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Regulated Expression of Homeobox Genes *Msx-1* Suggests a Role in Hormone Action and Epithelial–Stromal Interactions

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The murine homeobox genes *Msx-1* and *Msx-2* are related to the *Drosophila msh* gene and are expressed in a variety of tissues during mouse embryogenesis. We now report the developmentally regulated expression of *Msx-1* and *Msx-2* in the mouse mammary gland and show that their expression patterns point toward significant functional roles. *Msx-1* and *Msx-2* transcripts were present in glands of virgin mice and in glands of mice in early pregnancy, but transcripts decreased dramatically during late pregnancy. Low levels of *Msx-1* transcripts were detected in glands from lactating animals and during the first days of involution, whereas *Msx-2* expression was not detected during lactation or early involution. Expression of both genes increased gradually as involution progressed. *Msx-2* but not *Msx-1* expression was decreased following ovariectomy or following exposure to anti-estrogen implanted directly into the gland. Hormonal regulation of *Msx-2* expression was confirmed when transcripts returned to normal levels after estrogen was administered to ovariectomized animals. *In situ* molecular hybridization for *Msx-1* showed transcripts localized to the mammary epithelium, whereas *Msx-2* expression was confined to the periductal stroma. Mammary stroma from which mammary epithelium had been removed did not transcribe detectable amounts of *Msx-2*, showing that expression is regulated by contiguous mammary epithelium, and indicating a role for these homeobox genes in mesenchymal–epithelial interactions during mammary development.

INTRODUCTION

Mammary development and function are driven by a complex network of hormones acting systemically, which in turn influence peptide growth factors that regulate developmental events at the tissue level (Topper and Freeman, 1980; Dembinski and Shiu, 1987). Estrogen and progesterone are particularly crucial signals in growth and morphogenesis of the breast. It is likely that these hormones control regulatory genes that serve to coordinate developmental interactions and to specify pathway decisions in the developing gland. Because homeobox genes function as master regulators of embryonic events in a variety of organisms including the mouse (Morgan *et al.*, 1992; Balling *et al.*, 1989; Wolgemuth *et al.*, 1989; Ramirez-Solis *et al.*, 1993; Le Mouellic *et al.*, 1992), and because the expression of several *Hox* genes was recently reported in the mammary gland (Friedmann *et al.*, 1994), the mammary gland is a candidate target for hormone-regulated homeobox gene action.

In the mammary gland, genes regulating morphogenesis and growth are likely to be associated with epithelial–stromal interactions. With the onset of ovarian function at 3–4 weeks of age, the mouse mammary ductal system enters a phase of rapid growth and morphogenesis, in which continuing inductive interactions between mammary epithelium and contiguous stroma result in growth and patterning of a ductal tree that fills the adipose-rich stroma (Sakakura *et al.*, 1979). The ductal epithelial cells may follow one of several differentiative pathways depending on their position within the growth buds at the tips of the ductal branches, becoming cells for ductal walls, milk synthesis and secretion, or contraction (Williams and Daniel, 1983). In concert with this epithelial differentiation, the stroma adjacent to the growing duct becomes rich in fibrocytes, producers of

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materials of the fibrous extracellular matrix which encases mammary ducts. (Williams and Daniel, 1983).

The homeobox is a relatively conserved 183-nucleotide sequence encoding a DNA-binding domain found in many genes playing key roles in *Drosophila* embryogenesis. These genes are classified according to their homeobox sequence and chromosomal location. Thirty-eight mammalian homeobox-containing genes, the *Hox* genes, are found in clusters on four chromosomes. Gene disruption and gain-of-function mutations generated in mice have shown that improper expression of *Hox* genes leads to developmental defects (Chisaka and Capecchi, 1991; Kessel and Gruss, 1991; Lufkin *et al.*, 1991), many of which represent homeotic transformations (Morgan *et al.*, 1992; Ramirez-Solis *et al.*, 1993; Small and Potter, 1993; Le Mouellic *et al.*, 1992).

Other mammalian homeobox genes are not located within these clusters and form smaller classes based on sequences that relate them to other conserved motifs such as *Pax* and *Oct* (Herr *et al.*, 1988; Epstein *et al.*, 1991; Hill *et al.*, 1991; Palmieri *et al.*, 1994; Corcoran *et al.*, 1993). The *Drosophila melanogaster* muscle segment homeobox (*msh*) gene contains a homeobox which is markedly divergent from that of any other characterized *Drosophila* genes. In *Drosophila, msh* is mainly expressed in the central nervous system and in segmented striated muscles of the body wall. In the mouse there appear to be three distinct msh-like genes, named *Msx-1, Msx-2,* and *Msx-3,* which are found at separate loci and are not clustered (Hill *et al.*, 1989; Robert *et al.*, 1989; Monaghan *et al.*, 1991; Holland, 1991). Closely related versions of *Msx-1* and *Msx-2* have been identified in a variety of vertebrate species including zebrafish (Ekker *et al.*, 1992), *Xenopus* (Su *et al.*, 1991), and the chick (Coelho *et al.*, 1991).

Msx-1 and *Msx-2* show a closely associated, interactive pattern of expression throughout early embryonic development (MacKenzie *et al.*, 1991a, 1991b; Monaghan *et al.*, 1991). The earliest expression of both genes is detectable in primitive streak mesoderm, followed by expression in neural crest cells and their derivatives. Later expression patterns have been examined by *in situ* hybridization methods in the development of several organs, including the mouse and chick limb bud (Nohno *et al.*, 1992; Davidson *et al.*, 1991; Robert *et al.*, 1991), mouse tooth bud (Mackenzie *et al.*, 1991a, 1992; Jowett *et al.*, 1993), chick heart (Chan-Thomas *et al.*, 1993), and chick craniofacial development (Nishikawa *et al.*, 1994). The results suggested that the two genes play a role in epithelial–mesenchymal interactions in these developing organs.

In a previous paper we reported expression in the mammary gland of several genes from the four *Hox* clusters and the altered expression of some during tumorigenesis (Friedmann *et al.*, 1994). In this paper we describe the expression of *Msx-1* and *Msx-2* RNA in different stages of mammary development. To move closer to a functional analysis, we have examined the effects of experimentally altering tissue interactions and manipulating mammogenic steroids.

METHODS

Animals. C57BL/crl mice were used for collection of the inguinal mammary glands in all RNA preparations. Virgin mice were chosen randomly from multiple cages to minimize the chances of selecting animals in a particular stage of estrus. For involuting glands, pups were weaned 10 days after birth and the following day was counted as Day 1 of involution. Thoracic glands of virgin and pregnant C57BL/crl mice were used for *in situ* hybridization.

Surgery. Ovariectomy was carried out at about 5 weeks of age (animal weight 16–17 g) and glands were collected 4 weeks later to allow complete mammary regression. When estrogen implants were used, they were implanted subcutaneously 6 weeks after ovariectomy and glands were collected 4 days later. This staging was necessary because after estrogen replacement therapy, the mammary glands from ovariectomized animals more closely resemble developing glands in younger 6-week animals.

The inguinal fat pads were cleared of epithelium at 3 weeks of age by removing the portion of the gland containing epithelium and cauterizing the nipple area and associated blood vessels (DeOme *et al.*, 1959).

Implants. EVAc (Elvax 40P) was a gift from DuPont Chemical Co. (Universal City, CA). Anti-estrogen ICI 164,384 was a gift from ICI Pharmaceutical (Cheshire, England). 17 β-Estradiol is from Sigma (E-8875). Implant preparation is described in detail elsewhere (Silberstein and Daniel, 1982). Briefly, anti-estrogen was dispersed in 0.125 ml of EVAc that had been dissolved in dichloromethane (20% w/v). This mixture was quick-frozen and evaporated under vacuum, and the polymer matrix with entrapped chemical was then cut to form pellets containing 250 μg anti-estrogen and surgically implanted (typical implant weight, 0.5 mg). 17 β-Estradiol was mixed with dichloromethane and serially diluted to a final dose of 50 ng implants per animal. Recipient mice were anesthetized with an interperitonial injection of Nembutal (60 $\mu g\ g^{-1}$ body wt) and the number 3 mammary glands (in the case of anti-estrogen) were exposed by reflecting the skin from a midline ventral incision. A small pocket was made in the mammary fat pad using Dumont forceps, which were then used to insert the implant. Estrogen implants were inserted through a small incision subdermally at the back of the neck. The skin was then closed with wound clips and the animals were allowed to recover in an atmosphere of 95% O_2/5% CO_2.

RNA preparation and Northern hybridization. Inguinal mammary glands were frozen in liquid nitrogen immediately after removal, and total RNA was prepared by the guanidine isothiocynate (4 *M*), cesium chloride (5.7 *M*) method (Ausubel *et al.*, 1989). Total RNA from the glands (the number and age of animals used for each experiment are given in the figure legends) was isolated. In several cases poly(A)$^+$ RNA was purified by oligo(dt)–cellulose chromatography as described (Sambrook *et al.*, 1989). Five micrograms of poly(A)$^+$ enriched RNA, or 25 μg total RNA was electrophoresed in 1.0% agarose containing 2.3 *M* formaldehyde in Mops buffer (0.2 *M* morpholinopropane sulfonic acid, 50 m*M* sodium acetate, 5.0 m*M* EDTA, pH 7.0). RNA was transferred to a nylon transfer membrane (Magna NT, Micron Separation Inc., 0.45 μm) by the established procedure of Maniatis *et al.* (1982). Northern hybridizations were carried out under high stringency conditions, using ^{32}P-random-primed labeled (1×10^9 to 10×10^9 counts minute^{-1} μg^{-1}) murine *Msx-2* and *Msx-1* and human L7 cDNAs. The *Msx-2* fragment is ~400 base pairs (bp), derived from the 3′ end of the gene. It does not include the homeobox but does include ~200 bp of the 3′ UTR. The *Msx-1* fragment is ~850 bp, derived from the 3′ end

of the gene. It begins at the C-terminal half of the homeobox and includes ~550 bp of the 3′ UTR. Washes after hybridizations were in 0.1× SSPE/0.1% sodium dodecyl sulfate at 65°C. All Northern hybridizations were repeated at least twice using different blots.

In situ hybridization. Mammary glands from virgin and pregnant animals were fixed for 3 hr in 4% paraformaldehyde/PBS, dehydrated through a graded series of ethanols to xylene, and embedded in paraffin wax. Seven-micrometer sections were cut, floated on slides coated with 3-aminopropyltriethoxysilane (Sigma), and baked onto slides overnight on a slide warmer at 45°C. Sections were dewaxed through two changes of xylene and rehydrated through graded series of ethanols. Sections were then digested with proteinase K (1 μg ml^{-1} in 10 m*M* Tris–HCl, 5 m*M* EDTA, pH 7.5) at 37°C for 30 min and the reaction was stopped with two changes of H_2O and one wash in PBS for 2 min.

To avoid nonspecific binding of RNA probes, slides were prehybridized for 1 hr at 45°C with hybridization buffer (see below) that did not include the RNA probe. Slides were hybridized at 45°C for 16 hr under siliconized coverslips in a solution containing 50% formamide, 3 m*M* NaCl, 10 m*M* Tris–HCl (pH 7.5), 1 m*M* EDTA, 150 μg ml^{-1} tRNA, 1 mg ml^{-1} yeast total RNA, 10% dextran sulfate, 1% blocking solution (blocking reagent for nucleic acid hybridization, Boehringer-Mannheim Genius system kit), and 800 ng ml^{-1} digoxigenin labeled RNA probe. After hybridization, coverslips were removed in 2× SSPE and then slides were rinsed twice, for 1 hr each time in 0.2× SSPE at 50°C.

To avoid nonspecific binding of anti-digoxigenin antibody, slides were treated for 45 min at room temperature with 2% blocking solution in 100 m*M* Tris–HCl, pH 7.5, 150 m*M* NaCl and then for 45 minutes in BSA wash solution [1% BSA (Sigma A-7030), 0.3% Triton X-100, 100 m*M* Tris–HCl, pH 7.5, and 150 m*M* NaCl] at room temperature. Slides were incubated with alkaline phosphatase-conjugated anti-digoxigenin antibody in 2% blocking solution under siliconzed coverslips for 16 hr at room temperature. Coverslips were removed in BSA wash solution, followed by two more BSA washes, and then incubated in 2% blocking solution for 30 min followed by 2 min in a solution containing 100 m*M* Tris–HCl, pH 9.5, 100 m*M* EDTA, and 50 m*M* $MgCl_2$. To visualize probes, slides were incubated with a pair of colorimetric substances, NBT and x-phosphate, in the above solution (as described in Boehringer-Mannheim The Genius System User's Guide for Filter Hybridization, Version 2.0) for various times ranging from 5.5 to 24 hr. When the desired intensity was reached, the reaction was stopped in 10 m*M* Tris–HCl, 1 m*M* EDTA, pH 7.5. Slides were dehydrated through graded alcohols into xylene and coverslipped.

Riboprobes were labeled with digoxigenin labeling mix (NTP labeling mixture 10×, Boehringer-Mannheim Catalog No. 1277 073), using the appropriate SP6 or T7 transcription system. All *in situ* hybridizations were repeated at least four times.

RESULTS

Expression of Msx-1 and Msx-2 Transcripts Levels during Mammary Gland Development

The expression level of *Msx-1* and *Msx-2* transcripts in various stages of mammary development was evaluated by Northern blot hybridization to poly(A)$^+$ enriched RNA isolated from mouse mammary glands at several stages of development. The Northern blot was hybridized consecutively with probes for *Msx-1, Msx-2,* and L7 (as a control for the amount of RNA and its integrity). *Msx-1* (~2050 bp) and *Msx-2* transcripts (~1300 and ~2300 bp) were present in glands from virgin animals and glands from animals during early pregnancy (5–8 days post coitus) (Fig. 1). Transcript levels of both genes decreased substantially in glands from animals in late stages of pregnancy (15–18 days post coitus). In lactating glands *Msx-2* expression was not detected, while *Msx-1* transcripts were seen at low levels. The transcript size of *Msx-1* in glands from lactating animals was somewhat smaller (~1.9 kb) than the transcript size from other stages of the mammary cycle.

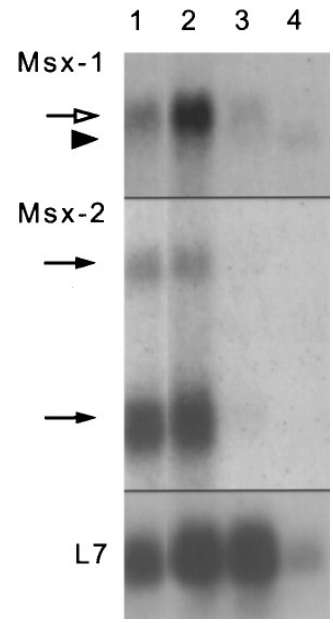

FIG. 1. RNA expression of *Msx-1* and *Msx-2* during stages of mouse mammary gland development. Lane 1: 25 immature virgin mice were taken; Lane 2: 7 mice were taken 5–8 days into timed pregnancies; Lane 3: five 15- to 18-days pregnant mice were taken; Lane 4: 3 animals were taken 3–4 days into lactation. Each lane contains 5 μg of poly(A)$^+$ RNA. L7 mRNA was used as a loading control. Open arrow points to *Msx-1* transcripts, solid arrows point to *Msx-2* transcripts. Arrowhead points to smaller *Msx-1* transcript observed only in lactating glands.

The final stage of the mammary cycle is involution, in which, following weaning, secretory tissue is destroyed by apoptotsis as the gland reorganizes to a form resembling its prepregnancy state. Total RNA was extracted from glands of mice that were lactating for 10 days before pups were weaned, and their glands were removed at several time points during involution. Figure 2 shows the expression of *Msx-1* and *Msx-2* during involution. *Msx-1* was expressed at low levels in the first 3 days of involution (lanes 2 and 3). In the fourth day of involution expression increased and remained at similar levels thereafter. *Msx-1* transcript size in glands that were involuting for 2 days was the same as in lactation. In the third day after weaning both transcripts

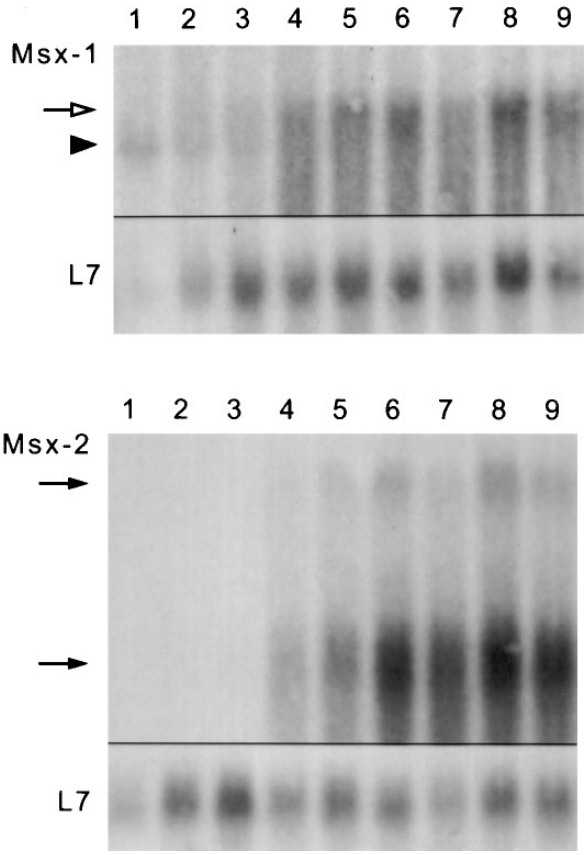

FIG. 2. Northern analysis of *Msx-1* and *Msx-2* in involuting mammary glands. Mice were lactating for 10 days before pups were removed. The next day is considered the first day of involution. RNA was extracted from glands involuting for different numbers of days. The Northern analysis was performed as described under Materials and Methods. Lane 1: lactating glands. Lane 2: 2 days involuting. Lane 3: 3 days involuting. Lane 4: 4 days involuting. Lane 5: 6 days involuting. Lane 6: 8 days involuting. Lane 7: 10 days involuting. Lane 8: 12 days involuting. Lane 9: 14 days involuting. Each lane contains 20 μg total RNA. Lanes 1–3: three animals were taken. Lanes 4–5: five animals were taken. Lanes 6–9: six animals were taken. Solid arrows point to *Msx-2* transcripts. Open arrow points to *Msx-1* transcript. Arrowhead points to *Msx-1* smaller transcript in lactating and early involuting glands.

were visible, after which the larger transcript size, that which was detected in other stages of gland development, was the predominant one. *Msx-2* transcripts could not be detected in the first 3 days of involution. Expression increased gradually in Days 4–8 and then reached a plateau.

Msx-2 Expression in Glands from Ovariectomized Animals and after Estrogen Replacement

To determine if *Msx-1* and *Msx-2* transcript levels are regulated by ovarian secretions, we isolated RNA and made a Northern blot from glands of animals that had been ovariectomized at two time points during the gland development. Ovariectomy was performed either at the age of 5 weeks, when the mouse initiates estrus cycles (puberty), or at the age of 12 weeks when the mouse is already mature and cycling (adulthood). After ovariectomy, mice were allowed to recuperate for 4 weeks, a time period that was determined adequate for ovarian steroids to be depleted from the tissues, when glands were taken.

The expression level of *Msx-1* in glands from ovariectomized mice was similar to that in glands from intact controls (Fig. 3). On the other hand, the expression of *Msx-2* was lower in glands from ovariectomized animals at both time points compared to glands from intact controls (Fig. 3), suggesting that *Msx-2* RNA level is up-regulated by ovarian secretions.

To further test this hypothesis, *Msx-2* was hybridized to mammary gland RNA from animals that were ovariectomized at 5 weeks of age, and in which estrogen was restored by subcutaneous implants at 11 weeks of age. Glands were collected 4 days after estrogen was implanted. When estrogen was replaced in animals that were ovariectomized, *Msx-2* levels (Fig. 4, lane 2) returned to levels found in glands from intact animals (Fig. 4, lane 3).

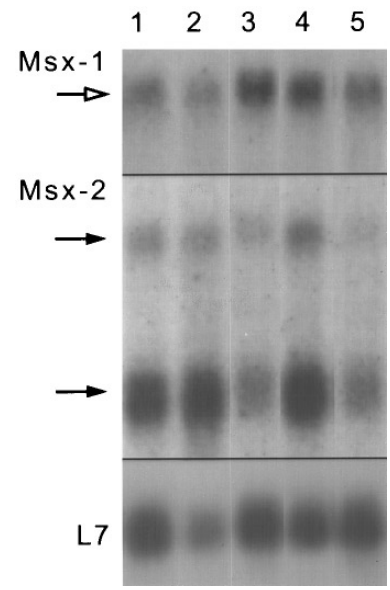

FIG. 3. RNA expression of *Msx-1* and *Msx-2* in the mammary gland in response to ovariectomy. Poly(A)$^+$ RNAs were extracted from glands of twenty 5-week-old endocrine intact mice (lane 1); fifteen 8- to 9-week-old endocrine-intact mice (lane 2); thirty 9-week-old mice that were ovariectomized at 5 weeks of age (lane 3); fifteen 16-week-old endocrine-intact mice (lane 4); twenty-five 16-week-old mice that were ovariectomized at 12 weeks of age (lane 5). Northern analysis was performed as described under Materials and Methods. Each lane contains 5 μg of poly(A)$^+$ RNA. Solid arrows point to *Msx-2* transcripts. Open arrow points to *Msx-1* transcript.

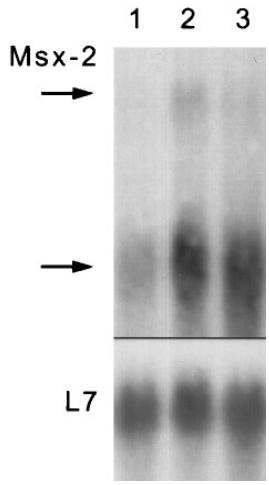

FIG. 4. Expression of *Msx-2* RNA in the mammary gland in response to estrogen replacement in ovariectomized mice. RNA was extracted from mammary glands of mice that were ovariectomized at the age of 5 weeks, let mature for 6 more weeks, when estrogen was added subcutaneously. Glands were collected 4 days later (lane 2). RNA levels are compared to RNA from glands of 6 weeks endocrine-intact animals (lane 3) and to glands from mice that were ovariectomized at 5 weeks of age and let mature for an additional 6 weeks prior to glands collection (lane 1). For each lane 10 animals were taken. Northern analysis was performed as described under Materials and Methods. Each lane contains 20 μg total RNA. Solid arrows point to *Msx-2* transcripts.

Expression of *Msx-2* RNA was also examined in glands that were treated with pure anti-estrogen in endocrine-intact animals. The pure anti-estrogens are estrogen antagonists without the estrogenic properties that are, paradoxically, associated with many conventional anti-estrogens. Pure anti-estrogens have been shown to have a highly localized inhibitory effect on mammary epithelial growth and morphogenesis when tested in a natural endocrinological and physiological milieu (Silberstein *et al.*, 1994). EVAc implants, containing the pure antiestrogen ICI 164,384, were implanted directly into the mammary gland in a concentration that was shown to influence only the implanted gland, leaving other glands in the same animal unaffected (Silberstein *et al.*, 1994). RNA was extracted from glands treated with anti-estrogen and from untreated contralateral glands. Glands that were treated with anti-estrogen show reduced levels of *Msx-2* RNA compared to the untreated glands (Fig. 5). These results support the hypothesis that *Msx-2* RNA is up-regulated by ovarian secretions, of which estrogen is a component.

Spatial Localization of Msx-1 and Msx-2 RNA in the Mammary Gland

In situ hybridization was performed on sections of mammary gland tissue using gene-specific probes. Figure 6A shows *Msx-2* RNA localized to the periductal stromal cells, where these cells are forming extracellular matrix (ECM) in coordination with ductal growth. ECM is maintained around the ducts in the quiescent gland of the nonpregnant mouse. In glands from pregnant mice *Msx-2* is localized to stromal cells adjacent to mammary ducts (Fig. 6B) and in most cases not around the developing secretory alveoli or away from gland.

To further test the involvement of *Msx-2* in epithelium–stroma interactions, we examined mammary gland-free fat pads from which the epithelial component had been surgically ablated in prepubertal mice (DeOme *et al.*, 1959). As adults these mice carry inguinal glands consisting solely of mammary adipose stroma which is devoid of any mammary epithelial component. RNA extracted from gland-free fat pads was probed with *Msx-2* and no transcripts were detected, even after a long exposure time (Fig. 7). *In situ* hybridization did not show any detectable *Msx-2* messages either (not shown). This indicates the essential role of epithelium in inducing mesenchymal expression of *Msx-2*.

Figure 6C shows *Msx-1* transcripts localized to the epithelium in glands from pregnant mice. Epithelial localization was found in glands from virgin mice as well (not shown).

DISCUSSION

In a previous paper (Friedmann *et al.*, 1994), we described the expression of *Hox* genes in mouse mammary gland development, in precancerous lesions, and in malignancy. Here we extend these experiments to include developmental studies on expression of two homeobox-containing

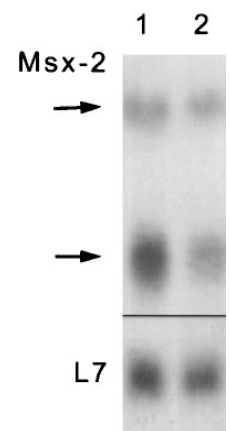

FIG. 5. RNA expression of *Msx-2* in the mammary gland in response to implanted anti-estrogen. RNA was extracted from glands of five pubescent mice that were implanted with 100 μg/gland of anti-estrogen ICI 164,384 for 4 days (lane 2) or from untreated contralateral glands (lane 1). Northern analysis was performed as described under Materials and Methods. Each lane contains 20 μg total RNA. Solid arrows point to *Msx-2* transcripts.

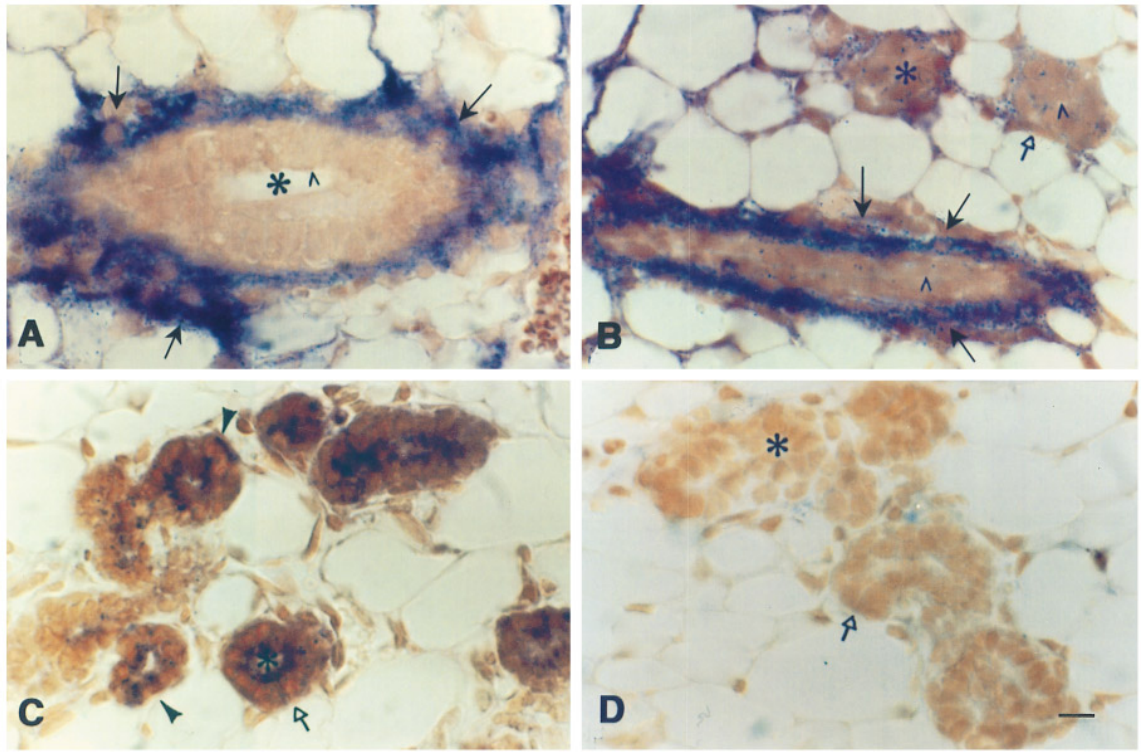

FIG. 6. Spatial expression of *Msx-1* and *Msx-2* mRNA in mammary gland. Riboprobes were labeled with DIG-11–UTP. Fragments labeled were the same as described for Northern blot hybridization (see Materials and Methods). (A) *Msx-2* expression in a gland of mature, virgin mouse. (B) *Msx-2* expression in a gland of a pregnant mouse. (C) *Msx-1* expression in mammary gland of a pregnant mouse. (D) *Msx-1* sense control probe in a gland of a pregnant mouse. It is representative of the controls for other stages of gland development for *Msx-1* and *Msx-2* sense probes. Solid arrowheads point to *Msx-1*-positive epithelial cells. ⟩ points to *Msx-2*-negative epithelial cells. Solid arrows point to *Msx-2*-positive periductal stromal cells. Open arrows point to lobule-alveolar structures in pregnant glands. Asterisks indicate lumens. Bar, 15 μm.

genes, *Msx-1* and *Msx-2*, which are located on chromosomes 5 (Hill *et al.*, 1989) and 13 (Bell *et al.*, 1993), respectively, and are not linked to other known homeogenes. *Msx-1* and *Msx-2* RNAs were expressed during mammary gland development in a stage-dependent manner, appearing in the virgin animal, declining during pregnancy and lactation, and increasing again at the later stages of involution. The expression patterns and the different expression levels of the two genes during the various stages of the gland development indicate differential regulation of *Msx-1* and *Msx-2*, as well as developmental regulation of their expression in the mammary cycle. Absence of detectable expression of *Msx-2* and low levels of expression of *Msx-1* during lactation may be due in part to high levels of milk protein transcripts that may dilute other mRNAs, as seen by the L7 loading control. The smaller size of *Msx-1* transcripts from lactating glands may indicate that an alternative protein with a possible different role is produced.

Increasing levels of expression of both *Msx-1* and *Msx-2* as involution progresses may indicate that both genes participate in the later stages of glandular reorganization, rather than being required for earlier apoptotic events. Though *Msx-1* is expressed from the beginning of involution, the expression level is low and the transcript size in the second day after weaning is the same as in the lactating gland. This indicates that the detected RNAs were probably leftover from lactation, and they may not participate significantly in the early stages of involution. *Msx-1* synthesis *de novo* appears to start only after 4 days postweaning, supporting a role for this gene in the later stages of involution. Both *Msx-1* and *Msx-2* transcripts showed a relatively high degree of degradation during involution on Northern blots. Because there is extensive tissue rearrangement during involution, this degradation is probably part of this process and may be specific to certain classes of transcripts, as the L7 control does not show the same degree of degradation.

The mammary gland is an endocrine target organ of considerable complexity. The ovarian steroids, estrogen and progesterone, are critically involved in the stimulation of mammary growth at puberty and during pregnancy (Lyons, 1958; Nandi, 1958), and genes that are involved in regulating tissue-specific responses to these hormones are likely to

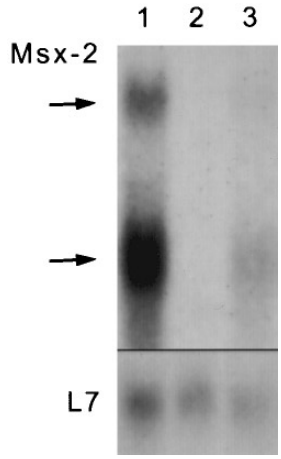

FIG. 7. RNA expression of *Msx-2* in gland-free fat pad. RNA was extracted from glands whose epithelial component had been removed in the 3-week-old mice. Glands were collected 2 months after surgery (lane 2). Expression is compared to levels in glands of epithelium-intact mature virgin mice (lane 1) and to glands of ovariectomized mice (lane 3). For lanes 1 and 3, 5 animals were taken; for lane 2, 15 animals were taken. The northern analysis was performed as described under Materials and Methods. Each lane contains 20 μg total RNA. Solid arrows point to *Msx-2* transcripts.

be directly or indirectly influenced by levels of circulating steroids. If *Msx-1* or *Msx-2* are involved in the development of the mammary gland, their activity is expected to be linked to mammogenic endocrine secretions, however indirectly. To test this, glands from ovariectomized animals were examined for the expression of *Msx-1* and *Msx-2* RNA. *Msx-1* transcript levels did not change conspicuously in response to ovariectomy. On the other hand, *Msx-2* transcripts levels decreased in glands from animals that were ovariectomized either at puberty or at adulthood relative to glands taken from same age intact animals, suggesting that ovarian secretions up-regulate *Msx-2* expression. When estrogen was replaced in ovariectomized mice, *Msx-2* levels returned to levels similar to those seen in glands in endocrine-intact mice.

This indicates that estrogen has the potential to regulate *Msx-2* expression, but the question of whether physiological, circulating levels of endogenous estrogen could do so remained unanswered. *Msx-2* expression was then studied in glands that were treated *in situ* with anti-estrogen. As with ovariectomized animals, transcripts levels declined, but in this case only the treated glands were affected, while untreated contralateral glands displayed normal levels of *Msx-2* expression. Because the anti-estrogen used belongs to a class of agents that lacks estrogenic activity (Wakeling and Bowler, 1988; Wakeling *et al.,* 1991), this experiment demonstrates that estrogen is required for and normally functions as a regulator of *Msx-2* expression. More work should be done to determine if the effect is direct or through downstream mediators.

Msx-1 and *Msx-2* belong to a family of genes which are related to the *Drosophila Msh* genes. Although a precise function has yet to be established for any of the *msh*-related genes, their spatial domains of expression, as well as features of their regulation, suggest that they are key participants in basic developmental processes. Msx-1 and Msx-2 are involved in epithelial–mesenchymal interactions in developing organs, including the mouse and chick limb buds (Davidson *et al.,* 1991; Robert *et al.,* 1991), mouse tooth development (Jowett *et al.,* 1993; Satokata and Maas, 1994), and mouse and human craniofacial bone development (Satokata and Maas, 1994; Liu *et al.,* 1994, 1995; Jabs *et al.,* 1993). Epithelium–mesenchyme interactions are crucial to the development of the mammary gland (Sakakura *et al.,* 1976; Sakakura, 1987), and localization of *Msx-1* and *Msx-2* transcripts by *in situ* hybridization was expected to provide insights into whether these genes play a morphogenetic role in mammary development.

In glands from virgin and pregnant animals, *Msx-1* was localized to the mammary epithelium. *Msx-2* RNA was found in stroma closely associated with epithelial elements, not in epithelial cells and not in stroma distant from epithelial elements. In pregnancy, *Msx-1* expression was detected in epithelium of the ducts as well as the developing alveoli. *Msx-2* expression was associated mainly with stromal cells surrounding ducts and not with cells in the less abundant stroma associated with lobule-alveolar structures. The close physical association of cells displaying *Msx-1* and *Msx-2* transcripts with mammary epithelium strongly suggests that these homeogenes play a role in the inductive interactions occurring between mammary epithelium and stromal cells of the mammary fat pad.

Msx-2 expression was not detected in epithelium-free fat pad. This indicates that mammary epithelium is required for the expression of *Msx-2* in contiguous periductal stroma. The absence of *Msx-2* expression in stromal cells associated with blood vessels indicates a degree of specificity in this tissue interaction. Interestingly, *Msx-1* expression and *Msx-2* expression, which are normally found in the mesoderm underlying the apical ectodermal ridge in developing chick limbs, are not maintained in a limbless mutant that is unable to form an apical ridge (Robert *et al.,* 1991). The mutant can be rescued by grafting normal ectoderm to the limb field, leading to expression of these homeobox genes. Our results indicate that mesodermal expression of *Msx-2* in the mammary gland also requires contiguous mammary epithelium. In the case of the breast, an additional regulatory element is indicated by the influence of endogenous estrogen on *Msx-2* expression.

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