Development 122, 2729-2737 (1996)
Printed in Great Britain © The Company of Biologists Limited 1996
DEV2046

Regulation of *Msx-1*, *Msx-2*, *Bmp-2* and *Bmp-4* during foetal and postnatal mammary gland development

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SUMMARY

Expression of the *Msx-1* and *Msx-2* homeobox genes have been shown to be co-ordinately regulated with the *Bmp-2* and *Bmp-4* ligands in a variety of developing tissues. Here we report that transcripts from all four genes are developmentally regulated during both foetal and postnatal mammary gland development. The location and time-course of the *Bmp* and *Msx* expression point to a role for Msx and Bmp gene products in the control of epithelial-mesenchymal interactions. Expression of *Msx-2*, but not *Msx-1*, *Bmp-2* or *Bmp-4* was decreased following ovariectomy, while expression of the human *Msx-2* homologue was regulated by 17β-oestradiol in the MCF-7 breast cancer cell line. The regulation of *Msx-2* expression by oestrogen raises the possibility that hormonal regulation of mammary development is mediated through the control of epithelial-mesenchymal interactions.

Key words: homeobox genes, *Msx*, *Bmp*, mammary gland, hormonal regulation, epithelial-mesenchymal interactions, mouse

INTRODUCTION

Foetal development in the mouse mammary gland can first be detected at embryonic day 10-11 (E10-11) as a line of epithelial thickening between the limb buds on both sides of the animal. The underlying fibroblastic mesenchyme then induces the epithelium to thicken and invaginate (E12-E14; Kratochwil, 1969; Sakakura et al., 1976; Sakakura, 1991). By E17-E18, the mammary epithelium has branched into a deeper adipocytic mesenchyme that (after birth) is referred to as the stromal fat pad. Three distinct periods of postnatal mammary gland development are driven by combinations of systemic hormones including oestrogen, progesterone and prolactin (Daniel and Silberstein, 1987). Ductal growth, which is dependent on the presence of oestrogen (Haslam, 1987; Lubahn et al., 1993; Korach, 1994), occurs from 6 to 8 weeks of age when epithelial 'end buds' ramify from the nipple throughout the fatty mesenchyme. Pregnancy initiates the second phase of development in which lobular-alveolar structures develop from the existing ductal system in a process that is driven by pregnancy-induced hormones and oestrogen (Nandi, 1958). The lobular system grows and differentiates to form alveoli in which milk protein synthesis takes place during lactation. Following weaning of the young, the mammary gland undergoes extensive remodelling, leading to the selective loss of the alveolar structures, in a process involving large scale apoptosis (Strange et al., 1992).

Although the major systemic hormones that drive mammary development have been characterised and the importance of epithelial-mesenchymal interactions has been demonstrated (Nandi, 1958; Sakakura et al., 1991; Cunha et al., 1992), the molecular mechanisms by which these processes function at the local level are poorly understood. Molecules that have been implicated include growth factors such as members of the EGF, Wnt, FGF, TGFβ, tachykinin families (Snedeker et al., 1991; Gavin et al., 1992; Bühler et al., 1993; Coleman and Daniel, 1990; Daniel and Robinson, 1992; Coleman-Krnacik and Rosen, 1994; Weber-Hall et al., 1994; Weil et al., 1995) as well as extracellular matrix components (Jones et al., 1993; Thesleff et al., 1995a,b). Recent data from null mutant mice have also shown that the High Mobility Group (HMG)-box transcription factor, Lymphoid Enhancer Factor 1 (LEF-1) and cyclin D1 are necessary for foetal and pregnancy stages of mammary development respectively (Van Genderen et al.,

1994; Sicinski et al., 1995). Results from tissue-recombination experiments, studies of morphological changes and patterns of gene expression suggest that mammary development shares common features with other systems in which epithelial-mesenchymal interactions underly morphogenesis (Sakakura, 1991; Thesleff et al., 1995). For example, both mammary and tooth development have both been shown to be arrested at the bud stage in LEF-$1^{-/-}$ mutant mice, underlining the similarity of these developmental programmes at this stage.

Two groups of molecules implicated in the control of tissue interactions are the Msx homeodomain-containing proteins (related to the *Drosophila* muscle segment homeobox gene *msh*) and the bone morphogenetic proteins (BMPs), in particular the closely related BMP-2 and BMP-4 (Lyons et al., 1990; Jones et al., 1991). Expression of *Msx-1*, *Msx-2*, *Bmp-2* and *Bmp-4* have been shown to be associated with sites of morphogenesis and are often colocalised in patterns that suggest they interact (Jones et al., 1991; Hogan et al., 1994; Davidson, 1995). Direct evidence for a role for BMPs in the regulation of *Msx* gene expression has been found in tooth development where addition of BMP-2 or BMP-4 can induce the expression of both *Msx-1* and *Msx-2* in tooth mesenchyme explants (Vainio et al., 1993). In addition, *Msx-2* expression in hindbrain neural crest tissue is rapidly induced in response to exogenous BMP-4 (Graham et al., 1994). The significance of the *Msx-1* gene for tooth development was recently demonstrated in mutant *Msx-1* null mice. These knockout mice died at birth and had teeth that did not develop beyond the bud stage (Satokata and Maas, 1994).

Here we report findings that point to a role for *Bmp* and *Msx* genes in the control of mammary gland morphogenesis. The finding that *Msx* and *Bmp* genes are expressed during the invagination of the ectodermal epithelium in both the tooth (reviewed by Davidson, 1995) and the mammary gland may suggest that common mechanisms underly epithelial-mesenchymal interactions in this type of development. Further studies show *Msx-2* expression is regulated by oestrogen in the postnatal gland and thus raises the possibility that the hormonal regulation of mammary development is mediated through the control of epithelial-mesenchymal interactions.

MATERIALS AND METHODS

Animals and tissues

In all experiments, the fourth abdominal mammary gland was taken from female Parks mice (MRC; outbred). Following lymph node removal, the tissue was snap frozen in liquid nitrogen and stored at –80°C until use. At least 6 mice (3 each for 17.5 day pregnant and lactating) were used per experimental point. Mice used for mating were between 6 and 8 weeks of age. Pregnancies were measured from the morning a vaginal plug was observed (day 0.5 post coitus (p.c.)), following overnight matings. The age of embryos are stated as embryonic day (E), where E0.5 is the morning a plug was observed. Days lactation were measured from the day pups were born (day 0). Days involution were measured from the day the pups were removed from their mothers (day 0). Fat pad mRNA was prepared from glands taken from 5-week old animals at which time epithelial growth had not extended far from the nipple (data not shown). To ensure no epithelial contamination, only tissue distal to the lymph node was taken. Ovariectomy was performed on 6-week old animals 2 weeks prior to killing and gland removal. Unless otherwise stated, poly(A)$^+$ mRNA was prepared from glands of 8-week old virgin mice that had been ovariectomised at 6 weeks of age. Visual confirmation of the ovariectomy was made at the time of gland removal. Embryos for the Bmp in situ hybridisation were from C57 mice.

RNA isolation

Total RNA was isolated by homogenisation of tissues in a guanidinium isothiocyanate solution followed by extraction with acidified phenol and ethanol precipitation (van de Vijver et al., 1989). Poly(A)$^+$ RNA was isolated from total RNA samples using the PolyA$^+$ Tract Kit (Promega) according to the manufacturer's protocol. Total RNA from cultured cells was isolated according to the method of Boehm (Boehm et al., 1988).

Northern analysis

Poly(A)$^+$ RNA (0.5 μg or 2 μg) or total RNA (12 μg) was fractionated in 1% agarose gels as previously described (Weber-Hall et al., 1994), transferred to ZetaProbe membrane (Biorad), and hybridised with specific cDNA probes. Random oligonucleotide DNA probes were generated as previously described (Ravdin, 1993) while [^{32}P]UTP-labelled RNA probes were synthesized as described by Melton et al. (1984). Control rat GAPDH (pRGAPDH-13) probes were as described previously (Weber-Hall et al., 1994), the 36B4 cDNA was donated by P. Darbre (Reading UK), the β-casein cDNA was donated by J. Rosen (Houston, TX) and the pS2 cDNA was described by Skilton et al. (1989; Md2 fragment). Murine *Bmp-2* and *Bmp-4* cDNA clones were kindly donated by Dr Mike Jones (Jones et al., 1991). *Msx-1*, *Msx-2* and human *MSX2* probes were provided by P. Sharpe (Hodgkinson et al., 1993; Jowett et al., 1993). DNA probes were hybridised as described previously (Weber-Hall et al., 1994), while for RNA probes, membranes were hybridised under more stringent conditions (60% formamide, 5× SSC, 5× Denhardts solution, 100 μg/ml ss salmon sperm DNA at 65°C) and were washed in 0.1× SSC, 0.1% SDS at 65°C.

Cell culture

MCF-7 cells (McGrath subline) were maintained in DMEM minus phenol red (Gibco BRL), 5% foetal calf serum, 6 ng/ml insulin, 10^{-8} M oestradiol, penicillin/streptomycin and passaged weekly. Prior to analysis, cells were plated for 24 hours in phenol red-free DMEM, 5% charcoal dextran stripped foetal calf serum (Darbre et al., 1983), 6 ng/ml insulin and antibiotics, media was subsequently replaced with or without the components described.

In situ hybridisation

For the postnatal in situ hybridisation, 7 μM frozen sections of fourth abdominal mammary glands were fixed for 5 minutes in 4% paraformaldehyde in PBS, then hybridised, washed and developed as described by Weber-Hall et al. (1994) with the following exceptions. The hybridisation buffer contained 70% formamide and yeast RNA at 10 μg/ml instead of random oligonucleotides. Hybridisation was with [^{33}P]ATP-labelled RNA probes that were prepared as described above and the posthybridisation washes included an RNase A digestion at 50 μg/ml, 45 minutes at 37°C. The sense control probe was generated from a 500 bp cDNA coding for the mouse *BRCA2* gene (A. Ashworth, ICR, London). Whole-mount in situ hybridisation was performed as described by Wilkinson (1992) with the following alterations: embryos were incubated only once in RNAse following hybridisation and were washed for only 30 minutes in TBST (140 mM NaCl, 2.7 mM KCl, 25 mM Tris-HCl, pH 7.5, 0.1% Tween) following incubation with antisera. ^{35}S radioactive in situ hybridisation (*Bmp-2* probe to embryonic fetal mammary glands) was performed as described by Davidson et al. (1988).

Generation and analysis of transgenic mice

The transgenic mice carrying the bacterial *lacZ* gene driven by the *Msx-1* promoter will be described in more detail (Hill, manuscript in

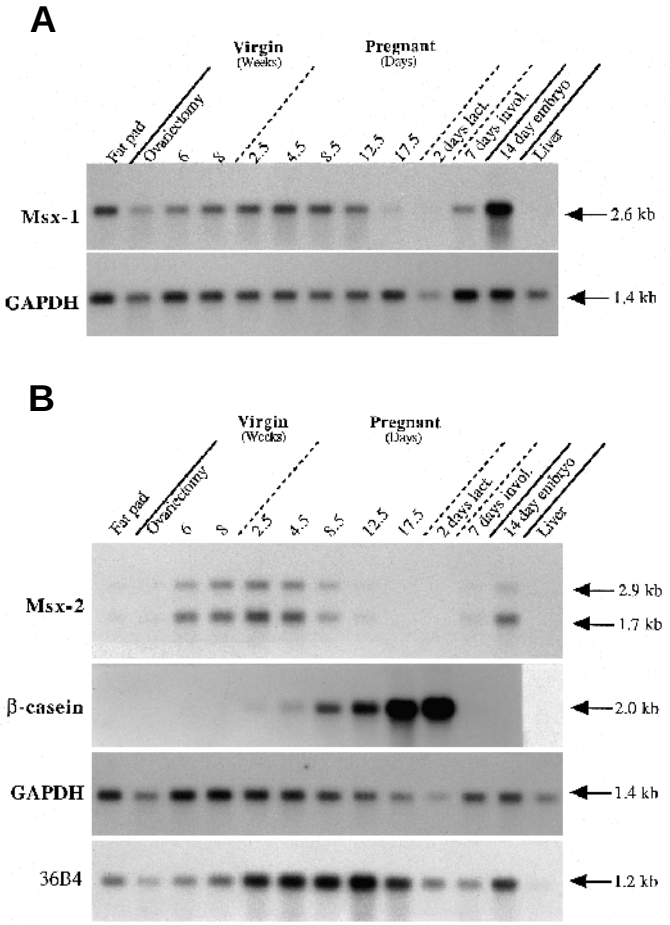

Fig. 1. Timecourse of *Msx-1* and *Msx-2* expression during postnatal mammary gland development. Poly(A)$^+$ RNA (0.5 μg) from mammary glands at the stages indicated were analysed by northern blot. lact, lactation; Fat Pad, epithelium-free mesenchyme isolated from 5-week virgin animals – ovariectomy was performed at 6 weeks of age and analysed at 8 weeks of age). (A) Northern blot probed sequentially with riboprobes for *Msx-1* followed by GAPDH. Exposure times were 5 hours and 30 minutes respectively. (B) Northern blot probed sequentially with riboprobes for *Msx-2*, GAPDH, 36B4 and β-casein. Exposure times were 5 hours, 30 minutes, 60 hours and 2 hours. 36B4 is a cDNA that has been reported not to be regulated by oestrogen (Darbre and King, 1988).

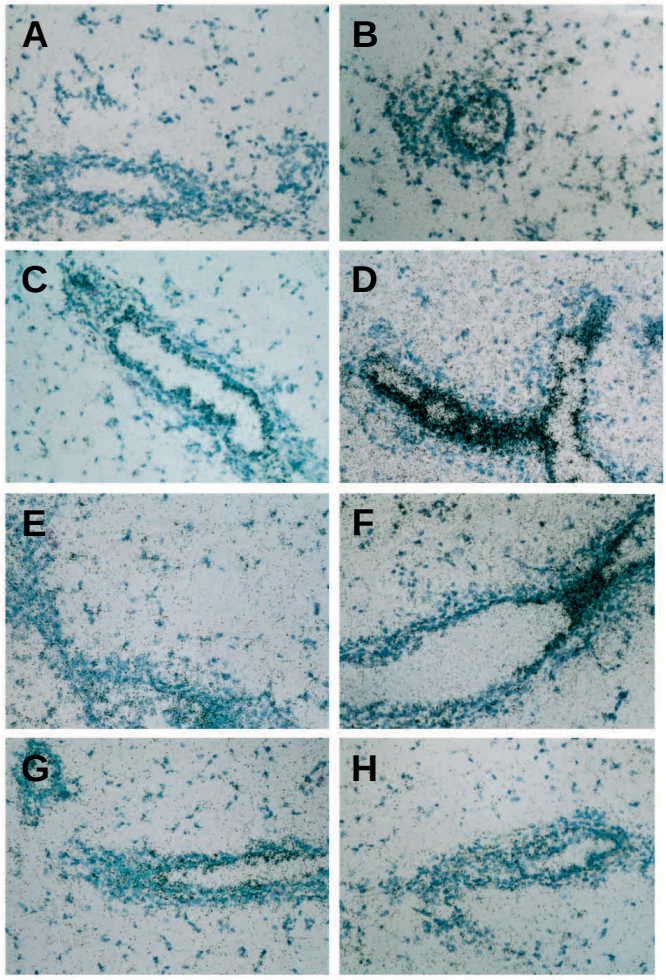

Fig. 2. *Msx-1*, *Msx-2*, *Bmp-2* and *Bmp-4* expression in postnatal mammary glands. In situ hybridisations using [^{33}P]ATP-labelled riboprobes to glands from 7-week virgin mice (A-C,E,G) or 4.5-day pregnant mice (D,F,H). (A) Control sense probe. (B) *Bmp-4* anti-sense probe. (C,D) *Bmp-2* anti-sense probe. (E,F) *Msx-1* anti-sense probe. (G,H) *Msx-2* anti-sense probe. Localisation was determined by silver grain deposition following autoradiography for 8 weeks. Magnification ×400.

preparation). Briefly, the *lacZ* gene from pCH110(rf) was subcloned to produce a fusion with a 4.9 kb *Nco*I genomic restriction fragment of the *Msx-1* 5′ upstream region. This DNA fragment extends into the coding region of *Msx-1* carrying the predicted start codon and the open reading frame encoding the first 41 amino acids (Monaghan et al., 1991). This is in frame with the coding region for β-galactosidase which will produce a fusion protein. This construct was used to make mice transgenic (Hogan et al., 1986) and nine independent mouse lines were established.

Male mice carrying the transgene were mated with Swiss wild-type females and the embryos were harvested at day 13.5 of development. Embryonic β-galactosidase expression was detected by the method of Beddington et al. (1989). The expression of β-galactosidase in the developing mammary glands was detected in the majority of the transgenic lines.

The *Msx-2-lacZ* mice carrying the −5.2-+3/*lacZ* transgene were generated as described previously (Liu et al., 1994). The embryos were stained as described previously (Sanes et al., 1986), dehydrated and paraffin embedded for sectioning.

The generation of the *Msx-1* null mice have been previously described (Satokata and Maas, 1994). Sections of glands were taken from 6 newborn *Msx-1* mutants and 6 wild type at day P0.

RESULTS

Msx-1 and *Msx-2* are developmentally regulated in the postnatal mammary gland

In this study we have correlated *Msx* mRNA levels with temporal changes in mammary gland morphology (Fig. 1A,B). *Msx-1* and *Msx-2* are both expressed in the glands of virgin females during ductal development and in the glands of early pregnant females during the proliferative phase of lobular development. Expression of both genes is down-regulated

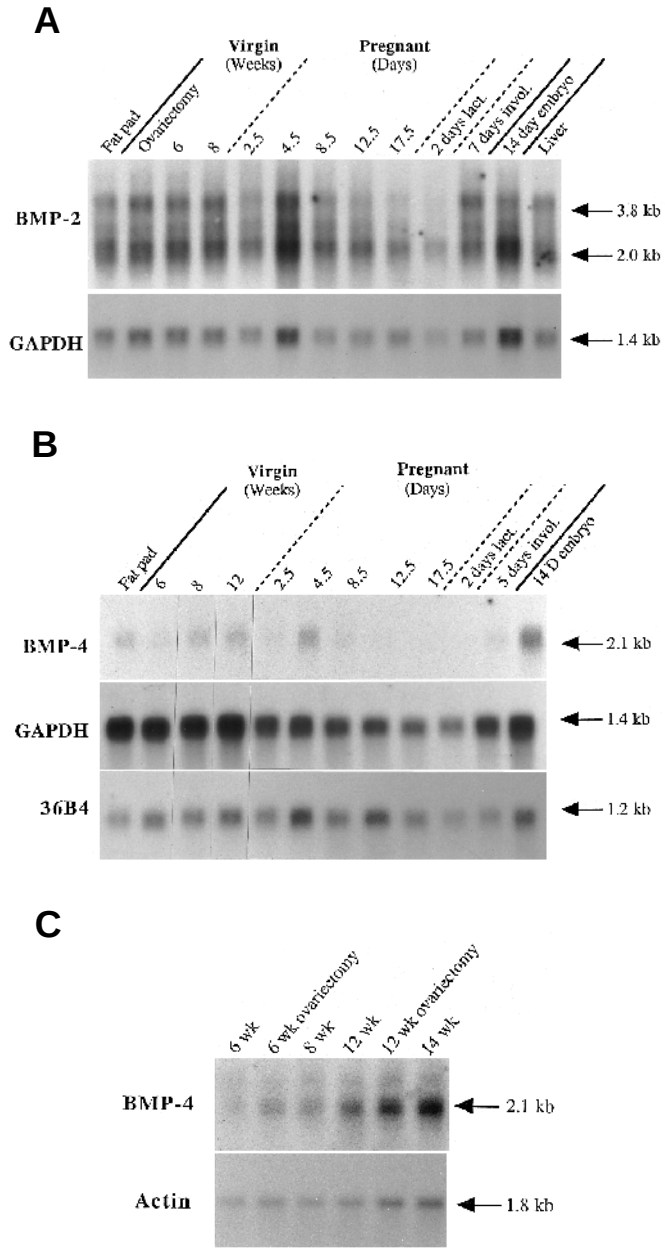

Fig. 3. Timecourse and regulation of *Bmp-2* and *Bmp-4* expression in the postnatal mammary gland. Poly(A)$^+$ RNA (0.5 μg) from mammary glands at the stages indicated were analysed by northern blotting. lact, lactation; Fat Pad, epithelium free mesenchyme isolated from 5-week virgin animals. (A) Northern blot probed sequentially with riboprobes for *Bmp-2* and GAPDH. Exposure times were 4 days and 60 minutes respectively. (B) Northern blot probed sequentially with riboprobes fror *Bmp-4*, GAPDH and 36B4. (C) Poly(A)$^+$ RNA (2 μg per track) was analysed by northern blot (mice were ovariectomised at either 6 or 12 weeks of age and the mammary glands removed for analysis 2 weeks later). The blot was probed sequentially with *Bmp-4* and actin. Exposure times 4 days and 30 minutes respectively.

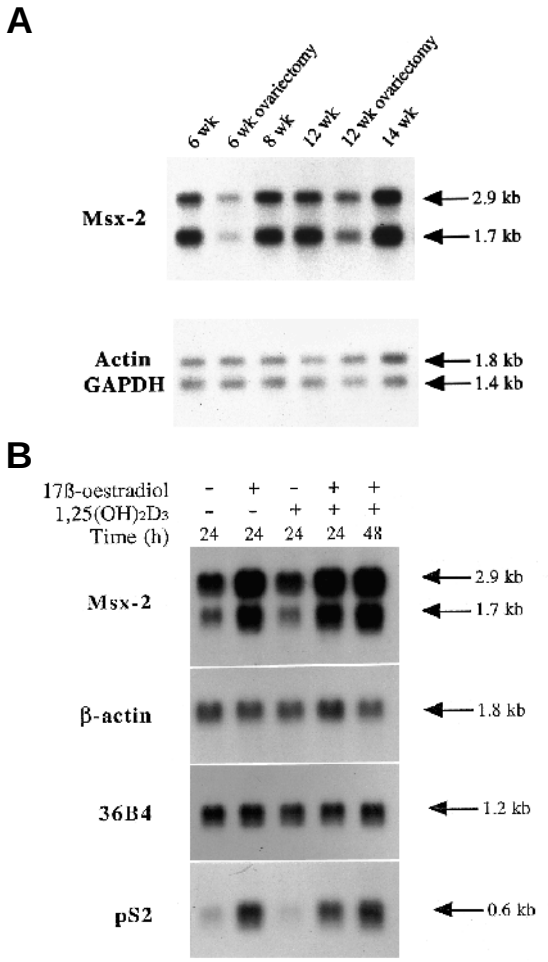

Fig. 4. Hormonal regulation of *MSX2*. (A) *Msx-2* expression is down-regulated as a consequence of ovariectomy in mammary glands from young and mature animals. Poly(A)$^+$ RNA (0.5 μg) from mammary glands of virgin mice, at the stages indicated, were analysed by northern blotting with [^{32}P]UTP-labelled riboprobes (*Msx-2*) or [^{32}P]dCTP-labelled DNA probes (actin and GAPDH). Exposure times were 21 hours and 90 minutes respectively. (B) MSX-2 regulation by oestradiol in the breast cancer cell line MCF-7. Northern analysis of *MSX2* expression in MCF-7 cells +/– 10^{-8} M 17β-oestradiol (E) and +/– 10^{-5} M 1,25(OH)$_2$D$_3$.(D3). The MCF-7 cells were seeded in control medium at 1×10^4 cells/cm^2, 24 hours later the cells were fed with fresh media ± hormones for a further 24 or 48 hours prior to harvesting. Total cellular RNA (12 μg per track) was sequentially hybridised with riboprobes for *MSX2*, pS2, 36B4 and β-actin. cDNA mapping showed that the 2.9 kb *MSX2* transcript had a longer 3′ untranslated region, although it is not known how this influences the oestrogen dependence of the transcript (Phippard, 1995).

(*Msx-2* prior to *Msx-1*) during late pregnancy and lactation, corresponding with a reduction of the proliferative index and the expression of terminal differentiation markers such as β-casein (Fig. 1B, (Zwierzchowski et al., 1984)). During the period of lobulo-alveolar apoptosis that characterises involution, both genes were partially reinduced.

Msx-1 but not *Msx-2* was expressed in isolated stroma (fat pads) taken from epithelial-free regions of glands prior to the ductal outgrowth (5 weeks old). These data show that expression of *Msx-1* occurs in the stroma at this stage, while expression of *Msx-2* is either present within the epithelium or requires the presence of the epithelium. To resolve these alternatives, expression of *Msx-1* and *Msx-2* was localised by in

situ hybridisation. In glands from 7 week virgin and 4.5 day pregnant mice, *Msx-1* and *Msx-2* expression was found in ductal epithelial cells (Fig. 2E-H). Northern analysis confirmed that *Msx-1* was expressed in purified mammary epithelial cells (data not shown). Taken together, these data strongly argue that *Msx-2* is expressed in the epithelium while *Msx-1* is expressed in the epithelium and stroma. However, given the limitations of the resolving power of radioactive in situ hybridisation, it is not possible to exclude additional *Msx-2* expression in stromal fibroblasts that are immediately adjacent to the epithelium.

Developmental regulation of *Bmp-2* and *Bmp-4* in the postnatal mammary gland

As *Bmp-2* and *Bmp-4* have been shown to be coordinately regulated with Msx genes during tooth development, we examined their expression patterns during postnatal mammary gland development. Both *Bmp-2* and *Bmp-4*, like *Msx-1* and *Msx-2* were expressed in the postnatal mammary gland at the earliest stage examined (Fig. 3A,B). While *Bmp-4* was down-regulated during late pregnancy and was not reinduced during involution, *Bmp-2* transcripts were not significantly regulated during development by comparison with the GAPDH. Levels of the GAPDH control mRNA reproducibly fell during lactation, possibly as a result of a dilution by milk protein mRNAs (Fig. 1B; Weber-Hall 1994; Gavin and McMahon, 1992; Bühler et al., 1993). Both *Bmp-2* and *Bmp-4* were expressed in the stromal fat pad in the absence of epithelium. Comparisons of *Bmp* and *Msx* expression profiles showed that the *Bmp-2* timecourse was most similar to *Msx-1* while the *Bmp-4* timecourse was most similar to *Msx-2* (Figs 1A,B, 3A,B). In situ hybridisation analyses showed that both *Bmp-2* and *Bmp-4* were expressed in the epithelial component (Fig. 2B-D). Taken together with the northern analyses, these data strongly argue that *Bmp-2* and *Bmp-4* are expressed in both the epithelial and stromal compartments.

Msx-2 is regulated by oestrogen

As the development of the mammary gland is under the tight control of ovarian hormones, we investigated the regulation of *Msx* and *Bmp* transcripts following ovariectomy. Ovariectomy during ductal development causes end buds to regress to blunt-ended ducts, although the ductal system itself is not lost (Nandi, 1958; Daniel and Silberstein, 1987). The effect of ovariectomy (at 6 weeks of age) on *Bmp* and *Msx* gene expression during ductal development was examined by northern analysis (Figs 1A,B, 3A,C). Both 6- and 8-week old unoperated mice were used as controls. Previous studies have shown that ovariectomy does not cause gross changes in content of mammary epithelium in this strain of mice (Weber-Hall, 1995). Following ovariectomy, levels of *Msx-2* expression were greatly reduced, while levels of *Msx-1*, *Bmp-2* and *Bmp-4* transcripts were unaltered, suggesting that *Msx-2* may be regulated by ovarian hormones. The regulation of *Msx-2* by ovarian hormones was confirmed by Northern analysis of mRNA derived from both 6- and 12-week old animals (Fig. 4A).

To determine the basis for the ovarian dependence of *Msx-2* expression, we used the oestrogen-responsive, breast cancer cell line MCF-7, to study the regulation of the human *Msx-2* homologue, *MSX-2*. As oestrogen has been shown to be the only ovarian steroid that is required for ductal epithelial development, we focused on the effects of 17β-oestradiol. To show specificity, we also studied the effects of the steroid vitamin D3 which has been shown to regulate *MSX-2* levels in osteoblasts (Hodgkinson et al., 1993). 24 hours after 17β-estradiol addition, quantitation with a phosphoimager showed that levels of *Msx-2* transcripts were induced 3-fold (1.7 kb transcript) and 2-fold (2.9 kb transcript) relative to controls (Fig. 4B; 36B4 and β-actin). The levels of *MSX-2* transcript induction by 17β-estradiol were comparable to the observed 4-fold induction of pS2, a gene previously demonstrated to be 17β-estradiol-dependent (Chalbos et al., 1993). The increased levels of *MSX-2* in the MCF-7 cells were not a secondary consequence of oestrogen-dependent changes in cell proliferation as no significant increases in cell numbers were observed within the time period of hormone treatment (24 hours). No changes in transcript levels were observed in the presence of vitamin D3 ($1,25(OH)_2D3$), suggesting that the induction of *MSX-2* expression by this steroid may be specific to osteoblasts. In addition, no changes in transcript levels were seen in the presence of progesterone or prolactin (unpublished data). Expression of *Bmp-2* and *Bmp-4* were not oestrogen-dependent in the MCF-7 cells, while expression of *MSX-1* was not detected (Phippard, 1995).

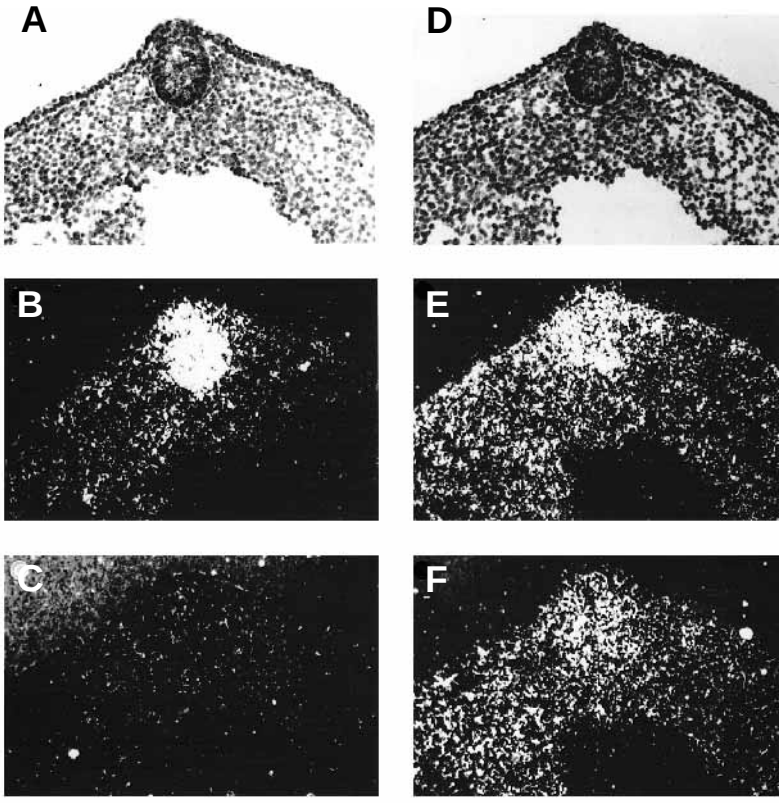

Fig. 5. In situ hybridisation of *Msx-1* and *Msx-2* to E13.5 embryonic mammary glands. Sequential sections of E13.5 foetal mammary gland. A and D show bright-field images; B, C, E and F are dark-field images following hybridisation to riboprobes for *Msx-1* antisense, *Msx-1* sense, *Msx-2* antisense and *Msx-2* sense probes respectively. Exposure times were 8 weeks. Magnification 100×.

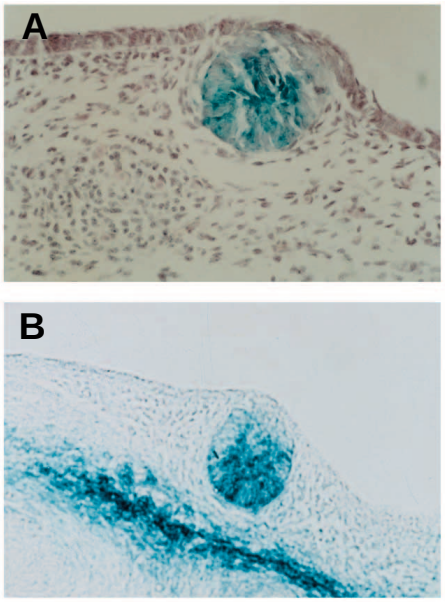

Fig. 6. *Msx-lacZ* fusion gene expression in the E13.5 mammary gland. Transverse sections of E13.5 embryos at the level of the number 4 foetal mammary glands from (A) *Msx-1-lacZ* transgenic female. *lacZ* is expressed only in the epithelium. Magnification 250×. (B) *Msx-2-lacZ* transgenic female. *lacZ* expression is detected in the epithelium and the underlying loose mesenchyme, but not in the condensed mesenchyme directly around the bud. Magnification 200×.

Msx-1 and *Msx-2* are expressed in the foetal mammary epithelium

As both *Msx-1* and *Msx-2* were expressed at the earliest stages of postnatal development examined, we examined whether they were also expressed in the foetal gland (Figs 5, 6). These studies focused on the E13.5 foetal gland because the mammary bud is relatively easy to detect at this stage and is analogous in structure to the bud stage of tooth development in which both *Msx* and *Bmp* expression have been detected (Thesleff et al., 1995). The epithelium of the mammary bud at E13.5 expressed *Msx-1* and *Msx-2* as shown by in situ hybridisation (Fig. 5A-F). *Msx-1* was not expressed in the fetal mesenchyme, but we were not able to exclude expression of *Msx-2* in the mesenchyme as relatively high levels of background were observed with the *Msx-2* sense RNA control.

To confirm the location of *Msx-1* and *Msx-2* expression, the control regions of the *Msx-1* (4.9 kb of 5′ flanking sequence) and *Msx-2* genes (5.2 kb of 5′ flanking sequence, the intron, both exons and 3 kb of 3′ flanking sequence) were fused to the *lacZ* marker gene and stably introduced into lines of transgenic mice (R. E. Hill, unpublished data; Liu et al., 1994). The lines of transgenic mice characterised have previously been shown to express β-galactosidase in areas of the embryo that correspond to those expressing endogenous *Msx* transcripts. At E13.5, β-galactosidase expression driven by both the *Msx-1* and *Msx-2* promoters was detected in the foetal epithelium (Fig. 6A,B), confirming the in situ hybridisation results and showing that the *Msx-1* and *Msx-2* promoters were sufficient to direct expression to the appropriate sites. No *lacZ* expression was detected in the mesenchyme immediately adjacent to the epithelial bud, but some expression driven by the *Msx-2* promoter was detected in underlying tissues that may be related to the appearance of the fat pad (Fig. 6B; Sakakura, 1991).

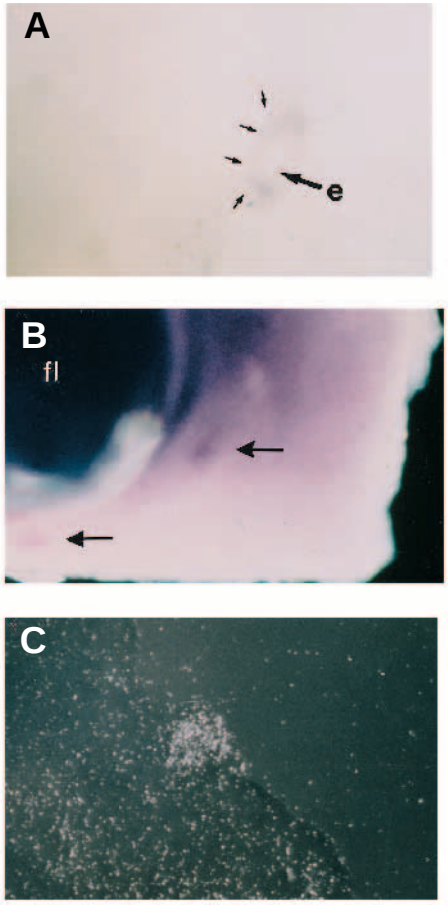

Fig. 7. Localisation of *Bmp-4* and *Bmp-2* expression in the E13.5 mammary gland. In situ hybridisation of E13.5 foetal mammary gland. (A) Whole mount probed with *Bmp-4* (magnification 18×); mesenchymal staining seen as an elliptical ring (arrows) around the clear epithelial bud (e). (B) Whole mount probed with *Bmp-2* (magnification 18×); the epithelial bud is indicated by arrows. fl, fore limb. (C) Thin section of E13.5 gland probed with ^{35}S-labelled *Bmp-4* probe (magnification 24×).

Msx-1 is not required for early mammary development

Although *Msx-1* expression was detected throughout mammary development (Figs 1A, 5B) it was not clear whether it was necessary for development. To investigate potential function(s) for *Msx-1* in mammary development, we examined mammary glands from six control and mutant *Msx-1*/*Msx-1* null mice. The mice were studied at day 0 (new born) since the mice die shortly after birth from respiratory problems (Satokata and Maas, 1994). Glands from all the homozygous null mice had a rudimentary ductal network and were found to be indistinguishable from normal or heterozygous littermates, showing that *Msx-1* function is not essential for normal mammary gland development up to this stage (data not shown).

Bmp-2 and *Bmp-4* are expressed in the foetal mammary gland

As both *Bmp-2* and *Bmp-4* were expressed at the earliest stages

of postnatal development examined (Fig. 3A,B), we examined whether they were also expressed in the foetal gland. In situ analyses localised *Bmp-2* expression to the epithelial bud of the E13.5 gland (Fig. 7B,C). By contrast, *Bmp-4* was expressed in the mesenchyme surrounding the epithelium, appearing as a ring structure as determined by whole-mount in situ hybridisation (Fig. 7A). Subsequent sectioning of the whole mounts confirmed that *Bmp-4* expression was localised in the mesenchyme underlying the mammary knob (data not shown).

DISCUSSION

Our findings show that mRNAs from the *Msx-1* and *Msx-2* homeodomain-containing genes and the *Bmp-2* and *Bmp-4* signalling ligands are expressed during both foetal and postnatal mammary gland development. As coordinate expression of these genes has been implicated in the regulation of organogenesis in other systems, this suggests that they may play similar roles in mammary gland development (Davidson, 1995; Thesleff et al., 1995b).

In the foetal mammary gland at E.13.5, *Msx-1*, *Msx-2* and *Bmp-2* were expressed in the foetal epithelium (Figs 5, 6), whilst *Bmp-4* was expressed in the mesenchyme. At E13.5, the mammary bud has a similar morphology to bud stages in other organs in which epithelia invaginate into underlying mesenchyme (Thesleff et al., 1995b). One of the best studied systems in which the Msx and BMP gene products have been implicated as regulators is tooth development. At the structurally analogous bud stage of tooth development (E12-13), *Bmp-4* and *Msx-1* are expressed in the mesenchyme, *Bmp-2* is expressed in the epithelium and *Msx-2* is expressed predominantly in the epithelium (Vainio et al., 1993; Thesleff et al., 1995b). This analysis suggests that there are significant differences between tooth and mammary gland development, however, a simple 'snapshot' of tooth development at the bud stage conceals a complex series of temporal and spatial expression patterns in which 'compartment switching' of *Msx* and *Bmp* expression precedes and follows the bud stage (MacKenzie et al., 1991a,b, 1992; Vainio et al., 1993). As *Msx-1* expression domains have been shown to undergo stage-specific expansions and restrictions in the developing limb bud (reviewed by Reginelli et al., 1995; Wang and Sassoon, 1995), it is reasonable to expect that detailed temporal studies of mammary development could reveal complex patterns of Msx expression while the use of soluble BMP factors may help elucidate regulatory BMP/Msx interactions. Interestingly, analysis of foetal glands from *Msx-2-lacZ* transgenic mice suggest that *Msx-2* is expressed in both the mesenchyme and epithelium at a day earlier than the data shown here (E12, unpublished data), suggesting that Msx expression patterns are dynamic. Following more extensive analyses, the differences between foetal mammary and tooth expression of *Msx* and *Bmp* genes may resolve into patterns that are consistent with conserved function or may be accounted for by tissue-specific processes.

While the importance of *Msx-1* expression for normal tooth development was recently demonstrated by the analysis of transgenic mice that lack a functional *Msx-1* gene (Satokata and Maas, 1994), no abnormal phenotype was detected in mammary glands from the same strain of mice in these studies (data not shown). The lack of a mutant/abnormal phenotype could be a result of functional redundancy in which the function of *Msx-1* is compensated for by a related gene or because *Msx-1* is non-functional in the foetal mammary gland. Given that both *Msx-1* and *Msx-2* are expressed in the E13.5 mammary epithelium, *Msx-2* could functionally compensate for loss of *Msx-1* expression at this stage. Support for this hypothesis has come from recent observations in *Msx-2* mutant and *Msx-1*/*Msx-2* double mutant mice in which defects in mammary development have been observed at E13 (R. Maas, unpublished data). In contrast to the mammary gland, during the bud stage of tooth development, *Msx-1* and *Msx-2* are predominantly expressed in different compartments (Vainio et al., 1993; Thesleff et al., 1995), perhaps explaining why a tooth phenotype was observed in the *Msx-1* mutant mice (Satokata and Maas, 1994). Although *Msx-1* may be redundant to *Msx-2* during foetal development, a unique function for *Msx-1* cannot be excluded during postnatal mammary development as *Msx-1* is expressed in the absence of *Msx-2* during late pregnancy (compare Figs 1A and 2A). Analysis of postnatal functions are not possible since *Msx-1*/*Msx-1* null mice die at birth. In a recent paper, *Bmp-4* mutant mice have also been described (Winnier et al., 1995), however neither the tooth nor the mammary phenotype of these animals could be judged because the animals die from multiple developmental defects between E6.5 and E9.5.

In the postnatal gland, *Msx-1*, *Bmp-2* and *Bmp-4* are expressed in the stromal fat pad while *Msx-2* is expressed in the epithelium (Fig. 1A,B 'fat pad', Fig. 2). The transition from foetal to postnatal development must involve the expansion of *Msx-1* and *Bmp-2* expression into the mesenchymally derived fat pad from the epithelium (Figs 5B, 7B,C). Thus, the postnatal functions of Msx and BMP molecules may be significantly different from those in foetal development. Studies in other systems have implicated *Msx-1* and *Msx-2* in the control of a diverse range of regulatory processes including the spatial definition of developing fields of cells (MacKenzie et al., 1991a,b, 1992; Jowett et al., 1993), cellular adhesiveness, epithelial morphology, cellular proliferation, apoptosis and an anti-differentiative/regenerative role (Reginelli et al., 1995; Thesleff et al., 1995). In addition to analogous functions for Msx and BMP proteins during foetal mammary and tooth development, some of these proposed roles may have particular relevance during postnatal mammary development since the gland retains a high proliferative and differentiative potential throughout adult life. Studies of limb regeneration and F3 myoblast differentiation have suggested that *Msx-1* (and possibly *Msx-2*) prevent terminal differentiation and maintain proliferation of undifferentiated cells (Song et al., 1992; Noveen et al., 1995). The continued expression of *Msx-1* and *Msx-2* in the mammary gland after birth (Fig. 1) may be related to the necessity to maintain a state of developmental competence in which the mammary epithelium can respond rapidly to changes in systemic hormones. During early pregnancy, the slight increase in expression of *Msx-1* and *Msx-2* may be related to an increase in cellular proliferation at that time and may prevent the lactational differentiation of lobular cells until the onset of lactation (Fig. 1A,B; Zwierzchowski et al., 1984). Alternatively, constant Msx expression in ductal but not lobular cells may prevent inappropriate lactational differentiation of the ducts. To distinguish between these possibilities,

a detailed analysis of the distribution of *Msx* expression throughout postnatal development will be required.

Msx-1, *Msx-2* and *Bmp-4* expression have been correlated with apoptosis of avian cranial neural crest cells and exogenous BMP-4 has been shown to induce both *Msx-2* expression and apoptosis in isolated hindbrain rhombomeres (Graham et al., 1994). As *Msx-1* expression precedes apoptosis at other locations including the limb bud, it is reasonable to suggest that the reinduction of *Msx-1* expression in the involuting gland may be involved in the control of the major apoptotic phase of mammary development. In this context, it will be particularly interesting to determine if *Msx-1* is expressed in the alveolar cells prior to apoptosis or in the ductal cells which are selectively retained.

In principle, members of the TGFβ, FGF, EGF, HGF/Scatter and Wnt families of secreted ligands could function in inductive interactions in the mammary gland as members from each family have been shown to be developmentally regulated during mammary gland morphogenesis (Snedeker et al., 1991; Gavin and McMahon, 1992; Robinson et al., 1992; Buhler et al., 1993; Coleman-Krnacik and Rosen, 1994; Niranjan et al., 1995). An important additional question is how the control of epithelial-mesenchymal interactions is integrated with the regulation of mammary development by hormones such as oestrogen, progesterone and prolactin. The observation that *Msx-2* is regulated by ovarian hormones in vivo (Fig. 1B) and that *MSX-2* expression is directly regulated by 17β-estradiol in vitro, raises the possibility that hormonal regulation may be mediated through existing epithelial-mesenchymal regulatory systems. During mammary gland evolution, key developmental control genes may have aquired hormone responsive control regions, thus imposing hormonal control on the entire network of epithelial-mesenchymal interactions. This may be a mechanism common to other genes in addition to *Msx-2* since expression of putative mammary control genes such as *Wnt-4* and *Hoxc6* were also affected by ovariectomy (Weber-Hall et al., 1994; Bradbury et al., 1995; Friedman et al., 1995). The mechanism by which *Msx-2* is regulated is not clear at present. In principle, oestrogen induction could be mediated by alterations in transcript stability or induction of transcription. Surveys of 800 bp of the murine *Msx-2* promoter, failed to detect canonical oestrogen-responsive elements (unpublished data), although this does not rule out further upstream elements or more indirect mechanisms of oestrogen regulation. An additional point of interest concerns the time at which the mammary epithelium becomes responsive to oestrogen and the time at which *Msx-2* expression becomes estrogen responsive. Studies in mutant mice that lack the oestrogen-receptor suggest that mammary development can proceed normally until postnatal stages of development when end bud development fails to take place (Lubahn et al., 1993; Korach et al., 1994). Future studies may be able to determine if *Msx-2* expression also becomes oestrogen responsive only at the onset of postnatal development.

The authors would like to thank Dr Cheryl Tickle for work on the analysis of *Bmp* expression and for critical reading of the manuscript. We would like to thank Dr Tahereh Kamalati and Dr Mike O'Hare for help in the culture of MCF-7 cells. We would particularly like to thank Duncan Davidson, Beatrice Howard and David Cook for useful comments and suggestions on the manuscript. Part of this study was funded by an MRC project grant (P. T. S.) and by the Cancer Research Campaign (T. C. D., M. S. N., H. J., S. W.-H. and D. P.).

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(Accepted 29 May 1995)