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Lactation is disrupted by α-lactalbumin deficiency and can be restored by human α-lactalbumin gene replacement in mice

(embryonic stem cells/gene targeting/lactose/milk)

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ABSTRACT **Mice carrying either a deletion of the murine α-lactalbumin (α-lac) gene (null allele) or its replacement by the human α-lac gene (humanized allele) have been generated by gene targeting. Homozygous null females are α-lac-deficient, produce reduced amounts of thickened milk containing little or no lactose, and cannot sustain their offspring. This provides definitive evidence that α-lac is required for lactose synthesis and that lactose is important for milk production. Females homozygous for the humanized allele lactate normally, indicating that human α-lac can replace murine α-lac. Mouse and human α-lac expression was compared in mice heterozygous for the humanized allele. The human gene expressed ≈15-fold greater mRNA and ≈14-fold greater protein than the mouse, indicating that the major determinants of human α-lac expression are close to, or within, the human gene and that the mouse locus does not exert a negative influence on α-lac expression. Variations in α-lac expression levels in nondeficient mice did not affect milk lactose concentration, but the volume of milk increased slightly in mice homozygous for the humanized allele. These variations demonstrated that α-lac expression in mice is gene dosage dependent.**

Milk is a complex mixture of proteins, lipids, carbohydrates, and inorganic components, the composition of which varies widely between species. The whey protein α-lactalbumin (α-lac) is thought to influence milk carbohydrate and fluid content through its role as a component of the lactose synthase complex (1). Lactose provides a major osmotic component of milk and determines milk volume by influencing water influx. It has been proposed that the lactose content of milk is directly related to the quantity of α-lac present (2), but a causative relationship has yet to be demonstrated *in vivo*.

We have used gene targeting in embryonic stem cells to produce null and replacement alleles at the murine α-lac locus (3). Mice in which the murine α-lac gene has been completely deleted (null allele) provide a definitive test of the role and importance of α-lac in lactation and mammary physiology. Mice in which the murine α-lac gene has been replaced by the human gene have been used (*i*) to test whether human α-lac can functionally substitute for mouse α-lac, and (*ii*) to investigate determinants of α-lac gene expression, since humans and mice produce markedly different amounts of milk α-lac.

MATERIALS AND METHODS

Mouse Lines. Derivation of mice bearing null and humanized α-lac alleles has been described (3).

RNA Analysis. Total RNA was prepared from abdominal mammary glands of female mice 5–6 days postpartum. RNase protection analysis used a [^{32}P]CTP-labeled antisense RNA probe transcribed from a 455-bp *Hin*dIII–*Bal* I mouse α-lac fragment (see Fig. 4*A*) cloned in pBluescript KS. Experimental conditions were as recommended by Promega.

Milk Composition and Yield Analysis. Milk samples were collected between days 3 and 7 of lactation. Milk fat content was measured as described by Fleet and Linzell (4). Defatted milk was assayed for protein (5), and lactose was measured by a method adapted from that of Bergmeyer and Bernt (6).

Milk yield was estimated using a tritiated water technique described by Knight *et al.* (7) in mice suckling young over a 48-hr period between days 3 and 6 of lactation.

Milk α-lac Analysis and Quantification. α-lac was detected on Western blots by absorption with rabbit anti-human α-lac antiserum (Dako), followed by goat anti-rabbit IgG peroxidase–antibody conjugate and chemiluminescence.

α-lac was purified and quantified from milk samples by calcium-dependent adsorption to phenyl-Sepharose (Pharmacia) (8). α-lac concentrations were calculated by comparing the OD_{280} of the α-lac fraction with a standard curve constructed using purified human α-lac (see Fig. 5).

Histology. Pups were removed for 2 hr from lactating mothers on the sixth day postpartum; mothers were sacrificed; and thoracic mammary glands were dissected, fixed, embedded, and hematoxylin/eosin stained using standard methods.

RESULTS

Mouse α-lac Gene Deletion. We have established a line of mice in which a 2.7-kb fragment covering the complete mouse α-lac coding region and 0.57 kb of promoter has been deleted and replaced with a 2.7-kb fragment containing a selectable marker gene (Fig. 1; ref. 3). Here we describe our analysis of animals carrying this allele, designated α-lac$^-$. The wild-type mouse α-lac allele is designated α-lac^m.

Northern blot analysis showed that α-lac mRNA was absent from lactating mammary glands of α-lac$^-$/α-lac$^-$ homozygotes (data not shown).

α-lac deficiency had no apparent effect in mice other than during lactation. α-lac$^-$/α-lac$^-$ homozygotes and α-lac^m/α-lac$^-$ heterozygotes of both sexes were normal in appearance, behavior, and fertility. However, α-lac$^-$/α-lac$^-$ homozygous females could not rear litters successfully. Their pups failed to thrive and died within 5–10 days but survived when transferred to wild-type foster mothers. Conversely, offspring from wild-type mothers transferred to homozygous α-lac$^-$/α-lac$^-$ mothers were not sustained. Table 1 shows that pups raised by α-lac$^-$/α-lac$^-$ mothers were approximately half the weight of those raised by α-lac^m/α-lac^m wild-type mice. Estimates of milk yield are consistent with this: α-lac^m/α-lac$^-$ heterozygotes produced similar quantities of milk as wild type, but the

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Abbreviation: α-lac, α-lactalbumin.

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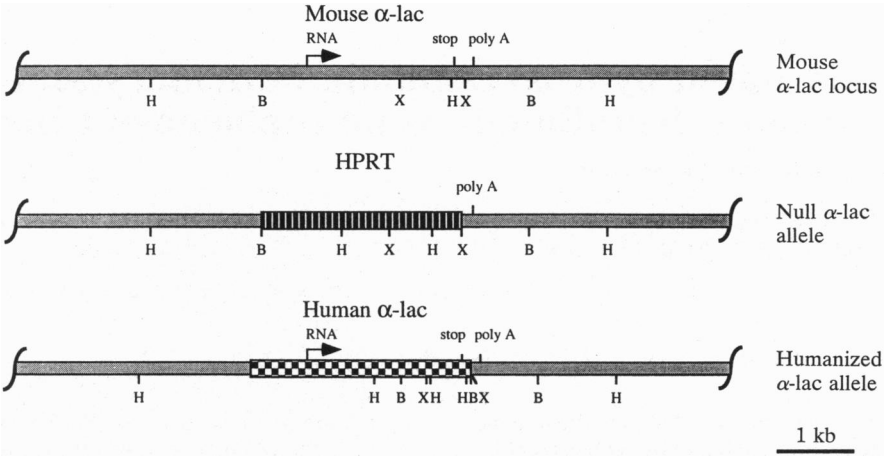

FIG. 1. Structure of null and humanized α-lac alleles. (*Top*) Wild-type murine α-lac locus. The position and direction of the transcribed region are indicated by the arrow; the translational stop site and RNA polyadenylylation sites are indicated. (*Middle*) Structure of the null allele. The striped bar indicates the hypoxanthine phosphoribosyltransferase (HPRT) selectable cassette. (*Bottom*) Structure of the human replacement allele. The checkered bar shows the human α-lac fragment. The transcription initiation, translational stop, and polyadenylylation sites are shown. Restriction enzyme sites shown are *Hin*dIII (H), *Bam*HI (B), and *Xba* I (X).

yield of α-lac$^-$/α-lac$^-$ homozygotes was severely reduced (Table 1).

Milk from α-lac^m/α-lac$^-$ heterozygotes was similar in appearance and composition to wild type (Table 1). In contrast, milk from α-lac$^-$/α-lac$^-$ homozygotes was viscous, difficult to express, and had a markedly different composition to wild type. Fat content was ≈60% greater than wild type, protein content was ≈88% greater, and lactose was absent. The apparent 0.7 mM lactose detected in α-lac$^-$/α-lac$^-$ females represents milk glucose content, since the lactose assay used involved the enzymatic conversion of lactose to glucose. Direct assay of glucose in wild-type milk indicated a concentration of 1.8 mM.

Western blot analysis of milk protein failed to detect α-lac in milk from α-lac$^-$/α-lac$^-$ homozygotes (Fig. 2, lane F). This was confirmed by phenyl-Sepharose chromatography (Table 2; see also Fig. 5). The α-lac concentration in α-lac^m/α-lac$^-$ heterozygote milk was estimated as 0.043 mg/ml, which is half that of wild type (Table 2).

α-lac deficiency had no apparent effect on mammary gland development. Table 1 shows that total mammary tissue weights of wild-type, heterozygous α-lac^m/α-lac$^-$, and homozygous α-lac$^-$/α-lac$^-$ lactating mothers were not significantly different. Light microscopy of mammary glands (Fig. 3) revealed that heterozygous and homozygous α-lac$^-$/α-lac$^-$ glands were histologically normal, but alveoli and ducts of homozygous glands were distended and clogged.

Replacement of Mouse α-lac by Human α-lac. We have generated mice carrying the human α-lac gene at the mouse α-lac locus. The 2.7-kb mouse α-lac deletion was replaced by a 2.97-kb fragment extending from 0.77 kb upstream of the human transcription initiation site to an *Eco*RI site 136 bp 3′ of the human translational stop site (Fig. 1; ref. 3). Here we describe our analysis of mice carrying this allele, designated α-lac^h.

α-lac^m/α-lac^h heterozygous and α-lac^h/α-lac^h homozygous mice were normal in appearance, fertility, and behavior. In contrast to α-lac$^-$/α-lac$^-$ mice, α-lac^h/α-lac^h homozygous mothers produce apparently normal milk and rear offspring similar in weight to those of wild-type mothers (Table 1). Therefore the human gene can functionally replace the mouse gene. Analysis of milk composition (Table 1) shows that lactose concentration in α-lac^m/α-lac^h and α-lac^h/α-lac^h milk is similar to that in wild type. Although both protein and fat concentrations seem reduced in α-lac^h/α-lac^h homozygous animals, only the fat reduction was statistically significant. These animals show an increase in milk volume over wild type (Table 1).

Relative Quantification of Human and Mouse α-lac RNA. Human milk contains considerably more α-lac (2.9 mg/ml) than murine milk (0.09 mg/ml; Table 2). We wished to determine what level of expression the human gene fragment displayed when it was placed at the mouse locus. α-lac^m/α-lac^h heterozygous mice provided an ideal means of addressing this question, as the expression of the human and mouse alleles could be directly compared within the same animal.

Fig. 4*A* shows how mouse and human α-lac mRNA were distinguished. α-lac^h mRNA contains a "tag" of 120 bases of untranslated mouse sequences at the 3′ end. A uniformly radiolabeled mouse RNA probe was used in an RNase protection assay to detect and distinguish human and mouse α-lac mRNA in the same RNA sample. The relative abundance of each mRNA was calculated from the isotope content of protected fragments.

Table 1. Milk composition, pup weight, mammary tissue weight, and milk yield in targeted mouse lines

Parameter	Genotype				
	α-lac^m/α-lac^m	α-lac^m/α-lac$^-$	α-lac$^-$/α-lac$^-$	α-lac^m/α-lac^h	α-lac^h/α-lac^h
Fat, % (vol/vol)	28.23 ± 1.65 (7)	29.6 ± 1.3 (6)	45.25 ± 2.15*** (6)	25.25 ± 1.36 (7)	21.2 ± 0.23* (4)
Protein, mg/ml	87.52 ± 5.82 (7)	95.81 ± 9.5 (5)	164.63 ± 13.92*** (8)	94.51 ± 5.97 (7)	77.07 ± 1.05 (4)
Lactose, mM	62.44 ± 9.27 (7)	42.7 ± 4.2 (6)	0.7 ± 0.34** (3)	42.40 ± 1.93 (7)	56.85 ± 3.8 (4)
Single pup weight, g	2.82 ± 0.25 (8)	3.14 ± 0.1 (7)	1.52 ± 0.12*** (10)	2.9 ± 0.15 (8)	3.4 ± 0.75 (4)
Mammary tissue weight per pup, g	0.34 ± 0.06 (7)	0.4 ± 0.1 (7)	0.35 ± 0.05 (8)	0.31 ± 0.04 (6)	0.51 ± 0.09 (4)
Milk yield, g/day	7.51 ± 0.44 (4)	6.7 ± 0.38 (6)	1.37 ± 0.48*** (4)	NT	9.94 ± 0.65* (5)

Values given are the means ± SE. Numbers in parentheses indicate the number of mothers analyzed. NT, not tested. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$ by unpaired t test.

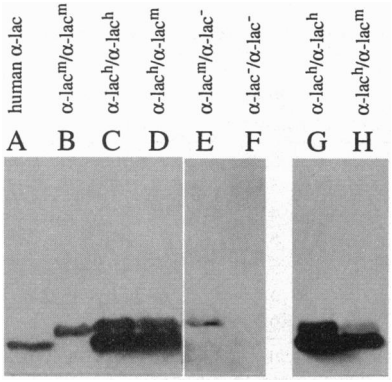

FIG. 2. Western analysis of α-lac from targeted mouse strains. Lane A, purified human α-lac. Lanes B–F show samples of milk from targeted mice; genotypes are indicated above the lane markers. Lanes G and H are a shorter exposure of lanes C and D.

The RNase protection assay is shown in Fig. 4*B*. Lane A shows undigested 455-base probe, and lane K shows yeast tRNA control. Wild-type mouse RNA protected a 305-base RNA fragment (lane B). Homozygous α-lac^h/α-lac^h gland RNA protected a smaller 120-base RNA fragment (lane C). Lanes D–J show a series of heterozygous α-lac^m/α-lac^h animals. Small and large protected fragments in each sample indicate the presence of both human and mouse α-lac mRNA. Protected fragments were excised from the gel, radioisotope content was measured and adjusted for size difference, and the ratio of human to mouse α-lac mRNA was estimated (Table 3). Although there was variation between individual animals, human α-lac mRNA was on average 15-fold more abundant than mouse mRNA.

Human α-lac Protein Expression. Fig. 2 shows a Western blot analysis of α-lac in targeted mouse lines. Human α-lac can be distinguished from mouse α-lac by its faster electrophoretic mobility (lanes A and B). The prominent lower band in α-lac^h/α-lac^h homozygotes and α-lac^m/α-lac^h heterozygotes (lanes C, D, G, and H) corresponds to the position of the human α-lac standard and was only observed in mice that express human α-lac, generated by either gene targeting or pronuclear microinjection (data not shown). This identity was confirmed by phenyl-Sepharose chromatography (data not shown, but see Fig. 5) and analysis of peptides released by cyanogen bromide cleavage (data not shown). The slower band, similar to mouse α-lac, is also a human α-lac gene product, the nature of which is unknown. This species varied in intensity with α-lac^h gene dosage (lanes G and H) and was also present in milk from human α-lac transgenic mice generated by pronuclear microinjection (data not shown).

The α-lac content of milk samples was quantified by phenyl-Sepharose chromatography. Fig. 5 shows superimposed absorbance profiles of column eluates of three illustrative milk samples including the α-lac^m/α-lac^h heterozygote and α-lac^h/α-lac^h homozygote shown in Fig. 2. Milk α-lac content was estimated by comparing integrated peak area with a human α-lac standard curve. Table 2 shows the concentration of α-lac in milk samples from mouse lines and lactating women. As milk volumes were broadly similar (Table 1), α-lac concentration represents the amount synthesized. α-lac^m/α-lac$^-$ heterozygotes (0.043 mg/ml) show an α-lac concentration half that of wild type (0.09 mg/ml); α-lac^m/α-lac^h heterozygotes express 0.65 mg/ml, and α-lac^h/α-lac^h homozygotes express 1.38 mg/ml. Thus the concentration of α-lac in milk is directly related to gene dosage. We also conclude that the total α-lac expressed by α-lac^m/α-lac^h heterozygotes is made up of human and mouse α-lac of 0.61 mg/ml and 0.043 mg/ml, respectively. The human protein is therefore 14-fold more abundant than mouse α-lac, a figure remarkably consistent with the relative proportions of mRNA.

Table 2. Milk α-lac content in humans and targeted mouse lines

Source	α-lac, mg/ml
Human	2.9 ± 0.1 (2)
α-lac^m/α-lac^m mice	0.09 ± 0.005 (6)
α-lac$^-$/α-lac$^-$ mice	0 (3)
α-lac^m/α-lac$^-$ mice	0.043 ± 0.004 (5)
α-lac^m/α-lac^h mice	0.65 ± 0.07 (4)
α-lac^h/α-lac^h mice	1.38 ± 0.12 (5)

α-lac content of milk samples was estimated by phenyl-Sepharose chromatography. Values are the means ± SE. Numbers in parentheses indicate the number of mothers analyzed.

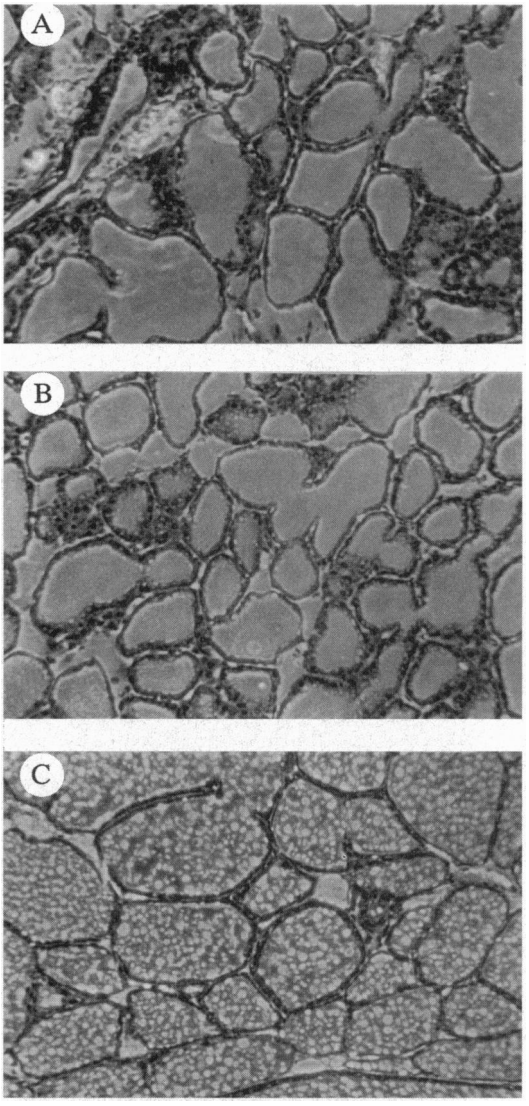

FIG. 3. Histological analysis of wild-type and α-lac$^-$ lactating mammary glands. The light micrographs shown are hematoxylin/eosin-stained sections of mammary tissue (original magnification, ×100). The genotypes of each gland are α-lac^m/α-lac^m (*A*), α-lac^m/α-lac$^-$ (*B*), and α-lac$^-$/α-lac$^-$ (*C*).

DISCUSSION

We have produced mice with a genetic deficiency of α-lac and then restored the deficiency by introducing a functional human α-lac gene at the mouse α-lac locus.

α-lac deficiency severely disrupted lactation in mice, unlike β-casein deficiency (9). α-lac-deficient animals produced small

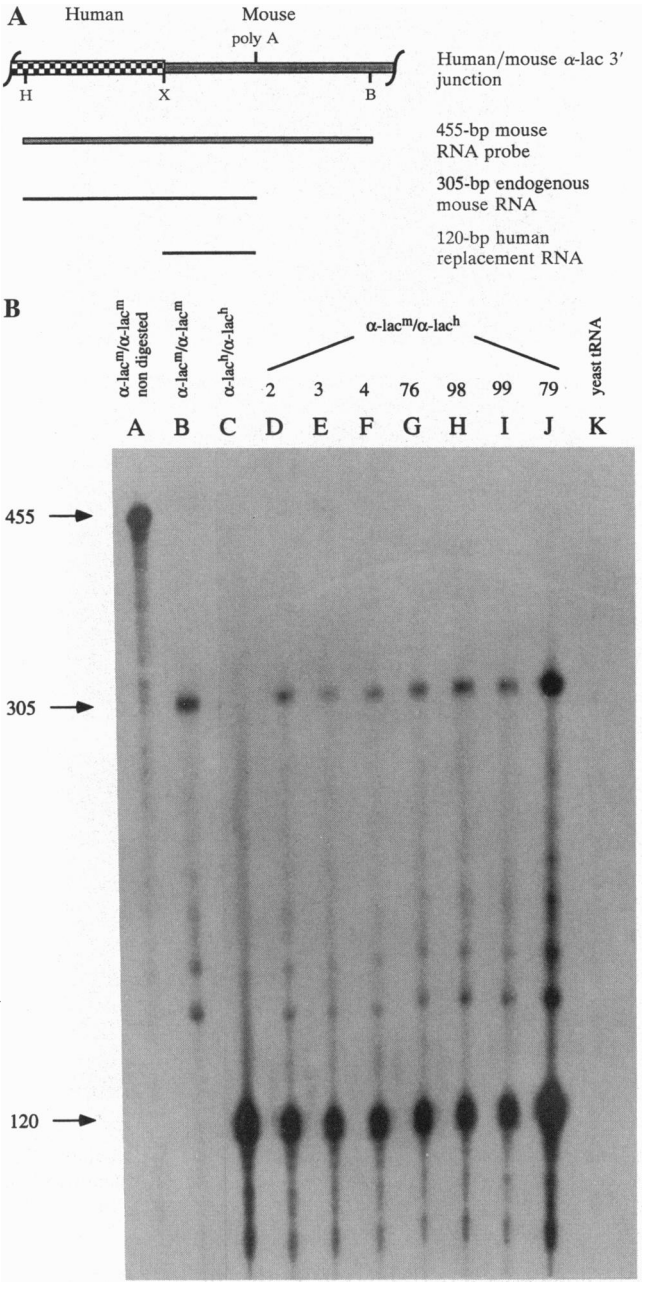

FIG. 4. (*A*) RNase protection assay used to distinguish human replacement and mouse α-lac mRNA. The 3′ junction between mouse and human DNA in the α-lac^h allele lies between the translational stop site and the polyadenylylation signal. Human α-lac mRNA contains 120 bp of mouse sequences in the 3′ untranslated region. Human replacement and mouse α-lac mRNA were detected by hybridization with a mouse RNA probe and distinguished by the size of RNA fragments protected from RNase digestion. Human sequences are indicated by the checkered bar, and mouse sequences are indicated by the shaded bar. Restriction enzyme sites shown are *Hin*dIII (H), *Bal* I (B), and *Xba* I (X). (*B*) RNase protection assay of mouse and human replacement α-lac mRNA. The autoradiograph shown is of a 5% polyacrylamide/urea thin layer gel. The source of RNA is indicated above the lane markers. Lane A, wild-type RNA hybridized to the mouse RNA probe undigested with RNase. Lanes D–J, RNA samples from α-lac^m/α-lac^h heterozygotes. The numbers indicate individual mice and are the source of the quantitative estimates given in Table 3. The predicted sizes of protected fragments are indicated.

amounts of thickened milk containing no lactose, which supports the widely held view that α-lac is a necessary component of the lactose synthase complex (2) and that milk lactose makes an important contribution to milk osmotic pressure and volume. These findings confirm those of a recent α-lac gene inactivation experiment (10).

Table 3. Relative quantification of human and mouse α-lac mRNA in α-lac^m/α-lac^h heterozygous mammary glands

Lane*	Mouse no.	120-base fragment†	305-base fragment†	Ratio of human to mouse RNA‡
D	2	5,957	1000	15:1
E	3	5,770	547	26:1
F	4	4,825	810	15:1
G	76	6,018	1077	14:1
H	98	5,206	1452	9:1
I	99	5,481	1117	12:1
J	79	26,858	3561	19:1

*Lane designations indicate the source of protected fragments and correspond to those shown in Fig. 4*B*.
†Numbers are expressed in cpm.
‡Ratio of cpm of 120-base fragment multiplied by 2.54 (to adjust for size difference) to cpm of 305-base fragment.

α-lac deficiency did not affect any aspect of mouse development or physiology other than lactation. This is consistent with previous reports that α-lac expression is restricted to lactating mammary glands (11). We observed no effect of α-lac deficiency or overproduction on mammary development or growth during lactation. It is therefore unlikely that α-lac acts as an inhibitor of mammary growth *in vivo*, as has been suggested (12).

It has been proposed that milk lactose content is a direct function of the amount of α-lac present (2). Comparison of milk from different species (5), breeds of cattle (13), and animals under different nutritional regimes (14) shows a correlation between levels of α-lac and milk lactose. The four genotypes of mice we describe here expressed a range of milk α-lac concentrations from 0 in α-lac$^-$/α-lac$^-$ homozygotes to

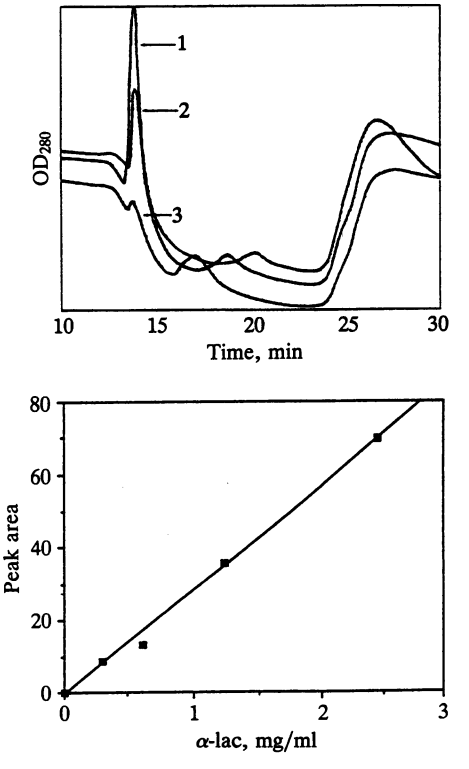

FIG. 5. Quantification of α-lac by hydrophobic interaction chromatography. (*Upper*) Phenyl-Sepharose elution profiles of three milk samples. 1, α-lac^h/α-lac^h homozygote; 2, α-lac^m/α-lac^h heterozygote (mouse 76); 3, α-lac^m/α-lac^m wild type. (*Lower*) Standard curve of known quantities of human α-lac plotted against integrated peak area.

1.38 mg/ml in α-lac^h/α-lac^h homozygotes. The expression levels correlated closely with gene dosage. Analysis of milk lactose concentrations in these mice did not indicate a direct relationship between α-lac and lactose content. All genotypes, apart from α-lac$^-$/α-lac$^-$ homozygotes, showed milk lactose concentrations similar to wild-type levels. Differences in lactose content were unlikely to be masked by changes in milk volume as only α-lac^h/α-lac^h homozygotes showed a slight increase in milk volume. It is uncertain whether the reduced fat content observed in α-lac^h/α-lac^h homozygotes was due to dilution or represented a real reduction in lipid synthesis. Clearly, larger numbers of mice must be analyzed to clarify the relationships between milk α-lac, lactose, and volume.

Comparative analysis of milk from different species has suggested an inverse relationship between milk sugar and fat content (15, 16). Milk compositions range from high sugar and low fat content in primates to low sugar and high fat content in marine mammals. We were interested in the effect of experimental reduction of milk lactose on milk fat content. Comparison of α-lac-deficient milk with wild-type milk showed a 60% increase in fat content and an 88% increase in protein content. These data probably reflect the overall concentration of milk constituents in α-lac-deficient milk and do not suggest any increased production of lipids. However, the fat content of α-lac-deficient milk obtained by manual expression may underestimate the level of lipid synthesis. The abundant droplets within alveolar lumina of α-lac-deficient mothers could indicate poor lipid transport out of alveoli and small ducts when milk is thickened.

The viscosity of α-lac-deficient milk was probably the principal cause of litter death, in that it prevented regular and effective milk removal by the pups. Milk secretion in other mammals is regulated by the frequency and completeness of milk removal through feedback inhibition by a secreted milk protein (17). A detailed histological study over the full course of lactation in α-lac-deficient mice has yet to be carried out, but we predict that impaired milk removal would progressively inhibit milk secretion, decrease mammary epithelial cell differentiation (18), and eventually precipitate tissue involution (19). Analysis of α-lac, lactose, and lipid synthesis during short-term mammary cell culture will provide a clearer indication of the direct metabolic consequences of α-lac deficiency or overexpression, distinct from effects due to milk viscosity and stasis.

Restoration of normal lactation by the human gene indicates that human α-lac can form a functional interspecies hybrid lactose synthase complex. While interspecific interactions between α-lac and β-1,4-galactosyltransferase have been suggested by studies *in vitro* (20, 21), our data provide definitive evidence of function. However, it is not known how efficiently human α-lac forms lactose synthase complex. Human and mouse α-lac can be compared directly by titrating purified protein in a cell-free assay of lactose synthase activity (22) using Golgi membrane preparations from α-lac-deficient mice.

Human milk has a higher α-lac content (2.9 mg/ml) than most other species, including mouse (0.09 mg/ml). We wished to investigate the factors responsible for such differences in expression level. Placement of a small human α-lac gene into the mouse locus provided a means of assessing the relative importance of regulatory elements close to or within the gene and more distant influences. Regions necessary for lactation-specific expression have been identified within 0.5 kb of the 5′ flanking region of the bovine α-lac gene (23). The human α-lac gene replacement removed 0.57 kb of mouse 5′ flanking region and replaced it with 0.77 kb of human 5′ flanking region; no human 3′ flanking region was included. This human α-lac fragment showed high mRNA expression. Although, in the absence of direct measurements of transcriptional activity, we cannot exclude different stability of each mRNA, our findings do indicate that all elements necessary for high α-lac expression are contained within the human fragment and that the mouse locus does not exert a negative influence on heterologous α-lac gene expression. Further experiments are clearly required to identify the precise regions necessary for high expression.

Double replacement gene targeting allows multiple gene replacements to be made at a single locus and the effects to be studied in an otherwise undisturbed genome (3). Thus, the analysis of α-lac gene expression can be continued by further replacements of the null allele (e.g., with genes carrying mutations in putative regulatory elements).

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