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Creation and phenotypic analysis of α-lactalbumin-deficient mice

(embryonic stem cells/homologous recombination/lactose/milk/transgenic mouse)

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ABSTRACT **α-Lactalbumin is an abundant milk-specific calcium metalloprotein which has an evolutionary relationship to lysozyme. It modifies the substrate specificity of a Golgi galactosyltransferase by forming the lactose synthetase binary complex. Lactose, together with other sugars and diffusible ions, is responsible for the osmotic pressure of milk. To assess the involvement of α-lactalbumin in lactogenesis, α-lactalbumin-deficient mice were created by disrupting the gene by homologous recombination in embryonic stem cells. Homozygous mutant mice are viable and fertile but females cannot feed their offspring. They produce a highly viscous milk that pups appear to be unable to remove from the mammary gland. This milk is rich in fat and protein and is devoid of α-lactalbumin and lactose. The phenotype of heterozygous mice was found to be intermediate, with a 40% decrease in α-lactalbumin but only a 10–20% decrease in the lactose content of their milk compared with wild-type animals. These results emphasize the key function of α-lactalbumin in lactogenesis and open new opportunities to manipulate milk composition.**

Mammary epithelial cells synthesize and secrete large amounts of fat, carbohydrates, and proteins. The main specific proteins in milk are the three calcium-sensitive α_{s1}-, α_{s2}- and β-caseins that associate with κ-casein to form casein micelles, β-lactoglobulin, α-lactalbumin (αlac), and whey acidic protein (WAP, found in rodent, rabbit, and camel milks). Milk composition varies quantitatively and qualitatively between and within species. Human milk lacks β-lactoglobulin, WAP, and α_{s2}-casein and contains only a small amount of α_{s1}-casein. Furthermore, a few females producing an atypical milk have been found in populations of domestic dairy ruminants that have not undergone an intensive selection; absence of α_{s1}- or β-casein in goats (1, 2) and of β-lactoglobulin in zebus (F. Grosclaude and M. F. Mahé, personal communication). In contrast, κ-casein and αlac seem to be ubiquitous milk proteins, which is in accordance with their known biological roles. κ-Casein is structurally important in the formation of micelles with the other caseins, thus preventing their precipitation by calcium, whereas αlac is involved in lactose synthesis (3). αlac modifies the substrate specificity of a Golgi UDP-galactosyltransferase (EC 2.4.1.22) by forming the lactose synthetase binary complex (4). As the predominant sugar, lactose is one of the key osmotic regulators of milk secretion (5), and it has been suggested that the origin of lactation was related to the molecular evolution of αlac from lysozyme (6). Lactose accounts for the majority of milk intolerance in humans and intestinal lactase deficiencies affect >80% of mankind (7).

Some mammals, however, secrete a milk that is apparently devoid of lactose, such as the California sea lion, *Zalophus californianus* (8), and the northern fur seal, *Callorhinus ursinus* (9). The milk of these sea mammals is characterized by high protein (10–14% by weight) and fat (30–50%) contents and a relatively high concentration of glucose (0.025%). Unfortunately, it is not known whether the lack of lactose is related to any αlac deficiency. In late lactation, the Tammar wallaby (*Macropus eugenii*) and a didelphid marsupial (*Monodelphis domestica*) produce milk that contains high concentrations of αlac (3 mg/ml, compared with 0.8 mg/ml in mouse milk), free galactose and glucose, but no lactose (10, 11). However, as suggested by those authors, the lack of lactose might arise from the observed concomitant increase in enzymes degrading UDP-galactose or lactose.

Furthermore, αlac was also described as a releasing factor for lysozomal enzymes (12, 13) in the early stage of mammary gland involution and as a growth inhibitory factor on epithelial cells (14). Since expression of the αlac gene begins in late pregnancy (15), any essential role of αlac in the early and middle development of the mammary gland seems unlikely.

To better understand the roles that αlac and lactose play in the lactation process of eutherian mammals, embryonic stem (ES) cells in conjunction with gene targeting were used to create an αlac-null allele, and subsequently mice were bred to be heterozygous or homozygous for this deficient gene. This approach was also used to assess the potential for modification of the protein and fat concentration and the sugar composition of the milk by generating αlac genotypes which might have important implications in terms of dairy technology and nutrition.

MATERIALS AND METHODS

Construction of αlac Targeting Vector. The targeting vector was derived in several steps from the mouse αlac-encoding gene (*Lalba*) of a BALB/c mouse (15) and both plasmids pPolyIII.I and pPolyII (16). Two *Bam*HI fragments of 4 kb and 5 kb were prepared from the 13.5-kb genomic insert (Fig. 1) containing the αlac gene. The 4-kb *Bam*HI fragment encompassing the transcription unit with 0.56 kb and 1 kb of 5′ and 3′ flanking regions, respectively, was subcloned into the *Bam*HI site of pPolyIII.I. The insert orientation was chosen so that a *Kpn* I digestion released a 1.4-kb internal fragment, encompassing the 0.56-kb 5′ flanking region and exons I and II of the transcription unit. By blunt-end ligation, a herpes simplex virus (HSV)–neomycin-resistance (Neo) cassette was introduced into the end-filled *Kpn* I-linearized recombinant plasmid. This plasmid, pPoly-Neo, was then linearized with *Sal* I. An *Xho* I–*Sal* I HSV–thymidine kinase (TK) cassette was then inserted into *Sal* I-linearized pPolyIII.I. The recombinant plasmid was linearized with *Bam*HI and the aforementioned 5-kb *Bam*HI genomic fragment corresponding to the 5′ flanking region of the αlac gene devoid of the last 0.56 kb was subcloned into it. The 6.8-kb insert released by *Xho* I was inserted into the *Sal* I-linearized

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Abbreviations: αlac, α-lactalbumin; ES cell, embryonic stem cell; HSV, herpes simplex virus; Neo, neomycin resistance; TK, thymidine kinase; WAP, whey acidic protein.

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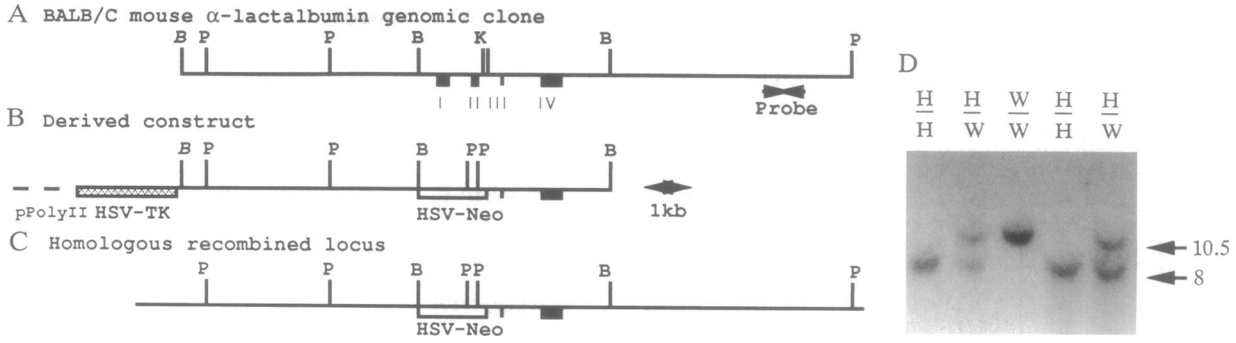

FIG. 1. Gene targeting the αlac locus. (*A–C*) Structure of the murine αlac genomic clone, of the derived construct (targeting vector), and of the recombined locus, respectively. Black boxes represent the four exons of the αlac gene. Location of the two cassettes and of the probe used in screening is indicated. P, *Pst* I; B, *Bam*HI; K, *Kpn* I; *B*, *Bam*HI site derived from the initial cloning vector (15). (*D*) Southern analysis of progeny from heterozygote matings. Genomic DNA prepared from tail biopsy samples was digested with *Pst* I. The probe hybridized with a 10.5-kb wild-type allele (*W*) fragment and an 8-kb homologous recombined allele (*H*) fragment.

pPoly-Neo. Finally, the *Xho* insert released from this last recombinant plasmid was cloned into *Xho* I-linearized pPolyII to allow subsequent linearization of the construct by *Sfi* I.

Production of Gene-Disrupted Mice. D3 ES cells (17), a gift from R. Kemler, were cultured on feeder cells (inactivated primary embryo fibroblasts) in Dulbecco's modified Eagle's medium supplemented with fetal bovine serum (10%), L-glutamine (2 mM), nonessential amino acids [1% (vol/vol) of 100% stock solution; GIBCO/BRL], 2-mercaptoethanol (0.1 mM), and leukemia inhibitory factor (1000 units/ml, prepared according to ref. 18). The αlac targeting vector was linearized with *Sfi* I and electroporated into D3 ES cells at passage 21 (30 μg of DNA per 2.5×10^6 cells; 800 V, 3 μF). After double selection [with G418 (100 μg/ml) and ganciclovir (2 μM)], 150 clones were isolated. Half of each clone was frozen and half was screened by Southern analysis of *Pst* I-digested genomic DNA and probed with an 800-bp 3′ external probe (Fig. 1). This probe identified a 10.5-kb germ-line band and an 8-kb homologous recombined band (Fig. 1). The seven ES clones with the targeted αlac allele were thawed 5 days prior to injection and 5–10 cells were microinjected into C57BL/6 blastocysts which were then reimplanted into 2.5-day-pseudopregnant (C57BL/6 × CBA)F_2 mice. All male chimeras were mated with C57BL/6 females, and agouti offspring were screened by Southern analysis. Heterozygotes were interbred to produce homozygotes.

Southern, Northern, and Milk Analyses. Genomic DNA preparation, Southern blot hybridization, and milk protein analysis (19) and isolation of total RNA and Northern analysis (20) were performed as described. Total milk protein content was estimated by the Lowry procedure (21) and total solid by lyophilization. Fat content was analyzed by using cholesterol (no. 139050) and triacylglycerol (no. 676519) automated analysis kits (Boehringer Mannheim) according to the manufacturer's instructions. Milk carbohydrates were analyzed by thin-layer chromatography with detection using an aniline phthalate spray (11). Milk viscosity was estimated on a coaxial viscometer (low-shear model 30; J. Lumy, Caluire, France) with a shear rate ranging from 0.1 to 100 s^{-1}.

Histological Analysis. Fragments of the mammary gland were fixed in 2% glutaraldehyde in 0.1 M cacodylate buffer (2 hr), postfixed in 1% OsO_4 in the same buffer (16 hr), dehydrated, and embedded in Epon. Thick sections were stained in toluidine blue for light microscopy. Thin sections, for electronic microscopy, were stained with uranyl acetate and lead citrate.

RESULTS

Creation of αlac-Deficient Mice. The targeting vector (Fig. 1) used for positive/negative selection (22) was constructed in several steps from the BALB/c mouse gene-encoding αlac (15) and from pPoly plasmids (16) as described in *Materials and Methods*. The construct contained a HSV-TK cassette upstream from the murine αlac gene, where a HSV-Neo cassette was substituted for the region spanning 0.56 kb of the proximal 5′ flanking region to the transcriptional region upstream from intron 2. Therefore, the uninterrupted 5-kb 5′ flanking region and 2.5-kb genomic sequences encompassing most of intron 2 down to a *Bam*HI site in the 3′ flanking region flanked the HSV-Neo cassette and were available for homologous recombination. The targeted αlac-recombinant allele (*H*) was unlikely to therefore produce any αlac mRNA.

D3 ES cells (17) were electroporated with the *Sfi* I-linearized vector and the results of two experiments are summarized in Table 1. From 5×10^6 electroporated cells, 150 colonies resistant to both G418 and ganciclovir were obtained, of which only 7 (4.7%) contained the targeted αlac gene as demonstrated by the occurrence of the homologously recombined 8-kb *Pst* I fragment. Cells from the seven identified colonies were injected into C57BL/6 blastocysts. Six ES lines generated chimeric males, which were tested for fertility and germ-line transmission (Table 2). Only males from lines 35 and 119 were found to be germ-line competent. Heterozygotes were mated to produce wild-type (*W*/*W*), heterozygous (*H*/*W*), and homozygous (*H*/*H*) mice.

Phenotype of αlac-Mutant Mice. *High milk viscosity disables milk ejection.* Heterozygote matings yielded wild-type, heterozygous, and homozygous offspring in the expected Mendelian ratios (13 *W*/*W*, 35 *H*/*W*, 12 *H*/*H*). All males and females appeared normal and all mice tested were fertile.

Females from each genotype were mated for subsequent study of their lactation. Offspring from *H*/*H* females appeared to suckle normally but were unable to remove milk from lactating glands (as judged by gut dissection) and were transferred to foster mothers. To maintain some stimuli, new pups were given to *H*/*H* females every 2 days. Offsprings of *H*/*W* appeared to suckle normally and no retardation in growth was observed. Milk samples were collected 5 days after parturition, as previously described (19), and two mice of each genotype were killed at this stage for further analysis.

Table 1. Homologous recombination at the αlac locus

Exp.	No. of cells electroporated	No. of G418^r and Ganc^r colonies	No. of targeted colonies
1	2.5×10^6	64	3
2	2.5×10^6	86	4
Sum	5×10^6	150	7

G418^r, G418-resistant; Ganc^r, ganciclovir-resistant.

Table 2. Production of chimeras and germ-line transmission

ES clone	No. of injected blasto-cysts	No. of progeny born	No. of chimeras		No. of fertile chimeric males	Germ-line trans-mission*
			Male	Female		
5	56	41	10/25	3/16	3/7	0/88
24	49	27	6/13	6/14	3/6	0/158
35	69	43	12/20	6/23	3/9	4/70
69	32	22	0	0		
74	65	31	11/17	9/14	2/8	0/148
97	30	19	9/15	0/4	2/5	0/147
119	37	22	7/11	6/11	4/6	42/52

*No. of newborns displaying the ES agouti coat color marker, from which about 50% were found to contain the targeted αlac allele.

Northern analysis reveals reduced level of αlac mRNA. Northern blot analysis of total RNA samples from lactating mammary gland of two mice of each genotype was performed as described (20). αlac mRNA was detected in mammary samples from *W/W* mice and in *H/W* mice at about 50% of the *W/W* level (Fig. 2). No αlac mRNA was detected in *H/H* mice even after overexposure of the blot. WAP, α-casein, and β-casein mRNAs were found in all samples. Dot blot analysis confirmed that αlac mRNA was present in lower amount in mammary samples from *H/W* mice than in those from *W/W* mice, but no other correlation could be found between the mouse genotypes and the relative amounts of the other milk-protein mRNAs tested (data not shown and Fig. 2).

Milk analyses. Fifty to 100 μl of milk was collected from *W/W* and *H/W* mice, whereas only 2–8 μl was obtained from *H/H* mice despite the use of additional oxytocin (10-fold, 3 units) and vigorous massaging of the mammary gland. The viscosity of the milk from *H/H* mice was found to be at least 10 times that of the milk from *H/W* mice, which in turn was 3 times that of the milk from *W/W* mice (Table 3). Furthermore, milk from *H/H* mice showed a shear-thinning behavior (data not shown).

Total solid content and fat and protein concentrations of milk samples from each genotype were estimated. These

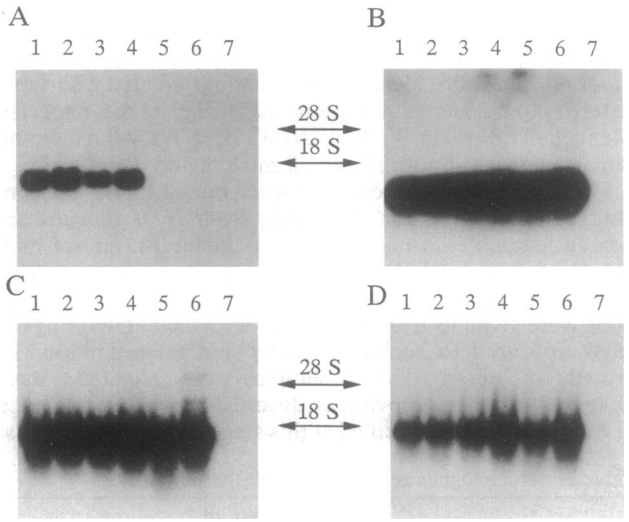

FIG. 2. Northern analysis of mammary RNA samples from lactating mice of different genotypes. Northern analysis was performed with 10 μg of total RNA from 5-day-lactating mice. Tissues analyzed were mammary gland of two *W/W* (lanes 1 and 2), *H/W* (lanes 3 and 4), and *H/H* (lanes 5 and 6) mice, respectively, and liver (lane 7) of *H/H* mouse. Probes were mouse αlac (*A*), WAP (*B*), β-casein (*C*), and α-casein (*D*) cDNA, respectively. Distances of migration of the 28S and 18S rRNAs are indicated.

Table 3. Milk composition of wild-type (*W/W*), heterozygous (*H/W*), and homozygous (*H/H*) mice

	W/W	*H/W*	*H/H*
Viscosity,* mPa·s	74	235	3224
Total solids, % (wt/wt)	39.6 ± 1.2	50 ± 0.5	67.4 ± 1.6
Total solids, % (wt/vol)	36.2 ± 1.2	42.5 ± 1.5	61.1 ± 2.4
Total proteins, mg/ml	83 ± 5	124 ± 10	180 ± 15
αlac, μg/ml	500	300	0
Triacylglycerol, μg/ml	83	112	216
Cholesterol, μg/ml	260	390	880

Data were calculated for pooled milk samples from six mice of each genotype.

*Measured at 20°C at a shear rate of 25.2 s^{-1} on milk samples which had been frozen only once, as freezing and thawing appear to alter the viscosity of the milk (data not shown).

concentrations were significantly higher in *H/H* than in *W/W* mice, whereas *H/W* mice were intermediate (Table 3). SDS/PAGE followed by Coomassie blue staining revealed a similar electrophoretic pattern of major milk proteins in all the samples tested. However, αlac was detected only by Western analysis in *W/W* and *H/W* milks (Fig. 3). The level of αlac detected in the milk of *H/W* mice was 40% lower than that in *W/W* mice (Table 3).

In the case of excessive alveolar distension, leakage of the tight junctions which normally cement together adjacent cells from the acini has been observed. This can lead to a partial equilibration of milk and extracellular fluid (23). Occurrence of milk proteins (i.e., caseins) in the blood was therefore tested by Western analysis. No difference could be seen between blood samples of 5-day-lactating animals of the three genotypes, with a minute amount of casein or possibly blood protein detected (data not shown).

Analysis of milk carbohydrates by thin-layer chromatography did not detect any lactose and derived-sugars in the

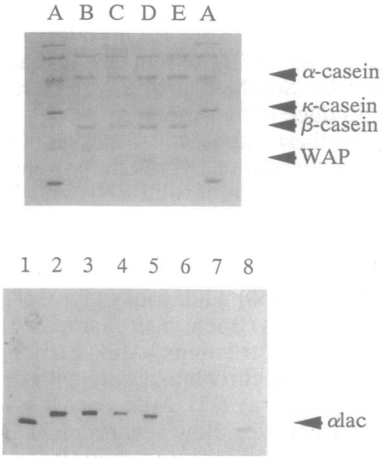

FIG. 3. Coomassie blue staining and Western blot analysis of milk proteins. (*Upper*) Coomassie blue staining of 270 μg of total milk proteins from *H/W* (lanes B and E), *H/H* (lane C) and *W/W* (lane D) mice. Lanes A; molecular size markers [from the top, phosphorylase *b* (94 kDa), albumin (67 kDa), ovalbumin (43 kDa), carbonic anhydrase (30 kDa), trypsin inhibitor (20.1 kDa), and bovine αlac (14.4 kDa)]. (*Lower*) Western blot analysis of milk proteins performed with an anti-bovine αlac polyclonal antibody previously shown to strongly crossreact with mouse αlac (20). Lanes 1 and 8, 40 ng and 10 ng of purified bovine αlac, respectively; lanes 2 and 3, 270 μg of total milk proteins from two *W/W* mice; lanes 4 and 5, 270 μg of total milk proteins from two *H/W* mice; lanes 6 and 7, 270 μg of total milk proteins from two *H/H* mice.

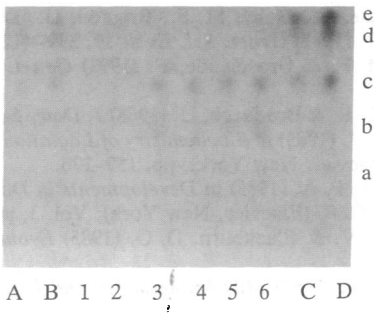

FIG. 4. Thin-layer chromatogram showing carbohydrates of 5-day-lactating mice of different genotypes. Lanes A–D contained 2, 5, 10, and 25 μg of lactose, respectively, mixed with 1, 2.5, 5, and 12.5 μg of glucose and of galactose, respectively. Samples (1 μl) of milk from two *H/H* (lanes 1 and 2), two *H/W* (lanes 3 and 4), and two *W/W* (lanes 5 and 6) mice were tested. At right, distance of migration of putative digalactosyllactose (a), putative galactosyllactose (b), lactose (c), galactose (d), and glucose (e) is shown.

milk of *H/H* mice (Fig. 4). No qualitative differences were observed between *H/W* and *W/W* milks, but the lactose content was 10–20% lower in *H/W* milk.

Mammary tissue from H/H *mice is characterized by a high content of lipids.* The structure of mammary epithelial cells appeared similar in *W/W* and *H/W* mice. However, cells from *H/H* mice were characterized by a reduced volume of their cytoplasm, secretory vesicles, and Golgi apparatus (Fig. 5). Furthermore, fat globules, restricted to the mammary acini lumen in *W/W* mice, were found to invade the extracellular spaces in *H/W* mice and even more so in *H/H* mice.

DISCUSSION

The gene-encoding αlac was inactivated by homologous recombination in ES cells, using a positive/negative selection protocol (22). The targeting frequency observed in two electroporation experiments was 4.7% of double drug-resistant colonies, making the use of an isogenic DNA construct unnecessary (24, 25). Furthermore, a targeting experiment involving this same locus was recently described (26). The vector used was overall similar to ours but based on isogenic DNA. The targeting frequency observed was only 2% of selected colonies. This lower percentage might reflect the shorter length of the 3′ homologous region, 0.85 kb compared with 2.5 kb (27, 28), or the use of a different gene for the selection, hypoxanthine phosphoribosyltransferase instead of Neo (29, 30).

While six out of seven ES lines generated chimeric mice, only two of them were found to be germ-line competent. This relatively low frequency might reflect the use of D3 cells at a high passage (21). Chromosomal analysis prior to injection of the ES cells into blastocysts might have enabled the rejection of selected clones with abnormal karyotypes. However, similar percentages of germ-line transmission are often obtained (31, 32).

Interbreeding of heterozygotes yielded heterozygous and homozygous offspring at the expected frequencies, and males and females from both genotypes are apparently healthy and fertile, suggesting that αlac is not required for normal development. However, lactating *H/H* females were unable to feed their pups and therefore this genotype would be lethal for the progeny. This inability is probably due to the incapacity of the mouse mammary gland to eject a highly viscous milk and emphasizes the extent of morphological adaptation required in sea mammals that secrete milk of very high protein and fat content. It also demonstrates that the osmotic function performed by lactose is not, or only very partially, compensated for by other potential osmotic factors such as monosaccharides or monovalent ions, despite their observed complementary relationship (5). Unfortunately, the amount of milk collected so far from the few available *H/H* mice is insufficient for a thorough analysis of milk composition.

Despite the fact that the pups appear to be unable to remove milk from the gland, the milk protein composition, the mammary structure, and the levels of WAP and casein mRNA observed in 5-day-lactating mammary gland of *H/H* mice appear normal. It demonstrates that a suckling stimulus

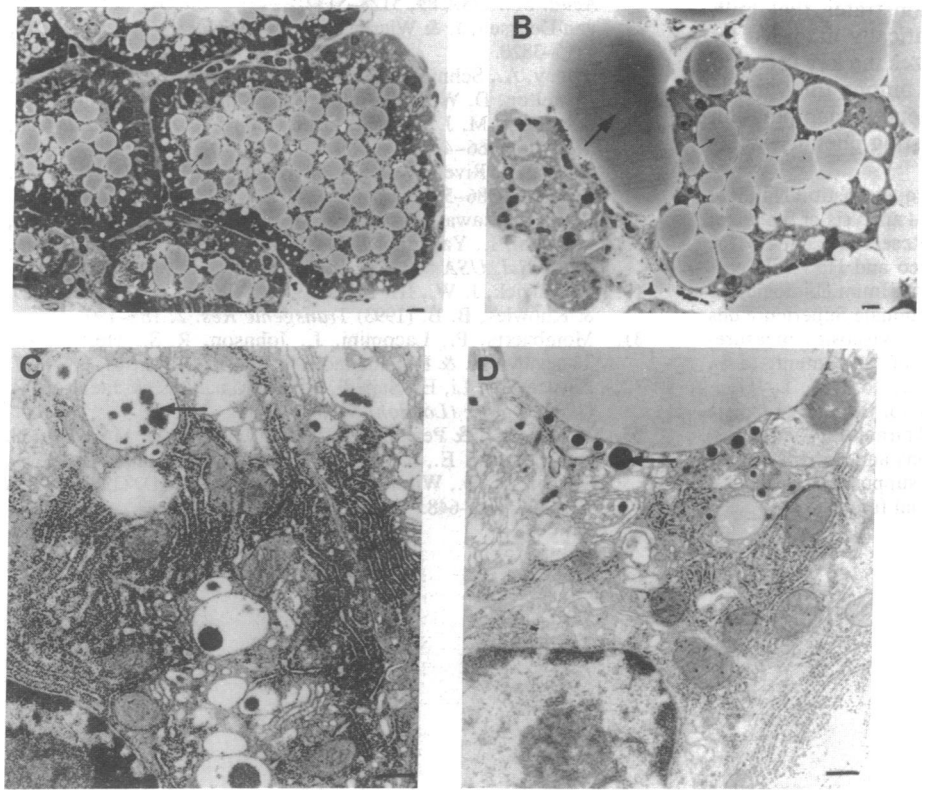

FIG. 5. Structural analysis of mammary sections of 5-day-lactating mice. (*A* and *B*) Sections of *W/W* and *H/H* 5-day-lactating mammary glands are shown, respectively. Fat globules, located inside the acini lumen (small arrow) or invading in *H/H* mice the extracellular spaces (large arrow) are indicated. (Bars = 5 μm.) (*C* and *D*) supranuclear regions of *W/W* and *H/H* 5-day-lactating mammary epithelial cells, respectively. The difference observed in the volume of the secretory vesicles (large arrow) is representative. (Bars = 0.5 μm.)

is sufficient to maintain this tissue in an apparently active state. The presence of fewer secretory vesicles in the epithelial cells of *H/H* mice, in conjunction with apparently normal tight junctions, suggests a partial inhibition of protein synthesis and secretion and/or an activation of intracellular protein degradation. The protein feedback inhibitor of lactation might partially explain this observation (33, 34). However, the structure of the endoplasmic reticulum, with elements arranged in parallel and not vesiculated or swollen (Fig. 5), does not appear consistent with this hypothesis.

Milk analysis revealed the occurrence of all major milk proteins at the expected levels, with the exception of αlac in *H/H* milk, a result in agreement with the RNA data. The overall protein concentration appears to be inversely proportional to the amount of αlac being produced (Table 3). The lactose concentration in *W/W* milk was slightly higher than that in *H/W* milk, whereas this sugar could not be detected in *H/H* milk. It suggests that the negative correlation observed between αlac and protein concentrations actually reflects that described between lactose and protein concentrations (23). However, the decrease observed in the lactose content in *H/W* milk is not proportional to that of αlac, which suggests that this protein, after the onset of lactogenesis, is in excess for the synthesis of lactose. The fat content of the milk is also related to the genotype, with a negative correlation between fat and αlac concentrations. Again, this phenotypic observation corroborates the described relationship of fat, lactose, and protein concentrations (23). It suggests that this relationship, observed between species and presented as an adaptation to the environment, results from a modification of carbohydrate metabolism, with glucose involved primarily either in lactose or in fat synthesis. A more detailed study of many mutant mice is needed to better understand the metabolic modifications induced.

It was recently demonstrated that αlac inhibits the growth of mammary epithelial cells in culture (14). The mammary gland structure observed in *H/H* mice appears normal in this respect. This suggests either that αlac does not have such a function *in vivo* in mice or that other endogenous proteins can compensate for its absence.

The knockout of the αlac gene demonstrates that milk composition and lactation can be dramatically modified by alteration of αlac expression. It emphasizes the key function of this protein but also the relationship among the various milk constituents and suggests that the genetic manipulation of αlac could create animals with interesting phenotypes.

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