Regulated Expression of Homeobox Genes Msx-1 and Msx-2 in Mouse Mammary Gland Development 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 Shi, 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 Mouellec 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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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 Moellic 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 (Her 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, 1991b; 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 involving 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 ovariec-tomized 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., 1999).

**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). 17β-Estradiol is from Sigma (E-8875). Implant preparation was 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). 17β-Estradiol was mixed with dichloromethane and serially diluted to a final dose of 50 ng implants per animal. Recipient mice were anesthetized with an intraperitoneal injection of Nembutal (60 mg/kg) and glands were collected 4 days later. This staging was necessary because after estrogen replacement therapy, the mammary glands from ovariec-tomized animals more closely resemble developing glands in younger 6-week animals.

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% O₂/5% CO₂.

**RNA preparation and Northern hybridization.** Inguinal mammary glands were frozen in liquid nitrogen immediately after removal, and total RNA was prepared by the guanidinium isothiocynate (4 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 mM sodium acetate, 5.0 mM EDTA, pH 7.0). RNA was transferred to a nylon transfer membrane (Glanza 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 32P-random-primed labeled (L × 10⁶ to 10 × 10⁶ 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 0.1x 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 ethanol 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 ethanol. Sections were then digested with proteinase K (1 μg ml⁻¹ in 10 mM Tris–HCl, 5 mM EDTA, pH 7.5) at 37°C for 30 min and the reaction was stopped with two changes of H₂O 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 mM NaCl, 10 mM Tris–HCl (pH 7.5), 1 mM EDTA, 150 μg ml⁻¹ RNA, 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 2x SSPE and then slides were rinsed twice, for 1 hr each time in 0.2x 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 mM Tris–HCl, pH 7.5, 150 mM NaCl and then for 45 minutes in BSA wash solution [1% BSA (Sigma A-7030), 0.3% Triton X-100, 100 mM Tris–HCl, pH 7.5, and 150 mM NaCl] at room temperature. Slides were incubated with alkaline phosphatase-conjugated anti-digoxigenin antibody in 2% blocking solution under siliconized 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 mM Tris–HCl, pH 9.5, 100 mM EDTA, and 50 mM MgCl₂. 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 mM Tris–HCl, 1 mM EDTA, pH 7.5. Slides were dehydrated through graded alcohols into xylene and coverslipped.

Riboprobes were labeled with digoxigenin labeling mix (NTP labeling mixture 10x, Boehringer-Mannheim Catalog No. 1277 073), somewhat smaller (1.9 kb) than the transcript size from Msx-2. In glands from lactating animals was in situ hybridization. In the fourth day of involution expression increased and Northern blot hybridization to 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 smaller Msx-1 transcript observed only in lactating glands.

Figure 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.

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.

The final stage of the mammary cycle is involution, in which, following weaning, secretory tissue is destroyed by apoptosis 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 invasion (lanes 2 and 3). In the fourth day of invasion 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
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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. 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.

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.

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 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.

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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 genes.
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 a 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. Open arrowheads point 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
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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 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 (Satokata 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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