THE CARBOXY-TERMINAL DOMAIN OF HUMAN SURFACTANT PROTEIN B IS NOT REQUIRED FOR SECRETION IN MILK OF TRANSGENIC MICE

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1. ABSTRACT

Previous studies in which human pulmonary surfactant protein B (SP-B) was targeted to the mammary gland of transgenic mice using the rat whey acidic protein (WAP) regulatory sequences resulted in secretion of only the unprocessed proprotein (42 kDa) in milk. To test the feasibility of producing a partially processed SP-B protein in milk, a new construct was designed in which the coding region for the carboxy-terminal domain was deleted. Expression of rWAP/SP-BΔ C mRNA was detected in all three transgenic lines generated, and the expected carboxy-terminal deleted SP-B molecule (28 kDa), identified by using domain-specific antibodies, was secreted in the milk. Histochemical examination of lactating mammary tissue from the transgenic line expressing the highest levels of WAP/SP-BΔ C mRNA revealed an inhibition of lobulo-alveolar development, and led to growth retardation in pups, apparently due to the decreased milk production. Mothers from this line tended to cannibalize litters in mid-lactation. This phenotype has been observed previously with several other WAP-based transgenes. This phenotype suggests that there may be an upper limit to the level of SP-BΔC which can be produced in milk.

2. INTRODUCTION

Previous attempts to produce recombinant hSP-B in the milk yielded only the proprotein (42

kDa) (1) suggesting that the mammary gland was unable to carry out the necessary post-translational proteolytic cleavage reactions required for the production of mature hSP-B. Analysis of SP-B expression in cell transfection experiments suggested that the amino-terminal domain of the proprotein is necessary for secretion, while the carboxy-terminal domain is not required (2). Therefore, we speculated that it might be possible to express a partially processed hSP-B protein in milk that could be used as a substrate for further *in vitro* processing to produce the mature hSP-B peptide. In order to test the feasibility of this approach, transgenic mice with a WAP/SP-BΔ C transgene were generated.

Use of the mammary gland as a bioreactor to produce heterologous proteins in milk is now well established (reviewed in (3-5)). In this study, the rat whey acidic protein (rWAP) promoter and 3' UTR sequences (6, 7) were employed to direct the expression of the SP-BΔ C transgene to the murine mammary gland. These regulatory sequences have successfully directed the expression of several heterologous transgenes to the mammary gland (8, 9, 1).

3. METHODS AND MATERIALS

Oligonucleotides

The oligonucleotides used in this study are shown in Table 1.

Construction of pWAP/SP-BDC

pHga33 was prepared by excising an HgaI fragment containing the SP-B sequence from pWAP/SP-B corresponding to gb:m24461 (4230-7700) (1) and subcloning it into the EcoRV site of pBluescript II (Stratagene, La Jolla, CA). pHga33

Table 1. Oligonucleotides

Name	Sequence
NcoStopForward	5'-CAT GGA TTA ACT GAC TAG CGC GC-3'
NcoStopReverse	5'-CAT GGC GCG CTA GTC AGT TAA TC-3'
SPBex7Forward	5'-CTA CTC CGT CAT CCT GCT CGA C-3'
WAP intron C Reverse	5'-CTC ACT GAA AGG ATA TCA CTG TAG GAG A-3'
WAP +1Forward	5'-ATC AGT CAT CAC TTG CCT GCC GCC G-3'
SP-Bex2Reverse	5'-TGA CCA GGG GGA AGT AGT CGT-3'

Table 1: Oligonucleotides Summary of names and sequences of oligonucleotides employed in PCR, sequencing and cloning are presented in table 1.

contained a single NcoI site [gb:m24461 (5546)], which is situated near the junction between the SP-B processed peptide and carboxy-terminal domains. Two complementary oligonucleotides (NcoStop Forward and NcoStopReverse; Table 1) were designed so that they could be annealed (10) to form NcoI compatible protruding 5' termini. In addition, the annealed oligonucleotides restored the final Asp residue of the SP-B processed peptide domain, contained stop codons in each of the 3 reading frames, and introduced a complete BssHII restriction endonuclease site 3' to the newly introduced stop codons.

pHga33 was digested with NcoI and the annealed NcoStop oligos were added by ligation to produce pHga33Stop. pHga33Stop and pWAP/SP-B were both then digested with BsmBI. The 2325 bp BsmBI fragment from pHga33Stop, containing the newly introduced stop codons, was ligated directly into the pWAP/SP-B vector. Since BsmBI cuts adjacent to its recognition site, this was a directional cloning step. The resultant pWAP/SP-B(Stop) clones were screened initially by BssHII digestion, and their identity was confirmed by sequencing using the SPBex7Forward oligonucleotide (Table 1) as a primer.

After confirming the presence of the NcoStop oligos and the preservation of the reading frame at the ligation junction by sequencing, the WAP promoter and SP-B(Stop) sequence encoding the amino-terminal and processed peptide domains were excised as a BssHII fragment, treated with Klenow (10), and then digested with Not I. The WAP 3' vector pWE3' (9) was digested with Not I and EcoRV, permitting directional cloning of the WAP promoter and SP-B(Stop) fragment to create the final construct pWAP/SP-BΔC.

Generation and screening of transgenic mice

The transgene was excised as a 6.5 kb Bssh II fragment, purified by adsorption to Qiaex II resin (Qiagen, Chatsworth, CA) and subjected to micro-injection (11). Genomic tail DNA was analyzed by PCR screening as previously described (1). PCR was conducted for 30 cycles of 1 min at 94ºC, 2 min at 60ºC, 3 min at 72ºC and samples were held at 4ºC until analysis. The forward primer was WAP +1Forward and the reverse primer was SP-Bex2Reverse (Table 1)

RNA preparation and Northern blot analysis

Mammary gland biopsies were performed on the female founder mice and the F1 females in mid-lactation under anesthesia (9). Total RNA was isolated by RNAzol (TEL-TEST , Friendswood, TX) according to the manufacturer's instructions. Fractionation of total RNA was on agarose gels containing formaldehyde (10). After transfer to ZetaProbe GT membrane (Bio-Rad, Hercules, CA), RNA was hybridized to ^{32}P-labeled human SP-B fragments generated using random hexanucleotide primers (10). Transfer, hybridization and filter stripping were performed according to the manufacturer's recommendations. Quantitation was performed using a Phosphorimager (Molecular Dynamics, San Francisco, CA) and the relative expression levels of SP-BΔC mRNA of transgenic lines were estimated.

Antibodies

Four polyclonal rabbit sera (previously prepared by TW) were employed in immune detection of western blots (2). R28031 reacts exclusively with epitopes in the fully processed SP-B molecule. R55522 reacts to epitopes in both the amino-terminal and the carboxy-terminal domains of the SP-B proprotein. R55019 reacts to epitopes only in the amino-terminal domain of the SP-B proprotein. R96189 reacts to epitopes only in the carboxy-terminal domain of the SP-B proprotein. These antibodies were employed in combination to determine which domains of the SP-B were produced by the transgene.

Lung homogenates

Lung homogenates were prepared for Western blot analysis as previously described (12) except that the primary antibodies were those described above.

Milk collection, PAGE, and Western blotting

Milk samples were collected during mid-lactation from anaesthetized mice using a constant vacuum apparatus (13). Polyacrylamide gel electorphoresis (PAGE) was performed according to the method of Schagger and von Jagow (14). 16.5%T/3%C Tris/Tricine gels were run overnight at 70v. Western transfer was for 90 min at 300 mA. Western blot analysis was performed as described previously (9) except that the primary antibodies were those described above. Visualization was by enhanced chemiluminescence (ECL, Amersham, Arlington Hts., IL)

Histochemistry

Mammary gland biopsies were obtained from anaesthetized mice on day ten of lactation. Mice were separated from their pups for 3 hr prior to biopsy to insure a buildup of milk in the alveoli. Tissue samples were fixed in 10% neutral buffered formalin for 8 hr at room temperature. Samples were rinsed twice in large volumes of deionized water and stored in 70% ethanol until embedding. Cubes of tissue from the central portion of the gland were dehydrated and embedded in paraffin. Sections (5 μm) were mounted on Probe-On Plus (Fisher Scientific, Pittsburgh, PA) slides. Sections were deparaffinized and rehydrated through an ethanol series prior to staining with hemotoxylin/eosin.

4. RESULTS

Expression of SP-BDC mRNA in transgenic mice

Five transgenic founders carrying the WAP/hSP-B Δ C transgene were identified by PCR analysis. Founders 4927, 4928 and 4931 were female while 4934 and 4945 were male. Biopsies of the female founders were performed and only line 4928 expressed SP-B Δ C RNA at a level detectable by Northern blot analysis (Fig. 1A). However, the other two female lines 4927 and 4931 did not transmit the transgene to any of their progeny suggesting that they were mosaic founders. These two lines were not characterized further. Female F1 progeny of the male founder lines 4934 and 4945 both expressed the SP-B Δ C transgene, although at variable levels (Fig. 1B). RNA samples from line 4934 required a 5 day exposure to X-ray film for detection of the SP-B Δ C transcript, while SP-B mRNA from lines 4928 and 4945 samples was visualized after only a one day autoradiographic exposure. Transcript levels in the 4945 line were approximately 2.5 times greater than those in the 4928 founder but this difference was not statistically significant due to variation within the 4945 line. Since expression of "housekeeping" genes is affected by lactation, and expression of transgenes in lactating mammary tissue may disrupt expression of endogenous milk genes, equality of loading was judged from Ethidium Bromide staining of gels (Fig.1 A and B) using multiple inputs of total RNA. All transgenic mice expressing the SP-B Δ C transgene displayed two transcripts (Fig. 1). The predominant mRNA corresponds to the expected transcript size of 1019 bp, while the minor transcript had an apparent size of 1800 bp perhaps due to inappropriate post-transcriptional processing.

SP-BDC protein in milk

Western blot analysis of milk from female 4928 showed the predicted 28 kDa protein (Fig. 2A) as well as a slightly smaller species. The 28 kDa SP-B D C was detected with antibodies R28031, 55019 and R55522, but not with R96189. This confirmed that the 28 kDa protein contains the amino-terminal and active peptide domains, but not the carboxy-terminal domain. A sample of milk from a SP-B transgenic female containing the previously characterized proprotein (42 kDa) (1) reacted with all four antibodies. SP-B proprotein in mouse lung tissues is rapidly processed to the mature peptide (8 kDA) and consequently the precursor protein is not detected (Fig. 2B, lane L).

Comparison of milk from SP-BΔC transgenic mice to SP-B derived from lung tissue (Fig. 2B) confirmed that the fully processed SP-B did not appear in the milk as a result of the carboxy-terminal truncation. The predicted 28 kDa species is clearly the major product of the transgene, although other bands are apparent, either as a result of proteolysis, or cross-reactivity of the primary antibodies with unrelated proteins in the milk.

Phenotype of line 4945

While females from lines 4928 and 4934 nursed normal-sized litters to weaning without incident, females of line 4945 routinely cannibalized all or part of their litters. Surviving pups from this line were retarded in growth, although they grew to normal size after weaning. Histochemical analysis of mammary gland tissue isolated on day 10 of the second lactation from 4945 F_1 females (Fig. 3) revealed both abnormally low amounts of secretory epithelium and an altered morphology of individual alveoli. Mammary gland isolated from day 1 of lactation from 4945 F_1 (third lactation) and F_2 (first lactation) females showed a similar pattern (Fig. 4).
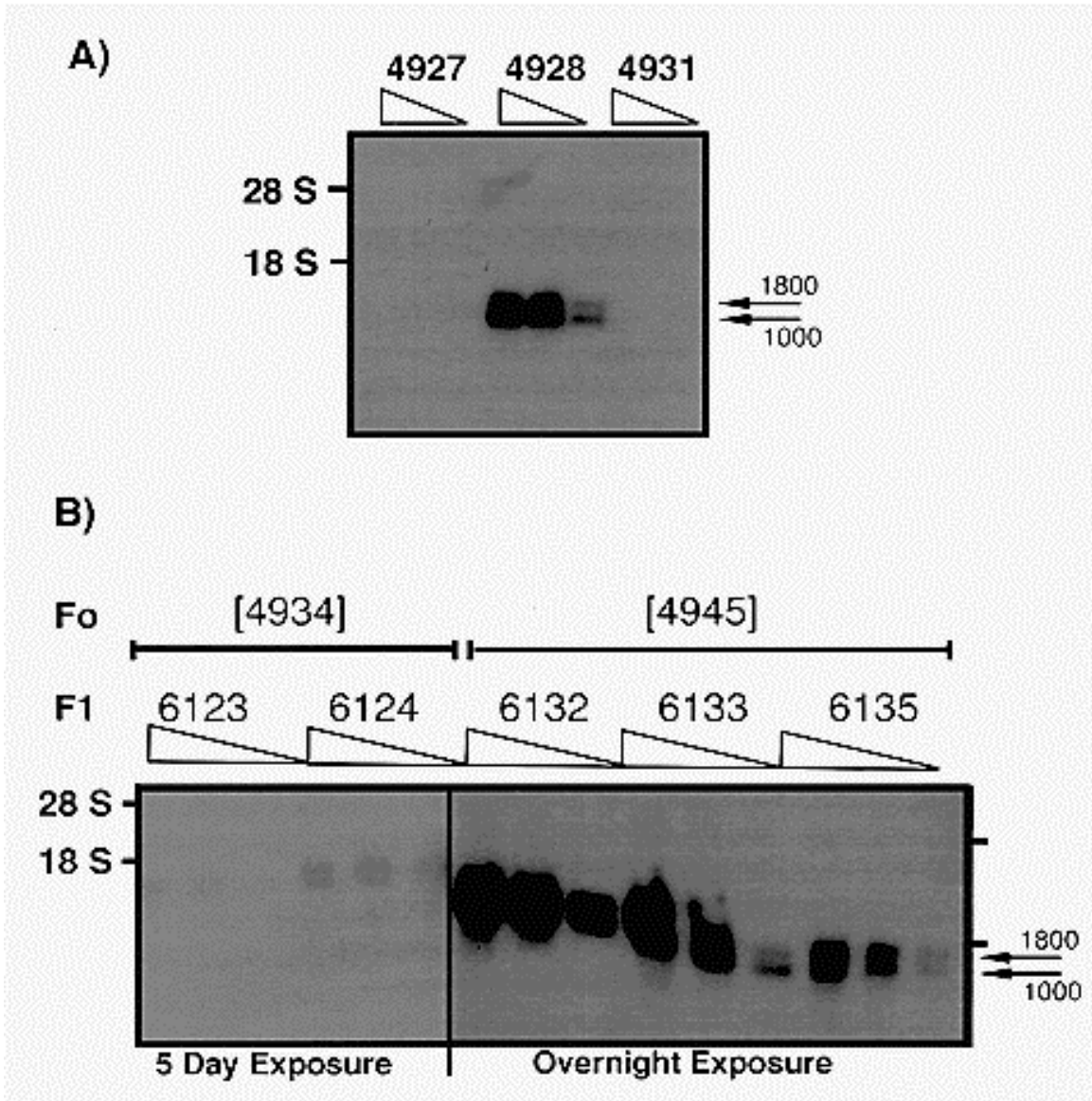
Figure 1: Northern blot analysis of WAP/SP-B lines. 10, 5, and 1 μg of total RNA from a mammary gland on day 10 of lactation were subjected to Northern blot analysis using a human SP-B probe (810 bp). A) Female founders 4927, 4928 and 4931. 4928 express the predicted 1019 bp transcript as well as an 1800 bp transcript. (overnight exposure) B) F1 females from the [4934] and [4945] lines all express the predicted 1019 bp transcript as well as a 1800 bp transcript (exposure time is 5 days for [4934] and overnight for [4945]).
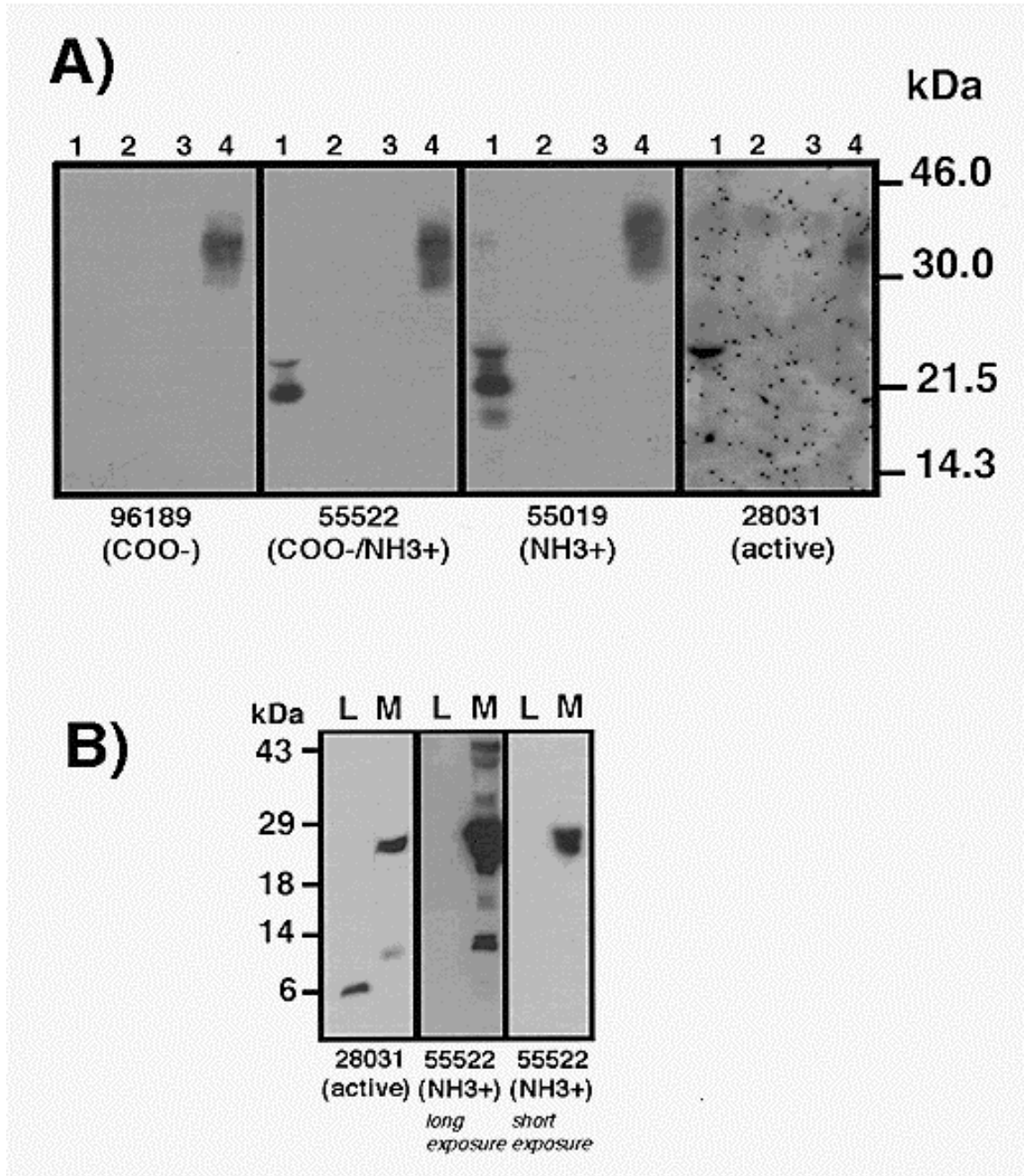
Figure 2: Western blot analysis of mouse milk and lung extracts. A) 1.0 μl milk/lane was subjected to PAGE and blotted to PVDF membrane (1; 4928), (2; 4931), (3; non-transgenic sample), (4; milk from a transgenic full length SP-B mouse). The blots were probed with the polyclonal rabbit sera (1:700 in TBS + 5% NFDM) with specificities to different domains of the SP-B proprotein. Sera and their specificity are indicated below each portion of the blot. Visualization of the antigen antibody complex was accomplished with biotinylated goat anti-rabbit serum, streptavidin-horseradish-peroxidase, and ECL reagents. B) Samples treated as in A. L= 25 μg of total lung homogenate and M= 1.0μl of milk from an F1 female of the [4945] line.
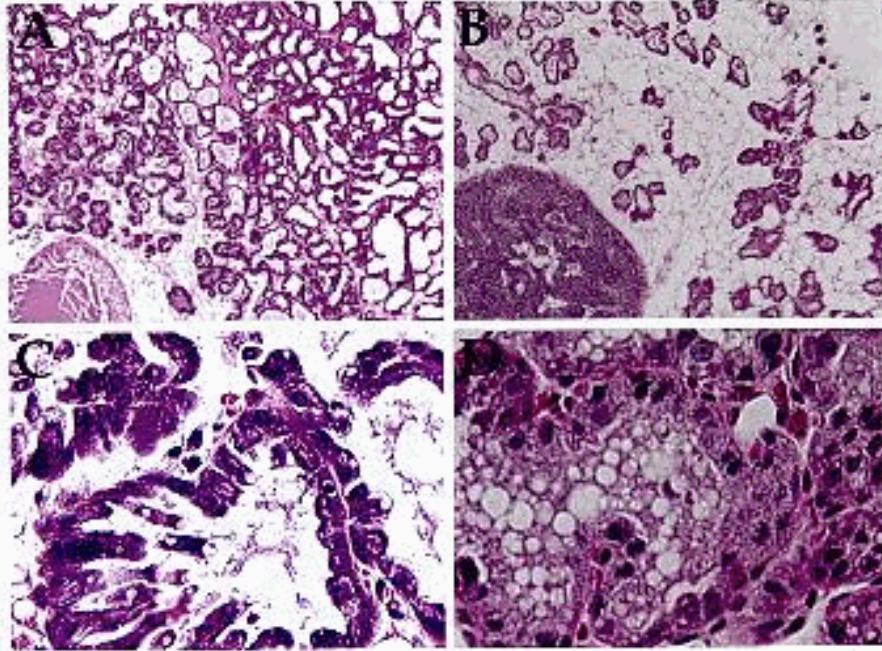
Figure 3: Histochemical analysis of mammary tissue in mid-lactation shows retarded development. Paraffin-embedded sections of normal (A and C) and transgenic line 4945 (B and D) mammary gland on day 10 of lactation were stained with haemotoxylin and eosin. (A and C) 40x (B and D) 400x .
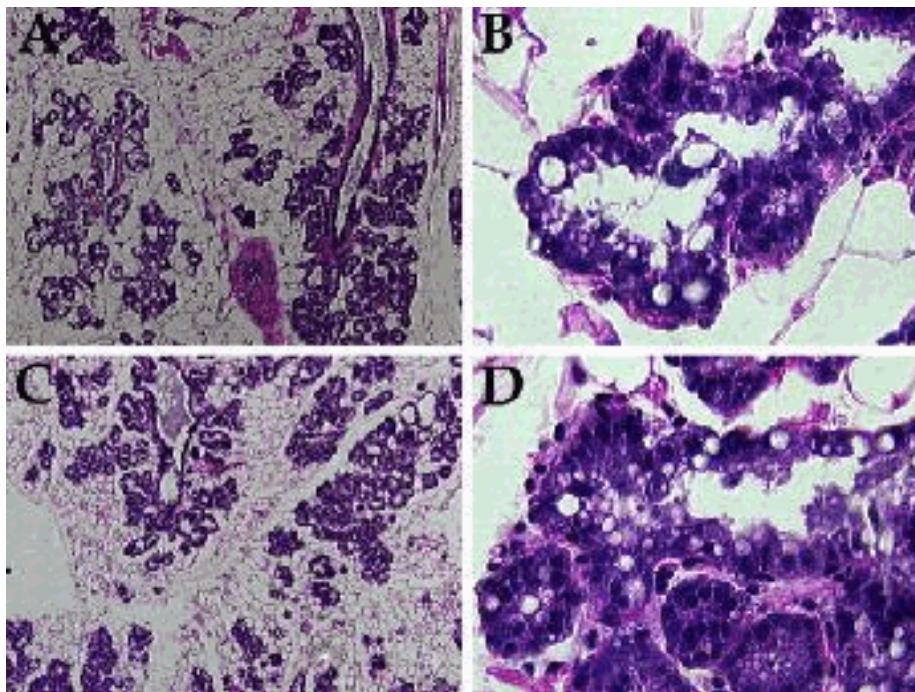

Figure 4: Histochemical analysis of mammary tissue in early lactation shows retarded development. Paraffin-embedded sections of transgenic mammary gland on day 1 of lactation were stained with haemotoxylin and eosin. All tissues are from [4945] line: (A, B);tissue from an F_1 female in third lactation, (C, D); tissue from an F2 female in first lactation. (A and C) 40x (B and D) 400x

The affected alveoli lack a clearly defined lumen and appeared to be surrounded by multiple epithelial cell layers. In addition, the amount of connective stromal tissue, relative to secretory epithelial tissue was greatly increased.

5. DISCUSSION

The expression of the WAP/SP-BΔC genomic construct in the mammary gland of transgenic mice has been demonstrated, and the expected SP-BΔC 28 kDa protein was secreted in the milk. This confirms the earlier observations in cell culture (2) and in transgenic mice (15) which suggested that the carboxy-terminal domain of SP-B is not required for secretion. The ability to produce partially processed hSP-B in milk should facilitate the attainment of the long term goal of this project, i.e. the production of fully processed SP-B for pharmaceutical use. These and the previously published results (1) suggest that the enzyme necessary for cleavage of the amino-terminal propeptide of SP-B is not available in the mammary gland, even in the absence of possible steric hindrance from the carboxy-terminal propeptide. In a recent report, SP-BΔC targeted to the lung was shown to be processed to an active molecule in that organ (15), ruling out the possibility that the carboxy-terminal domain is required for the correct folding of the active SP-B molecule. Since the enzyme responsible for the cleavage of the amino-terminal domain of SP-B remains unidentified, the next step toward production of the fully processed molecule will be insertion of a chemical or enzymatic cleavage site (via site directed mutagenesis) between the amino-terminal domain and the mature peptide domain (16).

One transgenic line expressing SP-BΔC produced an aberrant mammary gland phenotype characterized by decreased lobulo-alveolar development and led to the retardation of growth in the suckling neonates. Since little is known about the physical-chemical properties of SP-BΔC, its mechanism of action in producing the observed phenotype is unclear. No morphologic abnormalities result when SP-BΔC is targeted to the lung (17), but in that organ the SP-BΔC molecule is processed to mature SP-B. Similar phenotypes have been observed previously in both mice and swine overexpressing mouse WAP (18, 19), as well as transgenic mice expressing TGFβ1 and a mutant p53 gene (20-22). Transplantation studies were used to show that the observed phenotype in the WAP/TGFβ mice was due to a local effect of the transgene on the mammary epithelium (20, 21). The p53[arg-leu] transgenic mice apparently suffer from decreased lobulo-alveolar development because of increased apoptosis (22), which is consistent with p53's role as a tumor suppressor gene. Since the observed phenotype in the SP-BΔC occurred in heterozygotes, insertional mutagenesis seems unlikely. The SP-BΔC protein apparently exerts its effect during pregnancy, since the aberrant morphology is apparent at day one of lactation. This observation is supported by previous work using the same WAP vector employed in this study (6, 7, 9, 1, 8) in which low level expression of heterologous transgenes in midpregnancy, a critical period for lobulo-alveolar development, may result in impaired lactation. This phenotype has not been observed with all WAP-based transgenes, or even for all lines generated from a single transgene construct. This implies the observed developmental abnormalities are the result of the specific properties of the overexpressed proteins, and their concentrations, rather than transcriptional or translational interference by the transgene. It is interesting that such diverse transgene proteins result in similar phenotypes. The mammary gland is apparently quite sensitive to disruptions in development during mid-pregnancy.

The phenotype reported in this study is of interest primarily because it suggests that there may be an upper limit to the level of SP-BΔC which can be produced in milk. Hopefully, this will not impede development of production of SP-B for pharmaceutical use from milk.

6. ACKNOWLEDGMENTS

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