probe/ml. Final washes were in 0.15 $\times$ SSC at 65 $^{\circ}$ C. Blots were stripped of probe and reprobed with a radiolabelled mouse actin cDNA clone pAM91 (Humphries *et al.*, 1981), as a control to ensure that approximately equal amounts of RNA had been loaded in each gel track. Scanning densitometry of autoradiographs was performed using a Bio-Rad Model 620 Video Densitometer.

S1 mapping

Transcriptional start sites of the guinea-pig α -lactalbumin gene were mapped using the probe indicated in Fig. 1. An 848 bp *RsaI*-*HpaII* fragment from the 5' end of the gene was end-labelled using T4 polynucleotide kinase (BCL) and [γ -³²P]ATP (3000 Ci/mmol; New England Nuclear) as described by Patel *et al.* (1990). A 20 μ g portion of total RNA and 10⁴ c.p.m. of the probe were co-precipitated and the washed pellet was redissolved in 20 μ l of S1 hybridization buffer [80% (v/v) deionized formamide, 50 mM-Pipes, pH 6.4, 400 mM-NaCl and 1 mM-EDTA, pH 8.0]. After 15 min at 90 $^{\circ}$ C, the mixture was incubated at 52 $^{\circ}$ C for 15 h. Samples were then treated with S1 nuclease and the digestion products were analysed by 6% polyacrylamide/urea gel electrophoresis as previously described (Patel *et al.*, 1990). An M13mp18 sequencing ladder was used as a size marker.

Analysis of α -lactalbumin expression by Western blotting

Milk was expressed from lactating guinea pigs. Milking of lactating mice involved removal of the pups for extended periods, with unacceptably high mortality. Milk from mice was therefore obtained by decapitating one pup from each litter and pipetting out the stomach contents, which had the appearance of a white paste. This material was solubilized by boiling for 10 min in 100 μ l of 1% (w/v) SDS. Skin samples were dissected and 2 cm² portions were homogenized in 2 ml of 1% (w/v) SDS using a Polytron homogenizer. After boiling for 10 min, debris was removed by centrifugation and the supernatant was recovered. Protein concentrations were determined using the Pierce BCA protein assay. Protein samples (100 μ g) were subjected to SDS/PAGE (Laemmli, 1970) and transferred to Hybond-C filters (Amersham International) using a Bio-Rad Transblot cell. Filters were incubated for 1 h at room temperature in Tris-buffered saline (TBS; 10 mM-Tris/HCl, pH 7.4, 0.9% (w/v) NaCl) containing 2% (w/v) bovine serum albumin, and were then incubated for 15 h at 4 $^{\circ}$ C in a 1:100 dilution of rabbit anti-(guinea-pig α -lactalbumin) antiserum (Craig *et al.*, 1976) in TBS. Filters were washed twice for 10 min each, in 100 ml of

Fig. 3. Tissue-specific expression of the transgene

(a) Northern blots of total RNA were hybridized with the guinea-pig α -lactalbumin gene probe R205. Tissue was removed from lactating mice at 3 days *post partum*. RNA was isolated from lactating guinea-pig mammary gland (track a), lactating transgenic mouse mammary gland (b), kidney (c), thymus (d), small intestine (e), salivary gland (f), heart (g), lung (h) liver (i) and skin (j); virgin female transgenic mouse mammary gland (k) and skin (l); lactating normal non-transgenic mouse mammary gland (m) and skin (n); and male transgenic mouse mammary fat pad (o). (b) The blots shown in (a) were stripped of probe and rehybridized with a probe for mouse actin mRNA. Cross-hybridization of the mouse actin probe to guinea-pig actin mRNA is very weak (track a). Sizes of transcripts are indicated in nucleotides. (c) Relative levels of guinea-pig α -lactalbumin mRNA in the tissues tested, expressed in arbitrary units. Intensities of autoradiographic signals in (a) and (b) were estimated by scanning densitometry. The ratio between the signal intensity with the guinea-pig α -lactalbumin probe and the signal intensity with the actin probe was calculated for each tissue. Each of the ratios was multiplied by 1000 in order to give the values recorded. For tissues where no transcripts were detectable, an upper limit for the ratio is given (for example, < 3 in track o).

TBS/0.05% Nonidet P-40 and twice for 5 min each in TBS at room temperature, and were then incubated for 2 h at room temperature in a 1:25 dilution of horseradish peroxidase-conjugated goat anti-rabbit IgG antiserum (Dakopatts, Glostrup, Denmark) in TBS. After washing as before, the filters were rinsed in water and developed in a mixture of 50 ml of TBS, 10 ml of 4-chloro-1-naphthol (3 mg/ml) and 30 μ l of 30 vol. of H_2O_2 . Filters were then rinsed in water and photographed.

In situ hybridization

In situ hybridization to 2 μ m tissue sections was performed as previously described (Parkes *et al.*, 1988), using a 35 S-labelled RNA probe specific for guinea-pig α -lactalbumin transcripts. The probe, which contained sequences from part of exon 1, the whole of exons 2 and 3 and part of exon 4 of the guinea-pig α -lactalbumin gene, was prepared by first cloning a 438 bp *NaeI*-*Bam*HI fragment of the guinea-pig α -lactalbumin cDNA clone pgpK9 (Hall *et al.*, 1982) into *SmaI*-*Bam*HI-digested pGEM4 (Promega Corporation), yielding plasmid pgpK98. *EcoRI*-digested pgpK98 DNA was then used as a template for probe synthesis by T7 RNA polymerase, as previously described (Parkes *et al.*, 1988).

RESULTS

Generation of transgenic mice carrying the guinea-pig α -lactalbumin gene

Microinjection and transfer of 100 single-cell embryos yielded 46 founder mice. Blots of *EcoRV*-digested tail DNA were

hybridized with the guinea-pig α -lactalbumin gene probe R205. In *EcoRV*-digested guinea-pig liver genomic DNA, the α -lactalbumin gene was cut twice, generating a strongly hybridizing internal fragment of 1.7 kb and two larger flanking fragments which had minimal overlap with the probe and so hybridized only weakly (Fig. 1; Fig. 2, track o). The probe did not cross-hybridize significantly to normal non-transgenic (B10 $\times$ CBA/Ca/NIMR)F1 mouse genomic DNA, and the hybridizing 1.7 kb fragment was completely absent (Fig. 2, track p). A strongly hybridizing fragment of 1.7 kb was seen in DNA samples from two female founder mice, designated A1 (Fig. 2, track n) and A2 (results not shown), indicating that these carried the transgene. Comparison of the intensity of the hybridization signal with that seen with guinea-pig genomic DNA indicated that mouse A1 and its transgenic progeny each carried approximately five copies of the transgene (Fig. 2, tracks a, c, h, i and n), whereas mouse A2 and its transgenic progeny carried approximately two copies (results not shown).

Breeding lines were established from both transgenic founder mice by crossing them with male CBA/Ca/NIMR mice. In both lines, the multiple integrated copies of the transgene were transmitted as a single locus, in a Mendelian fashion and through both sexes (Fig. 2 and results not shown).

Tissue-specific expression of the transgene

Expression studies were performed on mice in the breeding line established from founder mouse A1. As shown in Fig. 3(a), high levels of the 750-nucleotide guinea-pig α -lactalbumin mRNA were found in lactating guinea-pig mammary gland

Fig. 4. Regulation of transgene expression during pregnancy

A Northern blot of total RNA was hybridized with the guinea-pig α -lactalbumin gene probe R205. RNA was isolated from 7-day-pregnant transgenic mouse mammary gland (track a), and 14-day-pregnant transgenic mouse mammary gland (b), liver (c), kidney (d), spleen (e), small intestine (f), heart (g), lung (h), thymus (i), brain (j) and skin (k). The size of the transcript is indicated in nucleotides (nt).

Fig. 5. S1 mapping of the transcriptional start site of the transgene

S1 mapping was performed using the probe indicated in Fig. 1. Track P contains undigested probe. The probe was digested after hybridization with total RNA isolated from lactating guinea-pig liver (track a) and mammary gland (b); lactating transgenic mouse mammary gland (c), skin (d) and liver (e); and lactating normal non-transgenic mouse mammary gland (f). Sizes of fragments are indicated in nucleotides (nt).

Fig. 6. Translation of transgene mRNA

An antiserum against purified guinea-pig α -lactalbumin was incubated with a Western blot of purified guinea-pig α -lactalbumin (track a), normal non-transgenic mouse milk (b), normal non-transgenic mouse skin (c), transgenic mouse milk (d, f) and transgenic mouse skin (e, g). Guinea-pig α -lactalbumin has an M_r of 14500.

Fig. 7. *In situ* hybridization to mammary gland sections

Sections are of single lobules of lactating mammary gland tissue taken 3 days *post partum* from a transgenic mouse (a and b) and a non-transgenic mouse (c and d). The lobules are surrounded by adipose tissue. Sections were hybridized with an RNA probe for guinea-pig α -lactalbumin transcripts and photographed under light-field (a and c) and dark-field (b and d) illumination. Magnification $\times 118$.

(track a) and in lactating transgenic mouse mammary gland (track b) and skin (track j). No homologous transcripts were detectable in the mammary gland or skin of virgin female transgenic mice (tracks k and l), in the mammary gland or skin of lactating normal non-transgenic mice (tracks m and n), or in male transgenic mouse mammary fat pad (track o). No transcripts were detectable in the kidney, thymus, small intestine, salivary gland, heart, lung or liver or lactating transgenic mice (tracks c-i), or in their brain or spleen (results not shown).

Overexposure of the autoradiograph revealed extremely low levels of mRNA in lactating transgenic mouse salivary gland, heart and lung, but transcripts remained undetectable in the other tissues tested (results not shown). No transcripts were detectable in the kidney, thymus, small intestine, liver, salivary gland, heart, lung, brain or spleen of virgin female transgenic mice, male transgenic mice or lactating normal non-transgenic mice (results not shown). Blots were reprobed with a radiolabelled mouse actin cDNA probe to assess the integrity and quantity of RNA in each track (Fig. 3b and results not shown).

The autoradiographs were analysed by scanning densitometry and, for each tissue, the ratio between the autoradiographic signals obtained with the guinea-pig α -lactalbumin probe and the mouse actin probe was calculated. This gave an indication of the relative levels of guinea-pig α -lactalbumin mRNA present in the tissues tested, as shown in Fig. 3(c). There are of the order of 10000 α -lactalbumin mRNA molecules per cell in lactating guinea-pig mammary glands at 3 days *post partum* (Burditt *et al.*, 1981). We estimate that lactating transgenic mice have similar levels in mammary gland and skin, of the order of 10

Fig. 8. *In situ* hybridization to skin sections

Sections were taken from the skin of lactating transgenic mice (*a, b, c* and *d*) and lactating normal non-transgenic mice (*e, f, g* and *h*) at 3 days *post partum*. They were then hybridized with an RNA probe for guinea-pig α -lactalbumin transcripts and photographed under light-field (*a, c, e* and *g*) or dark-field (*b, d, f* and *h*) illumination. Photographs (*a*) and (*b*) are of the same section and photographs (*c*) and (*d*) are of the same part of that section. Photographs (*e*) and (*f*) are of the same section and photographs (*g*) and (*h*) are of the same part of that section. The pilo-sebaceous apparatus comprises a hair follicle (*f*) and associated sebaceous glands (*s*), each consisting of a single acinus discharging directly into the follicle. Each acinus is lined with a single basal layer of proliferating undifferentiated epithelial cells. The progeny of these cells differentiate into mature holocrine cells, accumulating sebum vacuoles and migrating inwards the follicle until they lyse. *e*, epidermis; *h*, hair; *st*, stratum corneum. Magnification $\times 42$ (*a, b, e, f*) and $\times 84$ (*c, d, g, h*).

copies per cell in salivary gland, heart and lung, and less than 10 copies per cell in the other tissues tested.

The transgenic progeny of founder mouse A2 also expressed high levels of guinea-pig α -lactalbumin mRNA in lactating mammary gland and skin, with no detectable expression in other tissues (results not shown).

Regulation of transgene expression during pregnancy

Total RNA from tissues of virgin, 7-day-pregnant and 14-day-pregnant transgenic mice was analysed by Northern blotting as above. Guinea-pig α -lactalbumin mRNA was present at high levels in transgenic mouse mammary gland (Fig. 4, track b) and skin (Fig. 4, track k) after 14 days of pregnancy. Overexposure of the autoradiograph revealed extremely low levels of guinea-

pig α -lactalbumin mRNA in the transgenic mouse mammary gland after 7 days of pregnancy (results not shown).

Correct initiation of transgene transcripts

Since the transgene was unexpectedly found to be expressed at high levels in lactating transgenic mouse skin, it was possible that transcripts were incorrectly initiated. The 5' end of the guinea-pig α -lactalbumin mRNA was therefore mapped by S1 nuclease protection analysis. Hybridization of lactating guinea-pig mammary gland mRNA with the 848 bp S1 probe (Fig. 1) protected three fragments, the largest of which was 134 bp long (Fig. 5, track b; Laird *et al.*, 1988). As shown in Fig. 5, the guinea-pig α -lactalbumin transcripts in lactating transgenic mouse mammary gland (track c) and skin (track d) were correctly initiated.

Fig. 9. *In situ* hybridization to skin sections

Sections were taken from the skin of lactating transgenic mice at 3 days *post partum*, hybridized with an RNA probe for guinea-pig α -lactalbumin transcripts and photographed under light-field (a, c, d, f) or dark-field (b, e) illumination. Photographs (a) and (b) are of the same longitudinal section through a pilo-sebaceous unit and photograph (c) is of part of that section. Photographs (d) and (e) are of the same transverse section through a pilo-sebaceous unit, and photograph (f) is of part of that section. In panels (a), (b) and (c), the hair (h) is broken at the point of exit from the follicle (x). e, epidermis; f, follicle; s, sebaceous gland; ▲, proliferating, undifferentiated epithelial cells; △, mature holocrine cells. Magnification $\times 84$ (a, b, d, e) and $\times 252$ (c, f).

Translation of transgene mRNA

To determine whether guinea-pig α -lactalbumin mRNA was translated in the transgenic mice, Western blots of protein extracts were incubated with a rabbit antiserum raised against guinea-pig α -lactalbumin. As shown previously (Craig *et al.*, 1976), this antiserum reacted strongly with guinea-pig α -lactalbumin, which has an M_r of approx. 14500 (Fig. 6, track a). There was no detectable reactivity with any proteins in non-transgenic mouse milk (Fig. 6, track b) or in lactating non-transgenic mouse skin (Fig. 6, track c). Guinea-pig α -lactalbumin with an M_r of approx. 14500 was present at high levels in milk from transgenic mice (Fig. 6, tracks d and f), but was not detectable in lactating transgenic mouse skin (Fig. 6, tracks e and g).

Identification of the cell types within the lactating mammary gland and skin which synthesize α -lactalbumin transcripts

In order to determine the cellular site of guinea-pig α -lactalbumin synthesis, tissue sections were hybridized with a

radiolabelled RNA probe specific for guinea-pig α -lactalbumin transcripts. In lactating transgenic mouse mammary gland (Figs. 7a and 7b), there was hybridization to cells in the secretory epithelium of the acini. The strength of hybridization was not uniform across the lobule; some acini hybridized very strongly, others showed no detectable hybridization and others showed intermediate levels of hybridization. This indicates that the acini vary in the levels of guinea-pig α -lactalbumin transcripts which they contain. It is difficult to quantify this variation, but it could extend over at least two orders of magnitude. In the lobule shown, the hybridizing acini were generally located at the periphery, but this was not the case in other lobules (results not shown). There was no visible hybridization to the adipocytes surrounding the lobules.

There was no detectable hybridization to sections of lactating non-transgenic mouse mammary gland (Figs. 7c and 7d), demonstrating that the probe was specific for transcripts of the transgene.

In skin sections from lactating transgenic mice, hybridization was detectable only in association with the pilo-sebaceous

Fig. 10. Expression of β -casein mRNA in the skin of lactating normal non-transgenic mice

A Northern blot of total RNA was hybridized with a mouse β -casein gene probe. RNA was isolated from lactating normal non-transgenic mouse skin (track a), liver (c) and mammary gland (e); and from virgin normal non-transgenic mouse skin (b), liver (d) and mammary gland (f). The size of the major transcript is indicated in nucleotides (nt).

apparatus (Figs. 8a, 8b, 8c and 8d). The brightness of the stratum corneum in dark-field photographs (e.g. see Fig. 8b) was due to its refractile properties and not to hybridization of the probe. There was no detectable hybridization to sections from lactating non-transgenic mice (Figs. 8e, 8f, 8g and 8h), again demonstrating that the probe was hybridizing specifically to transcripts of the transgene. Figs. 8(a), 8(b), 8(c) and 8(d) and Figs. 9(a), 9(b) and 9(c) show longitudinal sections through the pilo-sebaceous apparatus. Hybridization was localized to basal layer cells at the periphery of the sebaceous gland and lining the hair follicle, and was particularly strong at the junction of the gland and hair follicle epithelium. There was no detectable hybridization to the differentiated holocrine cells, which are clearly visible in Fig. 9(c). Figs. 9(d), 9(e) and 9(f) show the pilo-sebaceous apparatus in transverse section. Here, two sebaceous glands associated with the hair follicle can be seen. As before, there was strong hybridization to cells in the basal layer of the glands, whereas there was no detectable hybridization to the holocrine cells. A further patch of hybridization below the hair follicle may represent part of a third sebaceous gland. No hybridization was seen to any other structures within the skin.

No detectable hybridization was seen to sections of liver, kidney, heart, lung, oesophagus, small intestine, salivary gland, spleen or thymus from both lactating transgenic and lactating non-transgenic mice (results not shown). It is difficult to give an accurate estimate of the sensitivity of the *in situ* hybridization technique, but it is clearly less sensitive than the Northern blotting analysis in which transcripts were detected in the salivary gland, heart and lung of lactating transgenic mice. We estimate that the limit of sensitivity in these *in situ* hybridization studies is between 10 and 100 copies per cell.

Expression of milk protein genes in the skin of normal lactating mice

The finding that guinea-pig α -lactalbumin mRNA expression in lactating transgenic mouse skin is restricted to the basal cells of sebaceous glands suggested that this expression might not be inappropriate, but that milk protein genes might normally be expressed in these cells. Since probes for mouse α -lactalbumin transcripts were not available, Northern blots of total RNA from

various tissues were hybridized with a radiolabelled mouse β -casein cDNA probe. As shown in Fig. 10, high levels of β -casein mRNA were indeed present in both mammary gland (track e) and skin (track a) of lactating normal non-transgenic mice.

DISCUSSION

In this report we have described the generation of two lines of transgenic mice containing the guinea-pig α -lactalbumin gene. The mammary glands and skin of lactating transgenic mice contained correctly initiated and processed guinea-pig α -lactalbumin mRNA at levels equivalent to those found in lactating guinea-pig mammary gland, which are of the order of 10000 copies per cell (Burditt *et al.*, 1981). These greatly exceed the levels of endogenous mouse α -lactalbumin mRNA expressed in lactating mouse mammary gland, as determined by translation of mammary gland mRNA *in vitro* (Takemoto *et al.*, 1980). This may indicate that mouse transcription factors interact more efficiently with the guinea-pig control sequences of the transgene than with the control sequences of the endogenous mouse α -lactalbumin gene. Alternatively, in view of the importance of post-transcriptional events in the control of milk protein gene expression (Bathurst *et al.*, 1980), there may be structural motifs within guinea-pig α -lactalbumin mRNA which promote mRNA stability in mouse cells.

The high level of transgene expression reported here contrasts with that found by Lee *et al.* (1988), who obtained expression of a rat β -casein transgene at 0.01–1% of endogenous levels, Andres *et al.* (1987), who obtained expression of a mouse whey acidic protein (WAP) promoter–c-Ha-ras gene fusion at 2% of endogenous WAP RNA levels, and Pittius *et al.* (1988), who obtained expression of a mouse WAP promoter–tissue plasminogen activator fusion at 1% of endogenous WAP RNA levels. Simons *et al.* (1987) generated transgenic mice carrying the entire sheep β -lactoglobulin transgene and obtained high-level expression, comparable with that found in sheep. These data also point to the importance of control sequences lying within the body of milk protein genes.

In the mammary gland, accumulation of guinea-pig α -lactalbumin transcripts to high levels was restricted to cells in the secretory epithelium, which are the normal sites of milk protein gene expression. Expression was not uniform across the mammary gland lobule; cells in some acini expressed much higher levels than cells in other acini. A similar asynchrony has been observed previously at the morphological level, with different acini developing at different rates (Mills & Topper, 1970).

Extremely low levels of guinea-pig α -lactalbumin mRNA (of the order of 10 copies per cell) were detected in the salivary gland, heart and lung of lactating animals, and it is possible that other tissues tested expressed transcripts at levels below the limit of detection. The WAP and β -casein transgenes noted above were also expressed at very low levels in tissues other than the mammary gland. This low-level 'leakage' probably results from the interaction of flanking host chromosomal sequences with the transgene, and is frequently found in transgenic mice (Palmiter & Brinster, 1986).

In the guinea pig, mammary gland development begins late in pregnancy; casein mRNAs appear at around parturition, and α -lactalbumin mRNA is only detectable from 2 days *pre-partum* (Burditt *et al.*, 1981). In contrast, the mammary glands of mice and rats develop relatively early (Anderson, 1978); casein mRNAs are detectable in mid-pregnancy (Takemoto *et al.*, 1980; Lee *et al.*, 1988) and rat α -lactalbumin mRNA is present from the first week of pregnancy (Nakhasi & Quasba, 1979). The levels of mouse α -lactalbumin in milk are very low, and mouse

α -lactalbumin mRNA is extremely difficult to detect (Takemoto *et al.*, 1980), but is likely to follow the temporal expression pattern of this gene in the rat. In the transgenic mice described here, guinea-pig α -lactalbumin mRNA was detectable at very low levels as early as 7 days after conception, and at high levels by day 14. The timing of mRNA accumulation therefore seems to follow the pace of the physiological development of the mouse rather than of the guinea pig, indicating that the interaction between mouse regulatory factors and the transgene's guinea-pig control sequences mediates correct hormonal regulation. Similar interactions presumably direct the high-level hormonally regulated accumulation of guinea-pig α -lactalbumin transcripts in the basal layer cells of the sebaceous glands. The high levels of transcripts found here suggest that this is not a leakage effect, and raise the possibility that milk protein gene expression in these cells is normal during lactation. The lack of a suitable mouse α -lactalbumin gene probe makes it impossible to test directly whether transcript of the endogenous mouse α -lactalbumin gene accumulate in skin during lactation. However, we have shown that transcripts of the endogenous β -casein gene are present at high levels in the skin of lactating normal non-transgenic mice. It is therefore highly likely that transcripts of the endogenous α -lactalbumin gene and other milk protein genes are also present in skin. This has not been reported previously. Of the many studies of milk protein gene expression in the mouse, only Pittius *et al.* (1988) examined mouse skin, and they did not detect WAP mRNA there. Interestingly, accumulation of WAP mRNA in mammary epithelial cells *in vitro* requires the formation of three-dimensional alveoli-like structures, whereas expression of caseins and α -lactalbumin by these cells does not (Chen & Bissell, 1989). The ability of sebaceous gland epithelial cells to express α -lactalbumin and casein mRNAs, but not WAP mRNA, parallels this finding and may therefore reflect differences in the structural organization of the sebaceous and mammary glands.

The finding that milk protein gene transcripts are present at high levels in both sebaceous glands and mammary glands is interesting in view of their common developmental origin. The specialized structures of mammalian skin, including the pilo-sebaceous apparatus, the sweat gland, the nail and the mammary gland, develop from thickenings of the germinal layer of the ectoderm, and the mammary gland has been described variously as a specialized sweat gland, sebaceous gland or hair follicle on the basis of morphological and developmental criteria (Sakakura, 1987). Our data indicate that in terms of their pattern of gene expression, the epithelial cells of the mammary gland are particularly closely related to those of the sebaceous gland, and to the undifferentiated rather than the holocrine component.

The undifferentiated basal layer cells of the epidermis resemble those of the sebaceous gland in their developmental origin and in that their programmed differentiation and death is required for proper skin function. However, these cells did not contain α -lactalbumin transcripts. One possible explanation for this is that the ductal organization of the sebaceous and mammary glands is necessary for α -lactalbumin and casein transcript accumulation. This is unlikely, however, since the formation of ductal structures by mammary epithelial cells *in vitro* is not a prerequisite for casein and α -lactalbumin gene expression, as it is for WAP gene expression (Chen & Bissell, 1989). Experiments *in vitro* have demonstrated that the accumulation of α -lactalbumin and casein mRNA to high levels in mammary epithelial cells is dependent upon interactions with the extracellular matrix and with non-epithelial cells (Medina *et al.*, 1987; Wiens *et al.*, 1987; Chen & Bissell, 1989). It is therefore possible that differences in the extracellular matrix associated with the different specialized structures of the skin exert an effect upon milk protein transcript accumulation.

In summary, it may be that sebaceous and mammary gland epithelial cells, but not other skin epithelia, experience those interactions with the extracellular matrix that permit accumulation of α -lactalbumin and casein mRNAs, but that only the particular three-dimensional structure of the mammary gland permits accumulation of WAP mRNA.

Whereas transgene mRNA in the mammary gland was translated efficiently, we were unable to detect guinea-pig α -lactalbumin protein in the skin. This may reflect the difficulty of extracting intact protein from this tissue. Alternatively, it may result from a specific block of translation (Casey *et al.*, 1988), although regulation of milk protein expression at this level has not been described previously. A third possibility is that α -lactalbumin is synthesized but rapidly degraded, either because sebaceous gland cells lack the apparatus for correct intracellular processing of nascent α -lactalbumin polypeptide chains, or as a result of the extensive protein degradation known to accompany the differentiation of sebaceous gland basal layer cells into holocrine cells (Wheatley, 1986).

Our finding of milk protein gene transcript accumulation in sebaceous gland cells opens up a new experimental system for studies of the regulation of milk protein gene expression. It will be of interest to examine skin samples from pregnant and lactating women in order to determine whether milk protein gene transcripts are present in their sebaceous glands. Analyses of the hormonal regulation of human milk protein production would be facilitated if such studies could be performed on skin biopsies. In addition, we have a novel experimental system in which to study the developmental biology of the skin adnexal structures *in vivo*. In particular, the guinea-pig α -lactalbumin gene will be a source of regulatory sequences with which to direct heterologous gene expression to the sebaceous glands.

We are grateful to the Medical Research Council for supporting part of this work, and would like to thank Dr. Chris Bunker and Dr. Len Hall for helpful discussions.

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Received 4 September 1990/19 November 1990; accepted 5 December 1990