The Breast Cancer-associated Stromelysin-3 Gene is Expressed During Mouse Mammary Gland Apoptosis

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Abstract. We have cloned from a mouse placenta cDNA library a mouse homologue of the human stromelysin-3 (ST3) cDNA, which codes for a putative matrix metalloproteinase expressed in breast carcinomas. The ST3 protein is well conserved between humans and mice, and the pattern of ST3 gene expression is similar in both species, and shows expression in the placenta, in the uterus, and during limb bud morphogenesis. We show that the ST3 gene can also be expressed in the normal mouse mammary gland. ST3 gene expression was not detected during mammary growth, neither in virgin nor in pregnant mice, but was specifically observed during postlactating involution of the gland, an apoptotic process associated with intense extracellular matrix remodeling. ST3 transcripts were found in fibroblasts immediately surrounding degenerative ducts, suggesting that ST3 gene expression may be associated with the basement membrane dissolution, which occurs during mammary gland involution. Since the ST3 gene is also specifically expressed in fibroblastic cells surrounding invasive neoplastic cells of breast carcinomas, we suggest that ST3 is implicated in extracellular matrix remodeling processes common to mammary apoptosis and breast cancer progression.

STROMELYSIN-3 (ST3)[1] (Basset et al., 1990) is a putative new member of the family of matrix metalloproteinase (MMP) enzymes whose substrates are extracellular matrix (ECM) components (Matrisian, 1990). ST3 substrate specificity is not known, but the ST3 sequence differs from those of the previously characterized MMPs in ways that suggest that it may be the first member of a new MMP subgroup (Basset et al., 1990; Murphy et al., 1991; Levy et al., 1992). It has been proposed, and to some extent demonstrated, that MMPs are involved in several physiological and pathological processes in which tissue remodeling is implicated, such as embryonic development, wound healing, and tumor invasion (Matrisian, 1990; Liotta and Stetler-Stevenson, 1990). In agreement with this concept, the ST3 gene is specifically expressed in fibroblastic cells immediately surrounding invasive neoplastic cells in several types of human carcinomas (Basset et al., 1990; and our unpublished results). The gene is also expressed in mesenchymal cells during limb bud morphogenesis, which suggests that ST3 may be a stroma-derived factor implicated in normal development that has been subverted in the cancer process.

In mouse, the mammary gland undergoes most of its morphogenetic and functional changes postpartum, under hormonal and growth factors that control both the epithelial and mesenchymal elements (Vonderhaar, 1988; Coleman et al., 1988; Robinson et al., 1991, and references therein). In vitro and in vivo studies have shown that growth and differentiation of mouse mammary gland are dependent on ECM remodeling (Vonderhaar, 1988; Silberstein et al., 1990; Talhouk et al., 1991), and that mammary gland involution is an apoptotic process at least partially directed by basement membrane dissolution (Wicha et al., 1980; Streuli et al., 1991).

To investigate whether the ST3 gene was expressed during mouse mammary gland development, we have cloned the mouse equivalent of human ST3 cDNA, and used the cDNA as a probe to detect ST3 gene expression. Our results show that the ST3 gene is not expressed in the mammary gland during gestation and lactation, but is specifically expressed during mammary involution, after weaning. ST3 RNA was detected in fibroblasts immediately surrounding degenerating mammary ducts, which suggests that ST3 gene expression in the mammary gland is associated with a basement membrane remodeling process common to apoptosis and cancer invasion.

Materials and Methods

Tissue Collection

Mammary glands at different stages of development were surgically excised from female mice and immediately frozen in liquid nitrogen until RNA ex-

1. *Abbreviations used in this paper:* ECM, extracellular matrix; MMP, matrix metalloproteinase; ST3, stromelysin-3.

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CCCGGGGCGGATGGCACGGGCCGCCTGTCTCCTCCGCGCGATTTCGGGGTGCCTCCTGCT 60
          M  A  R  A  A  C  L  L  R  A  I  S  G  C  L  L  L 17
CCCGCTGCCTCTGCTGCTCCTGTTGCTGCTGCTCCTGCCGTCGCCGCTGATGGCCCGGGC 120
  P  L  P  L  L  L  L  L  L  L  P  S  P  L  M  A  R  A 37
CAGGCCACCGGAGAGTCACCGTCATCACCCTGTGAAGAAAGGGCCTCGGCTCCTGCATGC 180
  R  P  P  E  S  H  R  H  H  P  V  K  K  G  P  R  L  L  H  A 57
AGCTCTGCCTAATACCTTGACATCTGTCCCCGCGTCTCATTGGGTCCCTAGTCCTGCCGG 240
  A  L  P  N  T  L  T  S  V  P  A  S  H  W  V  P  S  P  A  G 77
TAGCTCCAGGCCTCTACGATGTGGTGTGCCCGACCTGCCTGATGTACTGAATGCCCGGAA 300
  S  S  R  P  L  R  C  G  V  P  D  L  P  D  V  L  N  A  R  N 97
CCGACAGAAGCGCTTCGTCCTGTCAGGAGGACGCTGGGAGAAGACAGACCTCACCTATAG 360
  R  Q  K  R  F  V  L  S  G  G  R  W  E  K  T  D  L  T  Y  R 117
GATCCTCCGGTTCCCATGGCAGCTTGTAAGGGAGCAAGTCCGGCAGACAGTGGCAGAGGC 420
  I  L  R  F  P  W  Q  L  V  R  E  Q  V  R  Q  T  V  A  E  A 139
CCTCCAGGTATGGAGTGAAGTGACCCCACTCACTTTCACTGAGGTGCACGAGGGACGCGC 480
  L  Q  V  W  S  E  V  T  P  L  T  F  T  E  V  H  E  G  R  A 157
TGACATCATGATCGACTTCGCAAGGTACTGGGATGGTGACAACTTGCCGTTTGACGGGCC 540
  D  I  M  I  D  F  A  R  Y  W  D  G  D  N  L  P  F  D  G  P 177
TGGGGGCATCCTGGCCCATGGCTTCTTCCCTAAGACCCACCGAGAAGGGGATGTCCACTT 600
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  D  Y  D  E  T  W  T  I  G  D  N  Q  G  T  D  L  L  Q  V  A 217
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  A  H  E  F  G  H  V  L  G  L  Q  H  T  T  A  A  K  A  L  M 237
GTCCCCTTTCTACACCTTCCGCTACCCTCTGAGCCTTAGCCCAGATGACCGAAGGGGCAT 780
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CTGTGAGACTTCCTTCGACGCGGTTTCCACCATCCGAGGAGAGCTCTTCTTCTTCAAGGC 960
  C  E  T  S  F  D  A  V  S  T  I  R  G  E  L  F  F  F  K  A 317
AGGCTTTGTGTGGAGGCTGCGCAGTGGGCGACTGCAGCCCGGGTATCCTGCTTTGGCCTC 1020
  G  F  V  W  R  L  R  S  G  R  L  Q  P  G  Y  P  A  L  A  S 337
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  G  P  D  F  F  D  C  A  E  P  A  N  T  F  R  -              492
ATGCATTCAGGGGTACTGACTCCTGCCAGGGCACTTAGATCATGTAAGAGACCCACAGCC 1560
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CAATATTTCAGCTACCAATAATCCACAGTCAGCCTGGTTGCCCAAGGTCTCCTATCTCTG 1800
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GATTCAGACAAGTGCTTCCTGGAACTCAGGCCCCAAGGTACACAGCCAGCCAAGGAGGCA 2040
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GTATACAGTGTATAAAGACTTTTTTAAAAAAACAAAAAACCAAACCCCAAAAAAGCCAAG 2220
ACTGTCATTAAACATGAGTGTTTTCTAAAAAAAAAAAAAA                      2260
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Figure 1. cDNA and deduced amino acid sequences of mouse ST3. Nucleotides residues are numbered in the 5′ to 3′ direction, and amino acids in the open reading frame are designated by the one-letter code. The underlined nucleotide sequence corresponds to the poly(A) addition signal sequence. These sequence data are available from EMBL/GenBank/DDBJ under accession number Z12604.

traction. Some of the samples were fixed in formol before inclusion in paraffin for histological examination and in situ hybridization analysis. Mammary glands were collected from virgin mice, from pregnant and lactating mice, and during the involution of the lobulo-alveolar structures, which occurs after weaning (21 d postpartum).

RNA Isolation and Northern Blot Analysis

Total RNA used to construct the mouse placenta cDNA library was prepared by the guanidinium-cesium chloride procedure, and poly(A)$^+$ RNA was purified by oligo(dT)-cellulose chromatography. All other RNA samples were prepared by the method of Chomczynski and Sacchi (1987). RNAs were fractionated by electrophoresis in 1% agarose gels in the presence of formaldehyde and transferred to nylon membranes (hybond N; Amersham Corp., Arlington Heights, IL). Filters were acidified (10 min, 5% CH_3COOH) and stained (10 min, 0.004% methylene blue, 0.5 M CH_3COONa, pH 5.0) before hybridization, to check for integrity and amounts of transferred RNA. Blots were hybridized using cDNA probes ^{32}P labeled by random priming, for 18 h, at 37°C in the presence of 40% formamide (human ST3 cDNA probe; Basset et al., 1990), or at 42°C in the presence of 50% formamide (mouse ST3 cDNA probe, nucleotides 179–1505). In both cases, washings were performed in 2× SSC, 0.1% SDS at 22°C, then 0.1× SSC, 0.1% SDS at 55°C, followed by autoradiography.

Construction and Screening of Placenta cDNA Library

The first cDNA strand was synthesized with Avian Myeloblastem Virus

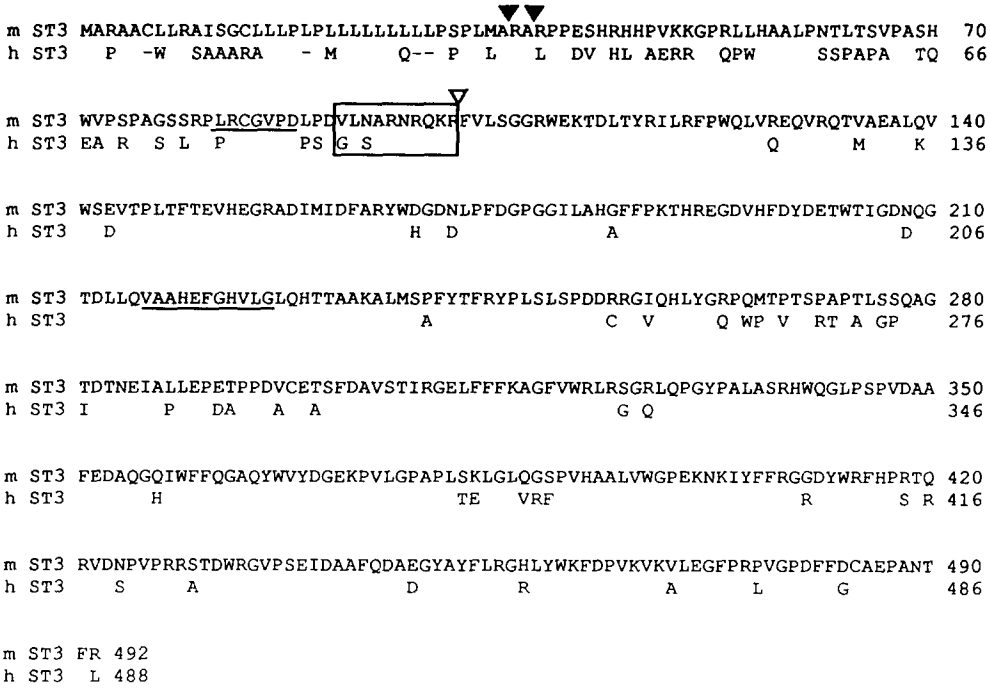

Figure 2. Comparison of mouse and human ST3 amino acid sequences. Only nonidentical amino acids are shown in the human sequence. Gaps (−) were introduced into the sequence to obtain maximal alignment of identical amino acids. The arrowheads denote putative signal peptide cleavage sites (Von Heije, 1986). The 10 amino acid residues specific for mouse and human ST3 are boxed. The open arrowhead points to the putative site of proenzyme cleavage, determined by comparison with other MMPs (Basset et al., 1990). Starting from the 5′ end, the underlined sequences correspond to the "cysteine switch" sequence characteristic of MMPs prodomain (Matrisian, 1990), and to the putative zinc-binding region (Vallee and Auld, 1990).

(AMV)-reverse transcriptase by using placenta poly(A)$^+$ RNA primed with oligo(dT) and random hexamers. The second strand was synthesized by using RNase H and DNA polymerase I (Gubler and Hoffman, 1983). Double-stranded cDNA was cloned into the EcoRI site of the λ gt10 vector after ligation to adaptators, according to the procedure of Sartoris et al. (1987). The ligation reaction was packaged in vitro and the resulting library was plated out on LB agar plates. 4×10^4 recombinant phages were screened using replica nylon filters (Biodyne A; Pall Corporation, East Hills, NY) hybridized with ^{32}P-labeled human ST3 cDNA (Basset et al., 1990), at 37°C in the presence of 50% formamide. Washings were performed in 2× SSC, 0.1% SDS at 22°C, followed by 0.1× SSC, 0.1% SDS at 55°C. After autoradiography, three positive plaques were detected.

Subcloning and Sequencing

The three purified recombinant phages detected with the human ST3 cDNA probe were amplified, and the phage DNA, prepared according to standard procedure, was digested with EcoRI enzyme. The longest cDNA insert (2.3 kb) was subcloned in M13 sequencing vector and DNA sequence was determined by the dideoxy chain termination method, using Sequenase and a deaza-dGTP reagent kit (U.S. Biochemical Corp., Indianapolis, IN). The sequence was analyzed with the PC/GENE software package.

In Situ Hybridization

Deparaffinized and acid-treated sections (6 μm thick) were treated with proteinase K and hybridized overnight with ^{35}S-labeled antisense transcripts from a mouse ST3 cDNA insert (nucleotides 1–1744), subcloned in pBluescript II (Stratagene, La Jolla, CA). Hybridization was followed by RNase treatment (20 μg/ml 30 min, 37°C) and two stringent washings (2× SSC, 50% formamide, 60°C, 2 h), before autoradiography using NTB2 emulsion (Kodak). Autoradiography was for 20 d, and after developing, the slides were counterstained with hematoxylin.

Results

Cloning and Sequencing of mouse ST3 cDNA

We assumed that the ST3 gene, which is expressed at a high level in human placenta (Basset et al., 1990), would be also expressed in mouse placenta. Indeed, a 2.4-kb transcript was detected by Northern blot analysis of mouse placenta poly(A)$^+$ RNA using a ^{32}P-labeled human ST3 cDNA probe (see Materials and Methods). A mouse placenta cDNA library was constructed in the λ gt10 vector and 4×10^4 clones were screened with the human ST3 cDNA probe. Three positive clones were obtained, and the longest insert (2.3 kb) was subcloned into a M13 vector and sequenced. The result of the sequence analysis showed that this insert corresponded to a potential full-length mouse ST3 cDNA of 2,260 nucleotides (Fig. 1). The open reading frame is predicted to encode a 492-residue protein exhibiting 80% sequence homology with the 488-amino acid residues of human ST3 (Fig. 2).

The putative mouse protein has an hydrophobic NH_2-terminal sequence (residues 1–35 or 1–37), which is a candidate leader sequence, and contains a Leu-Arg-Cys-Gly-Val-Pro-Asp (LRCGVPD, single-letter amino acid code; residues 82–88) similar to the PRCGVPD sequence characteristic of the prodomain of MMPs (Matrisian, 1990). The 10 amino acids characteristic of human ST3, at the junction between the ST3 prodomain and the domain of the putative mature enzyme (Basset et al., 1990), are also present in the mouse protein sequence (residues 92–101) and 80% conserved between mouse and human (Fig. 2). By analogy with the other MMPs, the mature mouse protein is predicted to start at phenylalanine 102 (Basset et al., 1990); it exhibits 89% sequence homology with the corresponding human ST3 form (starting at phenylalanine 98) (Fig. 2). An even higher homology (94%) is observed when the comparison is limited to the putative catalytic domain of both proteins (residues 102–254 and 98–250 for mouse and human ST3, respectively), which contains a Zn binding region identical in both species (residues 216–226 of mouse ST3) (Fig. 2).

That the mouse cDNA we had cloned corresponded to a mouse ST3 cDNA was further supported by studying the expression pattern of the corresponding gene in mouse tissues. Mouse ST3 transcripts were found to be expressed in the limb bud during morphogenesis, in the placenta and uterus, but not in the normal colon, intestine, liver, lung, kidney, heart, spleen, brain, and testis (data not shown), as expected from our previous results in human tissues (Basset et al.,

1990). However, the ST3 gene, which is not expressed in normal human skin (Basset et al., 1990), was expressed at low levels in mouse skin (data not shown).

ST3 Gene Expression in the Normal Mammary Gland

The branching growth of mammary ducts involves a complex interplay between epithelium and mesenchyme that is controlled by mammotrophic hormones. At ~4 wk of age, after the onset of ovarian secretion, small dense end buds appear at the ductal tips. These structures, consisting of layers of actively dividing epithelial and myoepithelial cells, serve as growth points for the elongation and branching of new ducts (Coleman et al., 1988; Vonderhaar 1988; Snedeker et al., 1991). At 8–10 wk postpartum, the entire mammary fat pad is filled with a highly branched network of epithelial ducts enveloped in a fibrous sheet of extracellular matrix (Silberstein et al., 1990). When the sexually mature female becomes pregnant, the mammary glands begin a cycle of lobulo-alveolar development that ultimately results in full functional differentiation and the production of milk (Topper and Freeman, 1980).

No ST3 gene expression could be detected by Northern blot analysis during the growth of mouse mammary gland. The gene was not expressed in virgin mice, nor in pregnant mice or during lactation (Fig. 3, lanes *1–7*). However, ST3 gene expression was detected 3 d after weaning (Fig. 3, lane *11*), in the involuting mammary gland. The expression was maximal 6 d after weaning (Fig. 3, lane *14*), persisted at lower levels for an additional 2 wk (Fig. 3, lanes *15–18*), and totally disappeared at the onset of the second gestation (Fig. 3, lanes *19* and *20*).

The results of in situ hybridization experiments were consistent with those obtained by Northern blot analysis. No ST3 RNA could be detected in mouse mammary gland sections during the first 2 d after weaning (Fig. 4, *a–c*), during which milk is still produced and distends the alveolar structures (Martinez-Hernandez et al., 1976). However, ST3 gene expression was observed between days 4 and 20 after weaning (Fig. 4, *d–l*), when ECM remodeling is prominent and apoptosis of epithelial cells is occurring in the involuting mammary gland (Wicha et al., 1980). At day 4 (Fig. 4, *d–f*) and at day 7 (Fig. 4, *g–i*), ST3 RNA was detected in fibroblasts immediately surrounding disorganized clusters of epithelial cells, but not in fibroblasts at a distance from epithelial cords, nor in the epithelial cells themselves. In the following days, ST3 RNA was still observed in fibroblasts surrounding neoformed mammary structures (Fig. 4, *j–l*), but the number of ST3-expressing areas decreased markedly between days 7 and 20 after weaning (data not shown).

Discussion

We have isolated and characterized the cDNA corresponding to the mouse equivalent of the human MMP ST3 gene. The sequences of the human and mouse putative proteins are well conserved and exhibit the structural characteristics of other members of the MMP family. The 10 amino acids that are specific to human ST3, and are located precisely at the putative proprotein cleavage site (Basset et al., 1990), are also present in mouse ST3 and well conserved, suggesting that this short unique sequence may be important in proST3 activation. The expression pattern of the ST3 gene is similar in

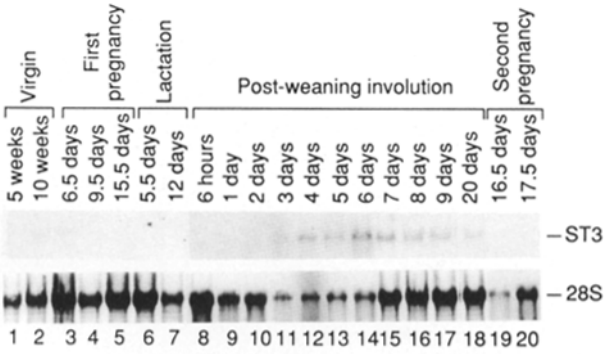

Figure 3. Northern blot analysis of ST3 RNA during growth and involution of mouse mammary gland. Each lane contained 10 μg of total RNA. Lanes *1* and *2*, virgin mice; lanes *3–5*, pregnant mice (first pregnancy); lanes *6* and *7*, lactating mice; lanes *8–18*, postlactating mice; lanes *19* and *20*, pregnant mice (second pregnancy). After electrophoresis and blotting, the filters were stained with methylene blue before hybridization, as described in Materials and Methods. A 2.4-kb ST3 transcript was detected in lanes *11–18*, corresponding to involuting mammary glands between days 3 and 20 postweaning. The methylene blue–stained 28S ribosomal RNA was used to reflect the amount of total RNA loaded on each lane.

mice and in humans, with high levels of expression in the uterus, in the placenta, and in the limb bud during embryogenesis.

ECM is an important regulator of mammary cell function in the mouse, and ECM remodeling accompanies the anatomical changes in the mammary gland during gestation, lactation, and involution (Wicha et al., 1980; Walker et al., 1989; Silberstein et al., 1990; Streuli et al., 1991, and references therein). Several proteinases have been implicated in these processes, including the urokinase plasminogen activator and a type IV collagenase (Ossowski et al., 1979; Talhouk et al., 1991). Urokinase activity is transiently increased after the initiation of mammary involution (Ossowski et al., 1979), while active type IV collagenase is absent during early involution, but appears 3–4 d after weaning, when massive restructuring of the ECM occurs (Talhouk et al., 1991). In this respect, there is a parallel between type IV collagenase expression and ST3 gene expression, which is also initiated 3–4 d after weaning. However, the active form of type IV collagenase is also abundant during pregnancy (Talhouk et al., 1991), while ST3 gene expression is specific to mammary gland involution.

It may be paradoxical that the ST3 gene, which is not expressed during mammary growth, is expressed both in breast carcinoma and during mammary involution, an apoptotic process characterized by epithelial cell death and commonly regarded as being the opposite of cell proliferation (Kerr et al., 1972; Gullino, 1980). However, it has been proposed that apoptosis and proliferation may use common molecular pathways (Evan et al., 1992), and, furthermore, another relationship between mammary involution and breast carcinoma is that both processes are characterized by basement membrane lysis. The basement membrane appears to play a central role in the function of normal mammary epithelial cells, and its dissolution during mammary involution correlates with functional regression of the mammary gland (Martinez-Hernandez et al., 1976; Wicha et al., 1980; Talhouk et al., 1991; Streuli et al., 1991). Thus, ST3 gene expression

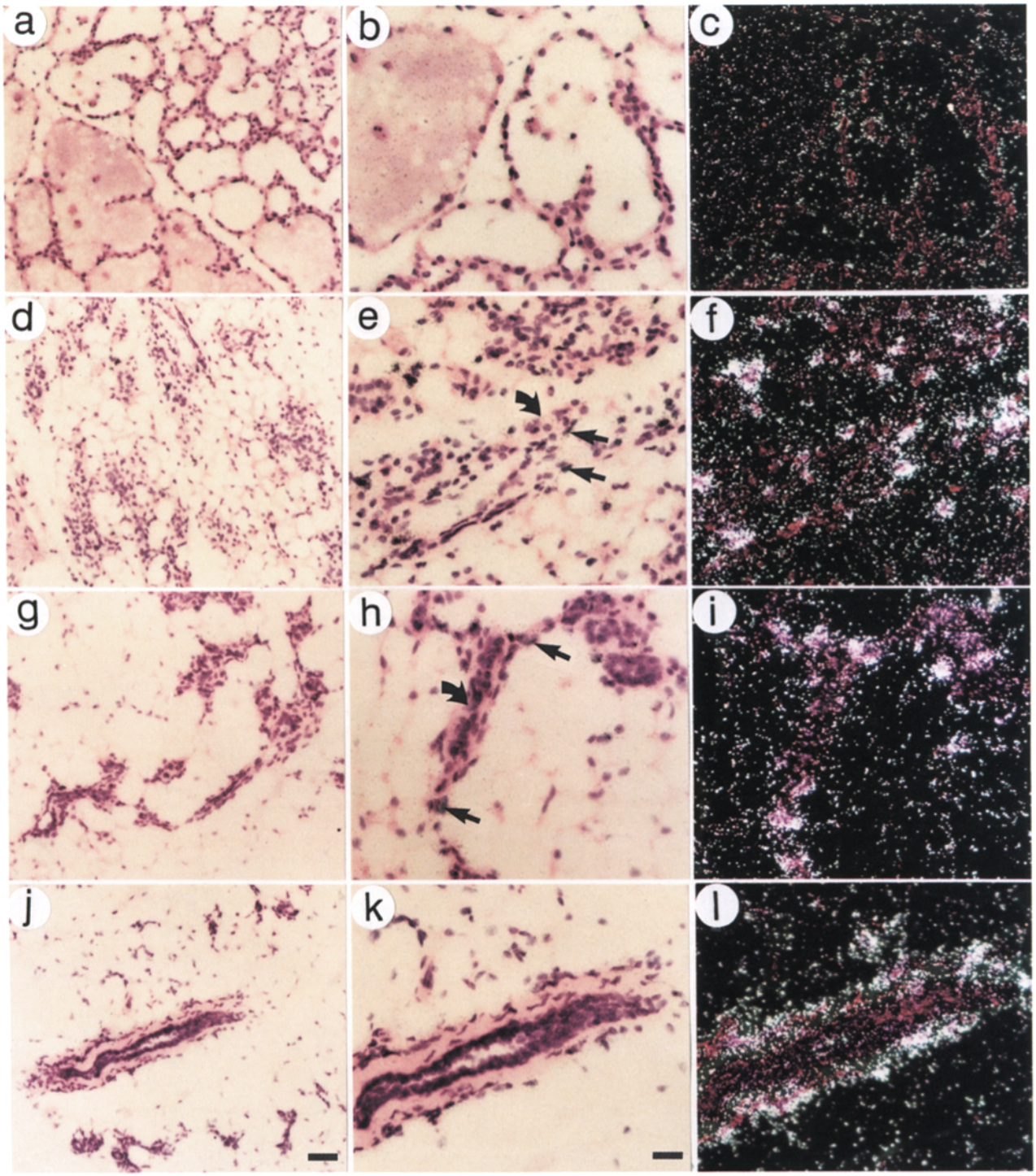

Figure 4. In situ hybridization of ST3 RNA on mouse mammary gland sections during postweaning involution. Analyses were performed at day 2 (*a–c*), day 4 (*d–f*), day 7 (*g–i*), and day 20 (*j–l*) after weaning. *a*, *d*, *g*, and *j* (×100), and *b*, *e*, *h*, and *k* (×200) are bright field micrographs; *c*, *f*, *i*, and *l* (×200) are dark field micrographs of the same sections where ST3 transcripts appear as white silver precipitate. In situ hybridization was carried out with a ^{35}S-labeled mouse antisense RNA ST3 probe. (*a–c*) Numerous secretory distended alveoli. No ST3 transcript could be detected above background. (*d–i*) Disorganized mammary tissue with few collapsed epithelial structures remaining present (*curved arrows*) and high proportion of fatty stroma. ST3 transcripts were detected in fibroblasts immediately surrounding degenerating ducts (*arrows*), but not in fibroblasts at a distance from epithelial cells, nor in epithelial cells themselves. (*j–l*) Neoformed mammary structure. ST3 transcripts were still detected in few fibroblasts, exclusively in the vicinity of epithelial cells. No significant labeling above background was found when using a sense ST3 RNA probe (data not shown). (*a*, *d*, *g*, and *j*) Bar, 40 μm; (*b*, *e*, *h*, and *k*) bar, 20 μm.

may be associated with basement membrane remodeling that occurs both during breast carcinoma progression and during mammary gland involution. Consistent with this hypothesis, in both processes, the ST3 gene is exclusively expressed in fibroblastic cells immediately surrounding epithelial cells.

It remains to be seen whether ST3 participates, possibly with other proteinases such as urokinase and type IV collagenase, in basement membrane remodeling or whether ST3 gene expression is secondary to basement membrane remodeling. In the latter case, ST3 gene expression may result from the transient contact between epithelial and stromal compartments, or from the action of components released during the degradation of the basement membrane itself, including growth factors known to be tightly associated with the basement membrane (Ruoslahti and Yamaguchi, 1991; Flaumenhaft and Rifkin, 1991). In any event, the observation that the ST3 gene is expressed both during breast carcinoma progression and during mammary gland involution further supports the concept that ST3 gene expression plays a role in normal processes and is subverted in breast carcinoma.

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