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Expression of human lactoferrin in milk of transgenic mice

GERARD J. PLATENBURG, ERIKA P.A. KOOTWIJK, PATRICIA M. KOOIMAN, SHELLEY L. WOLOSHUK, JAN H. NUIJENS, PAUL J.A. KRIMPENFORT, FRANK R. PIEPER, HERMAN A. de BOER and REIN STRIJKER*

GenePharming Europe B.V. Niels Bohrweg 11–13, 2333 CA Leiden, The Netherlands and Dept. of Biochemistry, Leiden University P.O. Box 9502, 2300 RA Leiden, The Netherlands (Fax: +31 71 21 6507)

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The expression of human lactoferrin (hLF) in the milk of transgenic mice is described. Regulatory sequences derived from the bovine αS1-casein gene were fused to the coding sequence of the hLF cDNA and several lines of transgenic mice were generated. Human LF RNA was detected exclusively in the mammary gland of lactating females and only after the onset of lactation. No aberrant RNA products could be detected using northern blotting and primer extension analysis. The hLF concentrations in the milk ranged from less than 0.1 to 36 μg ml^{-1}. Human LF thus expressed did not differ from human milk derived LF, with respect to molecular mass and immunoreactivity with monoclonal and polyclonal antibodies.

Keywords: human lactoferrin; αS1-casein; transgenic mice; tissue specificity; mammary gland

Introduction

Lactoferrin is the major iron binding protein in milk of many mammalian species (Masson and Heremans, 1971). Its concentration in human milk is about 1.7 mg ml^{-1}, which makes it one of the most abundant whey proteins. The crystal structure of hLF has been determined (Anderson *et al.*, 1989). The protein contains two highly homologous domains, each with a single iron binding site and an *N*-glycosylation site. These domains share extensive homology with those of other members of the transferrin family. LF can bind one ferric ion per domain with high affinity ($K_{app} = 10^{20}$), concomitant with the incorporation of one carbonate ion. Glycosylation of the LF protein appears not to be necessary for its high affinity iron binding property (Anderson *et al.*, 1990) or for its interaction with the hLF receptor (Kawakami and Lonnerdal, 1991). We (Rey *et al.*, 1990), and others (Rado *et al.*, 1987; Powell and Ogden, 1990), have cloned the human LF cDNA and have determined its DNA sequence.

The ratio of iron-free and iron-saturated hLF in human whey is approximately 20 to 1 (Fransson and Lonnerdal, 1980). In human milk, two main roles have been suggested for LF. The first role would be to inhibit growth of several groups of (potentially pathogenic) bacteria both in the intestinal tract of the suckling infant and in the mammary gland of the mother. This antibiotic effect can be exerted through at least two different mechanisms, namely, through direct binding to the outer membrane of Gram-negative bacteria (Ellison and Giehl, 1991) and through deprivation of iron thereby inhibiting growth of bacteria with a high iron demand (Stuart *et al.*, 1984). This latter mechanism is restricted to the iron-free form of the protein. The second role would be to mediate iron transport from the mother to the newborn (Saarinen and Siimes, 1979). This function is obviously restricted to the iron-bound form. It has also been reported that hLF produced by neutrophils can exert anti-inflammatory properties by inhibition of formation of toxic oxygen radicals (Kuizinga *et al.*, 1987), and by suppression of cytokine production (Broxmeyer, 1986) and complement activation (Kijlstra and Jeurissen, 1982). In view of the wide variety of properties, LF may have various nutritional and pharmaceutical applications.

The presence of both iron-free and iron-saturated LF in human milk is a prerequisite for the protein to fullfil both main functions described above. Therefore, it would be

*To whom correspondence should be addressed.

desirable that an expression system used to produce hLF would result in both forms. In addition, the protein should be available in large quantities. A suitable way to meet these criteria is expression via milk of transgenic dairy animals. Since structure and function of the mammary gland is very conserved between different species, it is likely that structural and functional properties of hLF expressed in milk of transgenic animals are similar to those of human milk-derived LF.

So far, several heterologous proteins have been expressed in the milk of mice (Gordon *et al.*, 1987; Hennighausen, 1990), rabbits (Buhler *et al.*, 1990), sheep (Wright *et al.*, 1991), goats (Ebert *et al.*, 1991) and pigs (Wall *et al.*, 1991) using regulatory sequences of various milk protein genes. To direct expression of hLF to the mammary gland, we have used regulatory sequences from the bovine αS1-casein gene. Bovine milk contains four different caseins which account for 80% of the total protein fraction. All four proteins are encoded by single-copy genes clustered in one locus (Ferretti *et al.*, 1990; Threadgill and Womack, 1990). The proteins with the highest concentration in bovine milk are αS1- and β-casein (about 10g litre^{-1} each); αS2- and k-casein are present at lower concentrations (about 3g litre^{-1} each).

As a model system for the production of hLF in the milk of transgenic dairy cows and to evaluate expression cassettes, we have generated transgenic mouse lines producing hLF in their milk. Human LF produced via this route was, by several criteria, indistinguishable from lactoferrin isolated from human milk.

Materials and methods

Cloning of the bovine αS1-casein flanking regions

For the isolation of bovine αS1-casein 5′ and 3′ flanking regions, probes were generated using the polymerase chain reaction. Bovine genomic DNA was used as a template for amplification. For the 5′ region, the sequence from position −681 to +180 relative to the major transcription initiation site was amplified (fragment I in Fig. 1), using two DNA-primers based on the published sequence (Lee *et al.*, 1986; sequence of forward primer: 5′-TCCATGGGGGTCACAAAGAACTGGAC-3′, sequence of the reverse primer: 5′-TGAAGCTTGCTAACAGTATATCATAGG). For the 3′ region, the sequence from position 730 to 1070 of the cDNA sequence was amplified (fragment II in Fig. 1) using two DNA-primers based on the published sequence (Stewart *et al.*, 1984; sequence of forward primer: 5′-GAGGGACTCCACAGTTATGG-3′, sequence of reverse primer: 5′-GCACACAATTATTTGATATG-3′). Amplified fragments obtained were partially sequenced to confirm their identity (data not shown). Fragment II consists of 1300 nucleotides, due to the presence of an intron of approximately 1000 nucleotides. After subcloning, fragments I and II were used to screen a bovine genomic library constructed in EMBL3 (kindly provided by Dr. M. Groenen, Agricultural University Wageningen, Netherlands). Two clones (GP1 and GP5) were isolated, the inserts were excised from the phage (*Sal* I digestion), subcloned in pKUN (Konings *et al.*, 1986), and characterized in detail. GP1 contains a region of about 14.2 kb upstream and extends to about 1.9 kb downstream of the transcription initiation site. GP5 contains a region of about 2 kb upstream and about 11.5 kb downstream of the stop codon of the bovine αS1-casein gene (Fig. 1).

Construction of the αS1-casein/hLF expression plasmid

To create the αS1-casein based hLF expression plasmid, two intermediate plasmids were constructed. The first intermediate plasmid containing the αS1-casein 5′-untranslated region and 6.2 kb 5′ flanking sequences was constructed as follows. To provide the 5′ αS1-casein sequences, the 1.6 kb *Eco* RI–*Bgl* II fragment from GP1 (−100 to +1500) was isolated from GP1. The *Bgl* II site is located at the junction of the first intron and the second exon of the bovine αS1-casein gene. A synthetic *Bgl* II–*Sal* I linker (sequence shown in Fig. 1) was ligated to the *Bgl* II site to facilitate subsequent cloning steps. To add more 5′ flanking sequences to this fragment, the partial *Eco* RI fragment (−6200 to −100) from GP1 was ligated to the *Eco* RI site at position −100. This resulted in the 7.7 kb *Eco* RI–*Sal* I fragment, which was subcloned into *Eco* RI–*Sal* I cut pKUN2 (a derivative of pKUN containing a *Not* I restriction site in the polylinker) thereby creating a *Not* I site 5′ to the *Eco* RI site at position −6200 resulting in pNE3BS.

The second intermediate plasmid, containing the hLF coding region fused at the 5′ end to the αS1-casein signal sequence and at the 3′ end to αSI-casein 3′ flanking sequences was constructed as follows. The hLF cDNA cloned in our laboratory (Rey *et al.*, 1990) encodes a protein which is unique at two positions compared to other published sequences (Rado *et al.*, 1987; Powell and Ogden, 1990). Whereas hLF as described in this study contains a threonine at position 130 and a cysteine at position 404, the other sequences encode an isoleucine and a glycine at these positions respectively. A synthetic DNA fragment, containing the bovine αS1-casein signal sequence (Stewart *et al.*, 1984) starting with an artificial *Xho* I site and ending with the last codon of the αS1-casein signal sequence (GCC), was (blunt) ligated to the first codon (GGC) of mature hLF coding sequence. The resulting sequence from the *Xho* I site on, including the αS1-casein signal sequence and the casein/hLF fusion is shown in Fig. 1. The 3′ end of the hLF coding sequence was fused directly downstream of the hLF stopcodon (TAA) to the 3′ αS1-casein untranslated and flanking sequences, using a synthetic linker. The 3′ αS1-casein untranslated region used in this construct starts at *GAG*

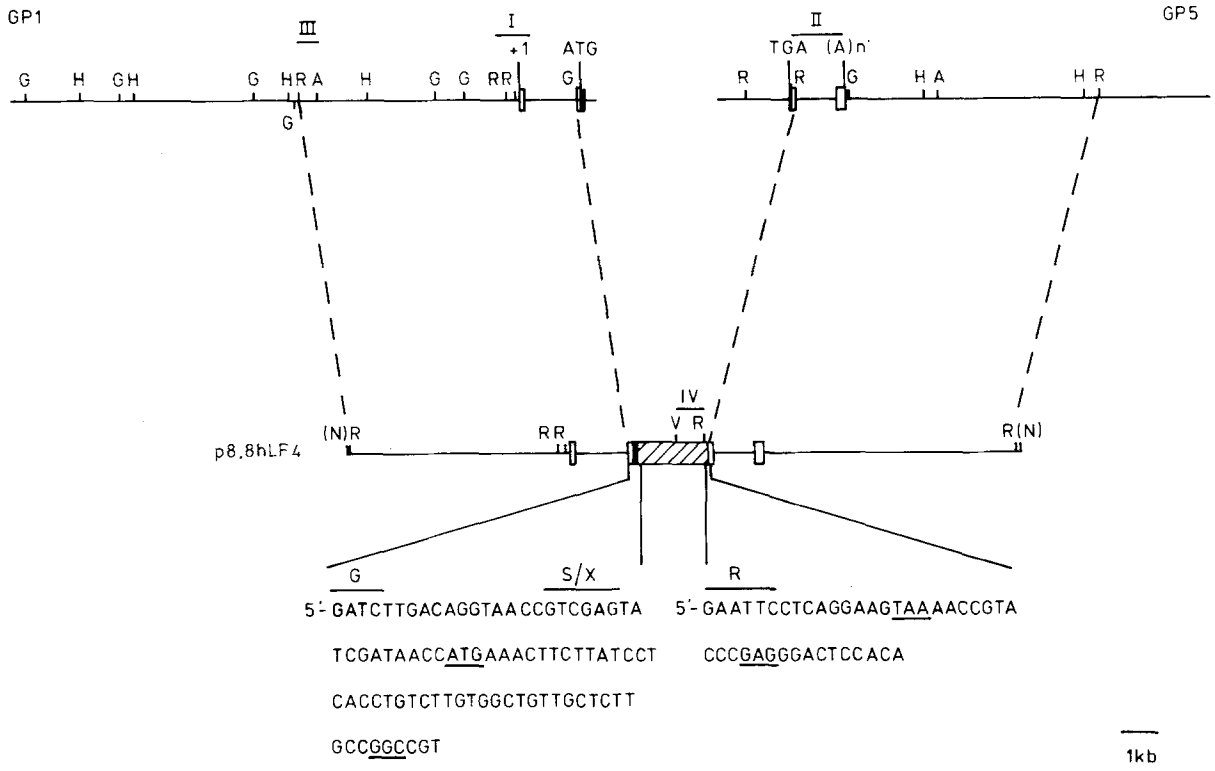

Fig. 1. Construction of the αS1-casein/hLF expression cassette. The structure of the bovine αS1-casein flanking clones (GP1 and GP5) is depicted at the top. The fragments used to construct p8.8hLF4 are indicated by a dashed line and the structure of the expression cassette is shown. Bovine αS1-casein flanking sequences are indicated by an uninterrupted line. Open boxes indicate bovine αS1-casein non-translated exons; the black box near the start codon represents the bovine αS1-casein signal sequence; the hatched box represents the hLF coding region. The sequences spanning the casein/hLF junctions are shown. The bovine αS1-casein transcription initiation site (+1), the translational start site (ATG), the stop codon (TGA), the polyadenylation site ((A)n), the first codon of the mature hLF protein (GGC), its translational stop codon (TAA), and the three first nucleotides of the bovine αS1-casein 3'-untranslated region used (GAG) are indicated. Restriction enzyme sites are abbreviated by: R: *Eco* RI; H: *Hin* dIII; G: *Bgl* II; A: Asp718; (N): *Not* I sites derived from pKUN2; S/X: *Sal* I site joined to Xho I site. The relevant probes used in this study (I, II, III, and IV) are indicated and described in materials and methods.

which is 18 nucleotides downstream of the αS1-casein stop codon. The resulting sequence from the (hLF) *Eco* RI site on, including the hLF stop codon and the hLF/casein fusion, is shown in Fig. 1. The fragment from the *Xho* I site to the *Eco* RI site 8 kb downstream of the αS1-casein stop codon was subcloned into a pUC118 derivative containing a *Not* I site downstream of the *Eco* RI site. The resulting plasmid was called phLF3'10.

To construct the final hLF expression vector, fragments of the described plasmids were fused. The *Not* I–*Sal* I fragment from pNE3BS was fused to the *Xho* I–*Not* I fragment from phLF3'10 and subcloned into *Not* I cut pKUN2, resulting in p8,8hLF4. DNA sequencing confirmed the fidelity of all regions containing synthetic DNA and all junctions of ligated fragments (data not shown).

Using the same approach, a second expression vector with 8.0 kb more 5' bovine αS1-casein flanking region, as compared to p8.8hLF4, was created. The 8.0 kb *Sal* I–*Eco* RI fragment (−14200 to −6200) was isolated from GP1 and subcloned in pUC19. The *Sal* I site was destroyed by inserting a synthetic oligonucleotide, creating a *Not* I site just 5' to the *Sal* I site. From this intermediate plasmid the 8.0 kb *Not* I–*Eco* RI fragment was isolated, ligated to the 7.7 kb *Eco* RI–*Sal* I fragment (described above) and subcloned in *Not* I–*Sal* I digested pKUN2. Subsequent cloning steps were as performed for p8,8hLF4 and resulted in p16,8hLF4. Transgenic mice containing the latter construct were used for primer extension studies.

Generation of transgenic mice

The expression cassette used for micro- injection was removed from plasmid sequences by *Not* I digestion and purified by 0.65% agarose gel electrophoresis and electroelution. Fertilized mouse eggs (CBA/BrA × C57Bl/6) were microinjected (with 100–200 copies of the fragment) and transferred into pseudo-pregnant females as described (Hogan *et al.*, 1986). Total genomic DNA was prepared from a short segment of mouse tail to check for integration of the injected DNA. Southern blot analysis of Asp718 digested tail DNA was done according to standard

procedures (Sambrook *et al.*, 1989). The probes used to check for integration and integrity of the DNA fragment, were the 600 bp *Eco* RI-*Asp*718 fragment isolated from GP1 (shown in Fig. 1 as fragment III) and the 758 bp *Eco* RV-*Eco* RI fragment isolated from the hLF cDNA spanning the 3′-half of the gene (shown in Fig. 1 as fragment IV). Both probes were labelled with ^{32}P using random hexanucleotide primers (Sambrook *et al.*, 1989).

Tissue- and stage-specific expression

From transgenic animals containing the expression cassette 8,8hLF4, total RNA was isolated from various tissues by the LiCl-Urea method (Auffray and Rougeon, 1980). Polyadenylated RNA was isolated from mammary glands using the Micro fasttrack mRNA isolation kit according to the procedures recommended by the manufacturer (InVitrogen). Ten to 20 μg of total RNA or 1 to 2 μg of polyadenylated mRNA was separated on 0.8% agarose formaldehyde gels (Sambrook *et al.*, 1989) and transferred to Hybond filters (Amersham). A 400 bp *Eco* RI–*Pst* I fragment containing the 3′-untranslated region (3′UTR) of the bovine αS1-casein cDNA (a kind gift from Dr. A.F. Stewart; Stewart *et al.*, 1984) was used as a probe to detect transgene expression in these Northern blot experiments. A 200 bp *Eco* RI–*Pst* I fragment of the murine β-casein cDNA, a 600 bp *Pst* I fragment from the murine WAP cDNA (both kind gifts from Dr. L. Hennighausen; Hennighausen and Sippel, 1982), and the 800 bp *Pst* I fragment from murine αS-casein (a kind gift from Dr. J. Rosen; Gupta *et al.*, 1982) were used to detect endogenous milk protein gene expression. To correct for loading differences, the blot was hybridized with a 1.1 kb *Pst* I-fragment of the murine gamma-actin cDNA (a kind gift from Dr. A.G. Jochemsen). Quantification of the hybridization signal was performed using a β-scope 603 blot analyzer counter (Betagen, Inc.).

Primer extension analysis

A 20-mer bovine αS1-casein signal sequence oligonucleotide primer (5′-GAGCAACAGCCACAAGACAG-3′) was used. The primer was end-labelled with ^{32}P using polynucleotide kinase (Gibco-BRL) to a specific activity of 10^8 cpm/μg. Total RNA was isolated from the mammary gland of a transgenic mouse containing the transgene 16,8hLF4. Total RNA (50 μg) of the transgenic mouse or 1 μg polyadenylated RNA (isolated with the Micro fasttrack mRNA isolation kit) of a lactating cow was co-precipitated with primer ($1\text{-}2 \times 10^5$ cpm), resuspended in 8 μl H_2O and 2 μl 5× annealing buffer (1.25 M KCl, 50 mM Tris-HCl, pH 8.0, 5 mM EDTA). The samples were incubated at 65 °C for at least 2 hours, then placed at 42 °C and supplemented with 23 μl extension mix (20 mM Tris-HCL pH 8.4, 10 mM $MgCl_2$, 10 μg/ml actinomycin C, 5 mM dNTPs) and 10 U AMV reverse transcriptase (Promega). The reaction was incubated for 1 hour at 42 °C. The samples were then ethanol precipitated, resuspended in 4 μl 0.1 mM NaOH/1mM EDTA, and analyzed on a 8% polyacrylamide/6M urea gel.

Milk collection and processing

Milk from lactating mice was collected 10 minutes after subcutaneous injection of 1 unit of oxytocin (PitonS, Organon). Milk samples were diluted ten-fold in phosphate buffered saline pH 7.4 (PBS) containing 10 mM EDTA, 0.05% (w/v) hexadimethrine bromide (Polybrene, Janssen Life Sciences Products), 10 mM benzamidine hydrochloride (Janssen) and 0.01% soya-bean trypsin inhibitor (STI, type I-S, Sigma). The tubes were centrifuged at 4 °C for 30 minutes at 11,000 g to separate the whey, casein (pellet), and fat fractions. Whey samples were stored at −20 °C until analysis.

Radioimmunoassay for hLF

Radioimmunoassay (RIA) analysis procedures were essentially as described (Nuijens *et al.*, 1992). A suspension of a monoclonal anti-hLF antibody preparation (mAB 13.17, a kind gift from Dr C.E. van der Schoot) coupled to CNBr-activated Sepharose (Pharmacia) was incubated with 50 μl serial dilutions of whey samples in 2 ml polystyrene tubes. HLF bound to Sepharose was quantified by incubating with affinity purified anti-hLF polyclonal rabbit antibodies (Sigma) labelled with ^{125}I. Bound radioactivity was measured in a 1261 multigamma gamma counter (LKB). Results were expressed as percentage binding of the labelled antibodies added. Levels of hLF in test samples were expressed in μg ml^{-1} using purified hLF (Sigma) as a standard (serial dilutions in PBS, 0.1% (w/v) Tween20, and 0.05% Polybrene). A similar RIA for hLF was also performed using Sepharose to which an affinity purified rabbit anti-hLF polyclonal antibody preparation (Sigma) was coupled.

SDS-PAGE and Western blot analysis of transgenic hLF

Procedures were essentially as described (Nuijens *et al.*, 1989). HLF from 20 μl of transgenic mouse milk was immunoprecipitated with the same Sepharose-bound monoclonal anti-hLF antibodies (described above) and was dissociated by adding 50 μl non-reducing SDS-sample buffer and boiling. Samples were analysed by SDS-PAGE (10% w/v) followed by immunoblotting with polyclonal ^{125}I-anti-hLF antibodies and autoradiography.

Results

Cloning of the flanking sequences of the bovine αS1-casein gene

Flanking regions of the bovine αS1-casein gene were isolated as described in Materials and methods, and as shown in Fig. 1. Two clones, GP1 and GP5 were shown to

be αS1-casein clones by partial DNA sequence analysis (data not shown). Comparison of the region from −103 to +180 and that spanning the second exon with the published sequence (Lee *et al.*, 1986) revealed no discrepancies. A comparison of the sequence of the first exon and the 5′-flanking region with the published sequence (Koczan *et al.*, 1991) also did not show any differences. Comparison of the sequence of the second exon with that of Koczan *et al.* (1991) revealed two differences, at positions 3581 and 3587 (numbering according to Koczan *et al.*). Our sequence contains a thymidine at position 3581, and a cytidine at position 3587; according to Koczan *et al.* these are cytidine and thymidine respectively. These differences did not affect the amino acid sequence. Consistent with the data of Koczan *et al.*, we found that the 3′ untranslated region of the αS1-casein gene, present in clone GP5, is interrupted by an intron of approximately 1.0 kb. Comparison of the sequence of the nineteenth exon with the previously reported cDNA sequence (Stewart *et al.*, 1984) and genomic sequence (Koczan *et al.*, 1991) revealed one nucleotide difference. This discrepancy, a C instead of a T is located at position 887 (numbering according to Stewart) or at position 19387 (numbering according to Koczan). The restriction map derived from GP1 and GP5 (Fig. 1) is identical to the map published by Meade *et al.* (1990).

Construction of hLF expression plasmids and generation of transgenic mice

The hLF expression plasmids contain the hLF cDNA fused to the αS1-casein signal sequence and flanked by the untranslated regions and flanking sequences of the αS1-casein gene. The plasmid constructions are outlined in the Materials and methods section.

Pronuclei of fertilized oocytes were microinjected with the complete inserts isolated from the described plasmids. Injections with 8,8hLF4 resulted in six transgenic mouse lines (numbered 7, 8, 9, 10, 11 and 13). In all cases the transgene had integrated in a head-to-tail fashion as judged from Southern blot analyses (see Materials and methods). The copy number of the transgene varied between 1 and 50 as estimated by comparing the intensity of the hybridization signal to that obtained with known amounts of human DNA. The founder mice were mated to non-transgenic mice to produce first generation transgenics (F_1). Five founders transmitted the transgene in a Mendelian fashion, whereas one founder (#9) appeared to be a germline mosaic. Lines #10 and #13 were randomly selected for hLF RNA expression studies.

Tissue-specificity of hLF expression

Northern blot analysis was performed to assess tissue-specificity of transgene expression (Fig. 2). Two lactating mice of lines #10 and #13 were killed at day 8 of lactation and total RNA from various tissues was isolated. Expression of transgene-specific RNA was observed exclusively in the mammary gland. Hybridization with a bovine casein-specific probe (containing the 3′-untranslated region) yielded two bands. The size of the larger band (2700 nucleotides (nt)) is in agreement with the size of the coding sequence of lactoferrin cDNA combined with the 5′- and 3′ non-translated sequences of the bovine αS1-casein gene. The smaller band has a length of about 700 nt and must be caused by cross-hybridization to a murine mammary gland-specific RNA as it is also present in the lane containing RNA from a non-transgenic mouse (Fig. 2, lane ma⁻). We have not characterized this cross-hybridizing RNA species further. Although there is a 69% identity between the murine and the bovine casein sequence in the region covered by this probe, this RNA is much smaller than the mouse α-casein mRNA (which has a length of about 1400 nt) and must therefore be derived from another (milk-specific) gene.

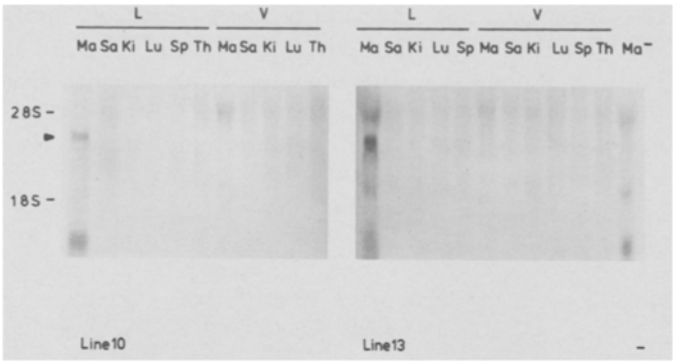

Fig. 2. Northern blot analysis of RNA from tissues of two independent mouse lines. Total RNA was isolated from several tissues of mouse line #10 (L, lactating female; V, virgin female) and from mouse line #13 and from mammary glands of a lactating non-transgenic mouse (Ma⁻). The tissues analysed were mammary gland (Ma), salivary gland (Sa), kidney (Ki), lung (Lu), spleen (Sp), thymus (Th). The position of the 2.7 kb hLF transcript is indicated with an arrow. The positions of the ribosomal RNAs are indicated on the left. Ten μg of total RNA was loaded in each lane. Hybridization was performed with the 3′UTR probe (see Materials and methods).

Total RNA was also isolated from various tissues of two virgin female mice from lines #10 and #13. Expression of the transgene was not detected in any of the tissues examined. Total RNA isolated from mammary gland of a non-transgenic lactating female mouse (lane Ma⁻) did not reveal hLF expression either. In addition, analysis of total RNA isolated from various tissues of transgenic males revealed no transgene expression in any tissue (data not shown).

Stage-specific expression

To determine at which stage of mammary gland development

the expression of the transgene is induced, polyadenylated RNA (shown to be intact based on a ethidium bromide stained gel; data not shown) was isolated from the mammary glands of offspring of line #10 at the later stages of pregnancy and lactation and subjected to northern analysis (Fig. 3). Expression was only detectable after the onset of lactation. The RNA levels of the transgene reached maximum levels around day 13 of lactation. The 700 nt band that is detected by cross-hybridization to the bovine αS1-casein probe has an induction pattern that is similar to that of the transgene. In this experiment, a drop in expression was observed in the mouse analysed around day 5 of lactation. However, a similar decrease in RNA levels was also detected for endogenous milk protein genes analysed in this experiment. This observation is not routinely seen. We do not have an explanation for this drop, but hybridization with the murine gamma-actin probe (panel F) confirmed that equal amounts of total RNA were loaded in all lanes, except for lane P19. An explanation for this observation would be that in the tissue sample taken at day 5 less epithelial cells were present than in the other samples. The RNA blot was reprobed with several cDNA probes specific for endogenous murine milk protein genes. The results are shown in Fig. 3 panels C, D and E and reflect the mRNA levels of αS-, β-casein and WAP respectively. It appears that all of these genes are already expressed at day 17 of pregnancy. Quantitative scanning of the blot revealed (after correction for differences in the gamma-actin signal) that at day 19 of pregnancy, as compared to day 17, the hybridization signals for αS-, β-casein and WAP were increased 1.7-, 2.0-, and 5.6-fold respectively. After parturition, the hybridization signals for αS-, β-casein, and WAP were increased another 1.8-, 1.3-, and 1.1-fold respectively. Similar to the expression pattern of the transgene, all three endogenous milk protein genes reach maximum message levels at around day 13 of lactation.

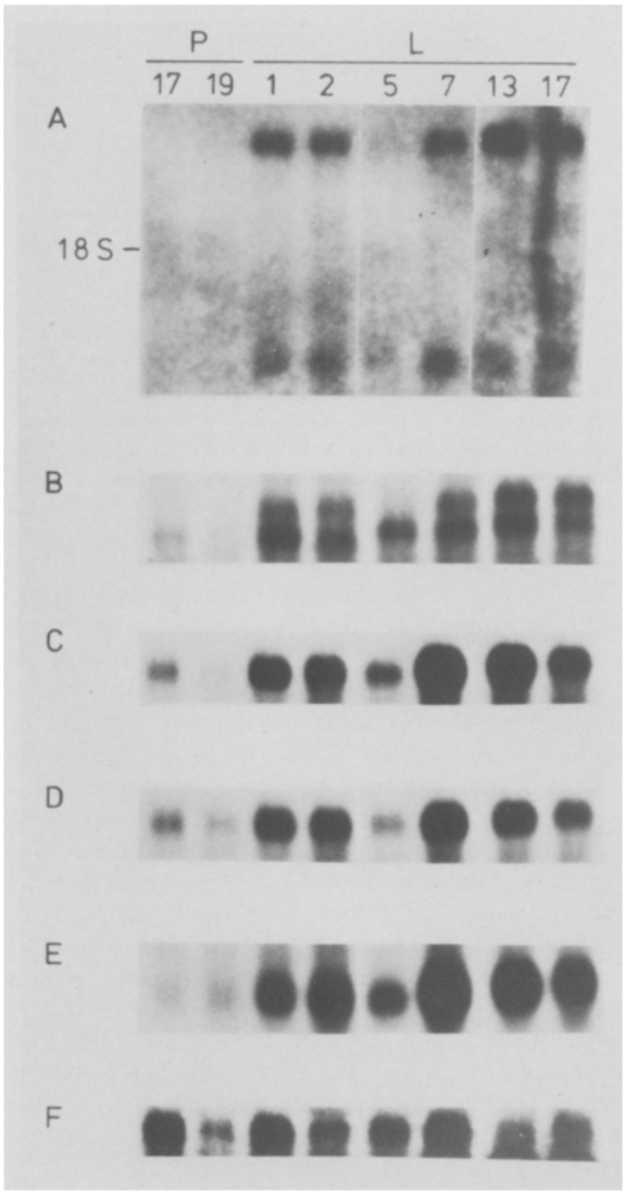

Fig. 3. Northern blot analysis of mRNA from mammary glands at different stages of lactation. Polyadenylated RNA was prepared from mammary gland tissue of transgenic offspring from line #10 isolated at the indicated days of pregnancy (P) or lactation (L). The position of the 18S ribosomal RNA is indicated on the left in panel A only. Two μg RNA was loaded per lane. For each sample lane mammary gland RNA from one mouse was used. Probes used were: panel A, bovine αS1-casein 3′ UTR; panel B, hLF cDNA probe IV; panel C, murine αS-casein; panel D, murine β-casein; panel E, murine WAP. To take account of loading differences, the blot was hybridized to a murine gamma-actin probe, panel F.

When the hLF cDNA was used as a probe on the same RNA blot (Fig. 3, panel B) the hLF RNA as well as the murine lactoferrin RNA (which is slightly smaller) could be detected. The latter species is clearly detectable at day 17 of pregnancy. This indicates that the induction of expression of the murine LF gene is not different from the other milk protein genes. Mouse LF is expressed at high levels in mouse milk. However, since the probe used in this experiment is specific for hLF, the mLF level is underestimated. The murine LF RNA level does not seem to be decreased at day 5 of lactation, in contrast to the other milk protein RNAs.

Transcription initiation site of the transgene

To determine whether the transgene uses the same transcription start site as the natural bovine casein gene, we performed primer extension experiments. Total RNA was isolated from the mammary glands of a lactating mouse, transgenic for 16,8hLF4, expressing hLF in the milk at a level of about 125 μg ml^{-1}. A 20-mer oligonucleotide (for sequence, see Materials and methods), complementary to part of the bovine αS1-casein signal sequence was used as a primer. The location of this primer is immediately down-

stream of the 21 nt synthetic linker that was inserted into the second exon of the casein/hLF construct. Therefore, in comparing extension products from the transgenic mouse RNA with those of the bovine RNA, a band 21 nt longer is expected. As can be seen in Fig. 4, bovine mammary gland RNA (lanes A, B, and C) yielded a major band with several minor bands. The size of the major band (105 nt) correlates well with the size that is to be expected upon transcription initiation at the downstream site described by (Stewart *et al.*, 1984), if the first intron is correctly removed during RNA processing. Primer extention on RNA isolated from the mammary gland of the transgenic mouse yielded indeed a major band that was approximately 21 nt longer. This result indicates that the transcription initiation site, as well as the splice-donor and acceptor sites, are the same in the transgene RNA and the natural bovine casein RNA.

Characterization of human lactoferrin expressed in milk of transgenic mice

To assess whether or not hLF produced in milk of transgenic mice is equivalent to human milk derived LF, milk from four founders, from their (F_1) offspring and from the (F_1) offspring of the male founders was analysed. Two types of radioimmunoassays (RIAs) were used. A dose response curve of transgenic mouse milk in the RIA with a monoclonal antibody is shown in Fig. 5a. The RIA using the polyclonal antibody yielded a similar picture. Both RIAs showed dose response curves of transgenic mouse milk that are parallel to that of the standard (purified hLF derived from pooled human milk). The parallel dose response curves obtained indicate that the accessibility of epitopes for both the monoclonal and polyclonal antibody are the same in transgenic and natural hLF, which suggests that both molecules are immunologically similar. Identical levels of hLF in transgenic mouse milk were measured, regardless of the assay used. SDS-PAGE and immunoblot analysis (Fig. 5b) revealed a relative molecular mass (M_r) of 80000 for both transgenic and human milk derived hLF, which corresponds well with published data (Kijlstra *et al.*, 1989).

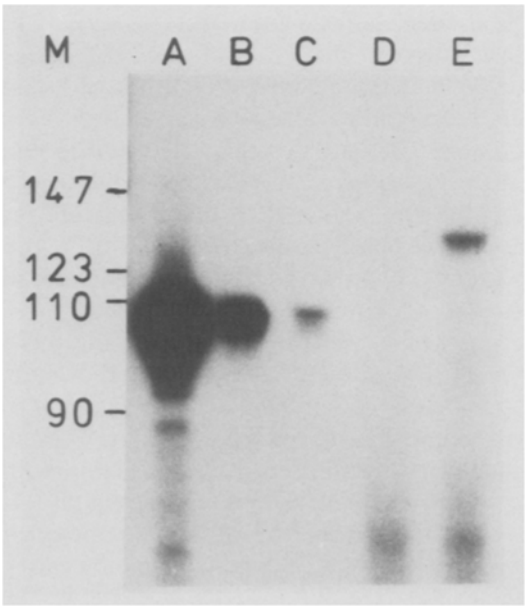

Fig. 4. Primer extension analysis of mRNA. 1, 0.1 and 0.01 μg of polyadenylated RNA from mammary gland tissue of a lactating cow (lanes A, B and C respectively), 50 μg of total RNA from mammary gland tissue of a non-transgenic mouse (day 8 of lactation, lane D), and from a transgenic mouse (day 8 of lactation, lane E) was annealed to a labelled 20-mer specific for the bovine αS1-casein signal sequence (sequence shown in Materials and methods). Extension was performed using AMV reverse transcriptase. The size marker (pBR322 cut with *Msp* I) is indicated on the left.

Expression levels of hLF in mouse milk samples

To determine hLF expression levels, milk samples were taken at different points during lactation, and analysed using the RIA with monoclonal anti-hLF antibody. All lines analysed expressed hLF in their milk. Individual mice (F_1) from three out of six lines showed similar expression levels: line #8 ranged from 4 to 24 μg ml^{-1}, line #10 from 4 to 29 μg ml^{-1}, line #11 from 0.3 to 36 μg ml^{-1}. Of the remaining three lines (F_1), two showed lower expression levels: line #7 ranged from 0.9 to 5.6 μg ml^{-1} and line #13 from 0.0 to 9.0 μg ml^{-1}. Founder #9 was a germline mosaic and showed an expression of 0.6 μg ml^{-1}. Its transgenic offspring also expressed at levels around 1 μg ml^{-1}. Considerable variation in the hLF-levels between different milk samples of one lactation was observed. No consistent patterns of fluctuation could be recognized although, in general, expression levels at mid-lactation were higher than begin-lactation, which is consistent with the RNA data shown in Fig. 3. There was no apparent relation between copy number and expression level of the hLF-transgene.

Discussion

This paper is the first report describing expression of recombinant hLF in the milk of a transgenic mammal. Other groups have reported the expression of recombinant hLF in fungi (Ward *et al.*, 1992) and baby hamster kidney cells (Stowell *et al.*, 1991). Our ultimate goal is to produce hLF in large quantities in milk of transgenic cows. We have reported earlier the feasibility of generating transgenic dairy cattle (Krimpenfort *et al.*, 1991). In view of their shorter generation time, mice serve as a useful model system to optimize expression cassettes and to study functional and structural characteristics of hLF thus produced. Several groups have reported that regulatory sequences of milk protein genes from other species are functional in transgenic mice (Hennighausen, 1990). Therefore, we

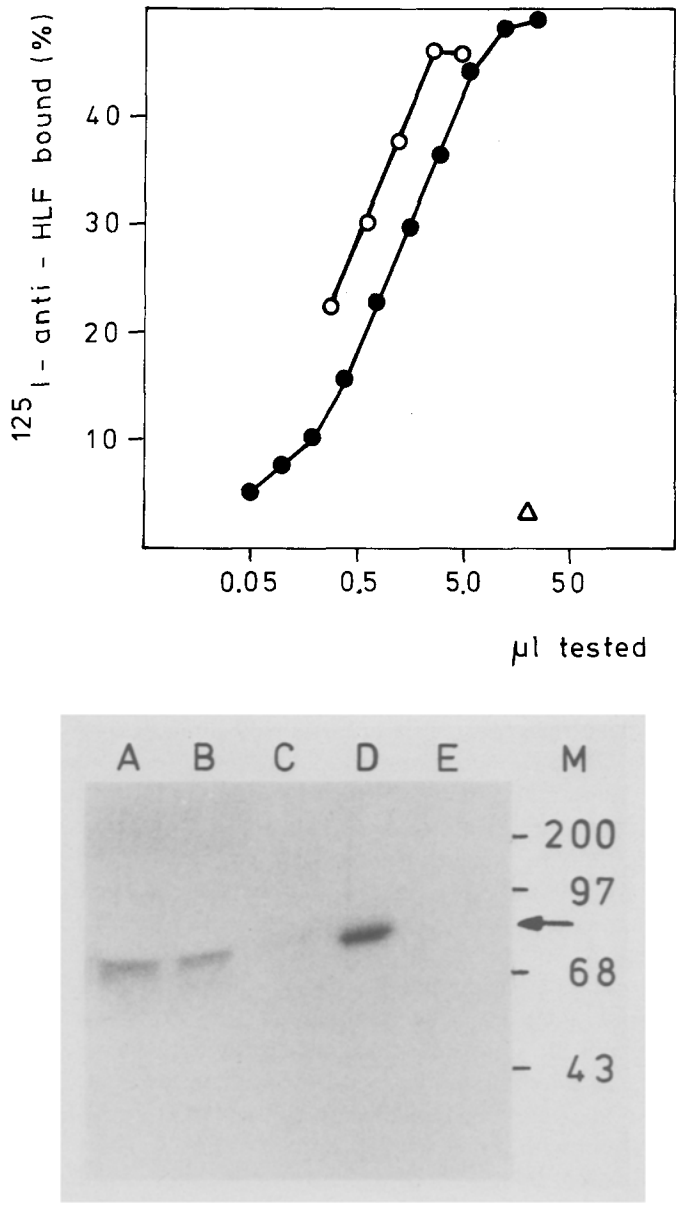

Fig. 5. Analysis of transgenic hLF by RIA and immunoblotting. Dose response curve of whey from milk of an individual mouse of line #10 (open circles) and human milk derived hLF (closed circles) with monoclonal (A) anti-hLF antibody coupled to Sepharose. The volume of whey tested is indicated on the abcissa. The negative control point (open triangle) was obtained using whey from milk of a non-transgenic mouse. B) Immunoblotting of transgenically produced hLF. Human LF was immunoprecipitated from milk of transgenic mouse #10 (containing 28 µg hLF ml^{-1}). The immunoprecipitate was resuspended in 50 µl of non-reducing loading buffer. In lane A, 15–20 ng of the immunoprecipitated hLF was loaded. In lanes B, and C, two-fold dilutions were loaded. Lane D contains 20 ng of hLF from human milk. Lane E contains 10 µl of immunoprecipitated non-transgenic mouse milk. Lane M contains molecular weight markers (Gibco). The position of hLF is indicated with an arrow.

expect that data derived from expression of hLF in transgenic mice can be extrapolated to larger animals such as cows.

To limit transgene expression to the lactating mammary gland, bovine αS1-casein regulatory sequences were incorporated in the expression plasmids. Indeed, transcription of the transgene described in this paper is restricted to the mammary gland of lactating female mice and is initiated most likely at the same transcription initiation site as compared to the bovine αS1-casein gene in the bovine mammary gland. The temporal expression of the transgene in the mouse is slightly different from endogenous milk protein genes: whereas all murine milk protein genes analysed in this study are already expressed at measurable levels during late pregnancy (which is in accordance with previously published data (Hennighausen *et al.*, 1991; Teng *et al.*, 1989)), expression of the transgene is induced around parturition. A similar delayed induction pattern was reported (Persuy *et al.*, 1992) for the caprine β-casein in transgenic mice. Goodman and Shanbacher (1991) have shown that induction of the casein genes in the bovine mammary gland also occurs around the day of parturition. Recently, this induction pattern was confirmed using another mouse line containing the transgene 16,8hLF4. No expression was detected at day 19 of pregnancy, whereas the endogenous WAP is already expressed at detectable levels. These data combined suggest that the bovine *cis*-acting signals involved in induction of expression of the αS1-casein gene are fully functional in mice and act in a species-independent fashion. The data described here further imply that all sequences required for tissue- and stage-specific expression of the bovine αS1-casein gene are present in the regions we used to construct p8,8hLF4 (i.e. about 6.2 kb upstream of the transcriptional start site and 8 kb downstream of the translational stop codon).

The expression levels in mice containing 8,8hLF4 are relatively low. Meade *et al.* (1990) have shown that 20 kb of the bovine αS1-casein flanking sequences, in combination with genomic urokinase sequences, can result in high level mammary gland-specific expression. Recently, we have obtained expression levels in the mg ml^{-1} range by using the same 6.2 kb 5′ αS1-casein promoter region as described here for p8,8hLF4 in combination with genomic hLF sequences [De Wit, I.C.M. and Pieper, F.R., unpublished]. This indicates that the relatively low expression levels obtained with 8,8hLF4 are the result of the use of the hLF cDNA rather than the lack of transcriptional capability of the promoter as such.

We have observed considerable variation in hLF levels of milk during the course of one lactation period and also between different lactations of one animal as well as between littermates. We also observed some variation in the hLF mRNA levels at different stages of lactation. The reason for the variation in protein levels is unclear. It

could be caused by differences at the gene expression level, or caused by differences in the health of the mammary gland at the time of milking or caused by differing conditions during drawing of the milk, both of which could have resulted in leakage of varying amounts of interstitial fluid into the milk.

Thus far, we did not observe any differences between hLF expressed in milk of transgenic mice and human milk derived LF with respect to immunoreactivity using two independent antibody preparations and molecular mass as determined by SDS-PAGE. Currently, we are investigating in more detail whether there are any structural or functional differences between the two protein species.

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