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ARTICLES

Expression of *Hoxa-1* and *Hoxb-7* Is Regulated by Extracellular Matrix-Dependent Signals in Mammary Epithelial Cells

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Abstract Homeobox-containing genes encode transcriptional regulators involved in cell fate and pattern formation during embryogenesis. Recently, it has become clear that their expression in continuously developing adult tissues, as well as in tumorigenesis, may be of equal importance. In the mouse mammary gland, expression patterns of several homeobox genes suggest a role in epithelial–stromal interactions. Because the stroma and the extracellular matrix (ECM) are known to influence both functional and morphological development of the mammary gland, we asked whether these genes would be expressed postnatally in the gland and also in cell lines in culture and whether they could be modulated by ECM. Using a polymerase chain reaction-base strategy five members of the *Hox* gene clusters *a* and *b* were shown to be expressed in cultured mouse mammary cells. *Hoxa-1* and *Hoxb-7* were chosen for further analysis. *Hoxb-7* was chosen because it had not been described previously in the mammary gland and was modulated at different stages of gland development. *Hoxa-1* was chosen because it was reported previously to be expressed only in mammary tumors, and not in normal glands. We showed that culturing the mammary epithelial cell lines SCp2 and CID-9 on a basement membrane (BM) that was previously shown to induce a lactational phenotype was necessary to turn off *Hoxb-7,* but a change in cell shape, brought about by culturing the cells on an inert substratum such as polyHEMA, was sufficient to downregulate *Hoxa-1.* This is the first report of modulation of homeobox genes by ECM. The results provide a rationale for the differential pattern of expression in vivo of *Hoxa-1* and *Hoxb-7* during different stages of development. The culture model should permit further in-depth analysis of the molecular mechanisms involved in how ECM signaling and homeobox genes may interact to bring about tissue organization. J. Cell. Biochem. 69:377–391, 1998.

Key words: homeobox; mammary gland; morphogenesis; basement membrane; gene expression

Extracellular matrix (ECM) profoundly influences cellular form and function during embryogenesis, as well as in the adult organism [Adams and Watt, 1993; Martins-Green and Bissell, 1995; Ashkenas et al., 1996]. This is particularly well documented for the mouse mammary gland, in which epithelial cells and the surrounding ECM interact dynamically throughout development [Bissell and Hall, 1987]. Unlike other organs, the mammary gland undergoes most of its growth and morphogenesis in the subadult and adult animal, and certain stages of the postnatal gland exhibit embryonic-like features [Sakakura et al., 1979; Cunha et al., 1992]. Regulation of pattern formation, cell differentiation, and epithelial–stromal interactions takes place after completion of embryogenesis, and recurs during each cycle of pregnancy, lactation, and involution. During branching morphogenesis in virgin animals, the ECM at the edge of the stroma influences end-bud development and ductal branching [Silberstein et al., 1992]. An intact basement membrane (BM) is required for epithelial morphology and functional differentiation during pregnancy and lac-

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tation [Aggeler et al., 1991; Sympson et al., 1994]. Finally, after weaning, involution is completed only if the BM is degraded by matrix metalloproteinases (MMPs) [Talhouk et al., 1992], an event that triggers programmed cell death, allowing the gland to return to its resting state [Strange et al., 1992; Boudreau et al., 1995].

A continuing, consistent body of work has demonstrated that the ECM plays a key role in mammary epithelial cell differentiation in culture as well [for review, see Roskelley et al., 1995]. Primary mouse mammary cultures, as well as various cell lines established from the glands of pregnant animals [Danielson et al., 1984; Schmidhauser et al., 1990; Desprez et al., 1993], when placed on a reconstituted BM, undergo a morphogenic process that leads to the formation of polarized three-dimensional structures similar to the alveoli observed in vivo [Aggeler et al., 1991]. These spheroids are able to synthesize and vectorially secrete large amounts of milk proteins in the presence of lactogenic hormones [Li et al., 1987; Barcellos-Hoff et al., 1989].

From *Drosophila* to mammals, homeobox-containing genes have been identified as master regulators of cell fate and pattern formation during development [McGinnis and Krumlauf, 1992; Kenyon, 1994; Krumlauf, 1994; Lawrence and Morata, 1994]. These genes are characterized by a highly conserved 183-bp sequence encoding a 61-amino acid domain (homeodomain), which includes a helix-turn-helix DNA-binding motif [Gehring et al., 1994]. Despite extensive literature on their role in embryogenesis, their expression in the adult has been reported only recently in a few tissues, including kidney, intestine, testis, and the mammary gland [Wolgemuth et al., 1987; James and Kazenwadel, 1991; Wolf et al., 1991; Friedmann et al., 1994]. Furthermore, their regulation and function in these tissues is poorly understood.

In the mouse mammary gland, the expression of *Hoxc-6* is differentially regulated during postnatal development and is influenced by mammogenic hormones. *Hoxc-6* transcripts are present in normal glands and absent in mammary neoplasia. By contrast, *Hoxa-1* is detected only in mammary tumors of epithelial origin, but not in the normal gland or in precancerous outgrowths [Friedmann et al., 1994].

We hypothesized that a regulatory crosstalk may exist between some homeobox genes and the ECM and that this interaction may play a role in different stages of mammary gland development. To test this hypothesis, we used degenerate primers and reverse transcription-polymerase chain reaction (RT-PCR) and identified several *Hox* genes that are expressed in two cell lines isolated from a functional mouse mammary cell strain that are ECM responsive [Schmidhauser et al., 1990; Desprez et al., 1993]. Here, we focus our studies on the expression and regulation of *Hoxa-1* and *Hoxb-7* for the reasons described in the summary. The former was previously identified only in mammary adenocarcinomas [Friedmann et al., 1994] and the latter has not been described previously in the mammary gland. This report shows that functional nontumorigenic mammary cells cultured on tissue culture plastic express these genes, but a reconstituted BM suppresses the expression of both genes. The mechanism of suppression, however, differs between *Hoxa-1* and *Hoxb-7* such that their mode of expression in vivo in normal gland and in tumors may be explained by ECM-dependent signals.

MATERIALS AND METHODS

Cell Culture

CID-9 cells [Schmidhauser et al., 1990] were derived from the mouse mammary cell strain COMMA-1-D [Danielson et al., 1984] by an enrichment of the epithelial subpopulation. As a result, the percentage of cells able to produce β-casein in response to ECM and lactogenic hormones was increased from 8 to more than 43. Homogeneous epithelial cells, SCp2, were isolated from the heterogeneous CID-9 cell line by limiting dilution cloning. SCp2 do not deposit their own BM, but more than 90% of these cells are able to produce β-casein in response to an exogenous BM-like ECM or purified laminin and lactogenic hormones [Desprez et al., 1993].

Each cell line was routinely grown in a mixture of Dulbecco's modified Eagle's medium (DMEM) and Ham's F12 medium (F12) (1:1) (GIBCO-BRL, Grand Island, NY) containing 5% heat-inactivated fetal bovine serum (FBS) (Sigma, St. Louis, MO); insulin, 5 μg/ml (Sigma); and gentamicin, 10 μg/ml (UC, San Francisco). At the onset of the experiments, 5×10^4 cells/cm^2 were plated on different substrata in DMEM–F12 medium supplemented with insulin, 5 μg/ml (Sigma), and the lactogenic hormones hydrocortisone, 1 μg/ml (Sigma) and prolactin, 3 μg/ml (National Institute of Diabetes

and Digestive and Kidney Disease, Bethesda, MD), as well as 0.25–2% FBS. After 24 h, the medium was replaced with serum-free DMEM–F12 plus insulin and lactogenic hormones, and the cells were maintained under these conditions for the times indicated for each experiment.

For studies on the influence of cell proliferation on *Hoxa-1* expression, the medium was changed to serum-free DMEM–F12 plus hydrocortisone and prolactin (as indicated above), but with increasing amounts of insulin, 0, 0.25, 1.25, or 5 µg/ml, 24 h after plating. Cells were maintained for another 24 h. This included a 12-h labeling period with 5-bromo-2′-deoxyuridine (BrdU), as described below.

Cell Proliferation Assay

DNA synthesis was measured by BrdU incorporation using a BrdU Labeling and Detection Kit (Boehringer Mannheim, Germany). Cells were incubated with 10 µM BrdU for 12 h, and labeled nuclei were detected according to the manufacturer's instructions with the addition of a blocking step before the incubation with anti-BrdU antibody. Blocking was performed by incubating with 0.1% bovine serum albumin (BSA), 10% goat serum in 1× phosphate-buffered saline (PBS) for 1–2 h. Nuclear labeling indices were determined by counting at least 300 cells from randomly selected visual fields and calculating the percentage of cells with labeled nuclei.

Substrata

When indicated, 100-mm tissue culture plates were precoated with 1–2 ml of reconstituted BM-like ECM purified from the mouse Engelbreth-Holm-Swarm tumor (EHS matrix). EHS or "factor-free" EHS was prepared as described by Kleinman et al. [1986] or was obtained from Collaborative Biomedical Products (Matrigel) (Collaborative Research, Waltham, MA).

For studies on the influence of cell shape and of cytoskeleton, tissue culture plates were coated with the nonadhesive substratum poly(2-hydroxyethylmethacrylate) (polyHEMA) (Sigma) as described by Folkman and Moscona [1978] and Roskelley et al. [1994], using an initial concentration of 10 mg/ml in 95% ethanol before drying.

Animals

For RNA preparation, CD-1 mice were used as a source of normal mammary glands. Hyperplastic glands and tumors were collected from transgenic animals made in a CD-1 background. For involuting glands, pups were weaned at 9 days after birth; the next day was counted as day 1 of involution. C57Bl/crl mice were also used in parallel with CD-1 mice and yield similar results as the CD-1 mice. Thoracic glands of virgin and pregnant C57BL/crl mice were used for in situ hybridization.

RNA Isolation, cDNA Synthesis, and PCR Amplification

Mouse mammary glands were frozen in liquid nitrogen immediately after removal. Total RNA was prepared using TRIzol reagent (GIBCO-BRL) according to the manufacturer's instructions. Total RNA from cultured cells was prepared as described by Chomczynski and Sacchi [1987].

Poly A^+ mRNA was purified from 1 mg total RNA from cultured cells by chromatography on oligo(dT)-cellulose column as described by Maniatis et al. [1989]. cDNA was synthesized from 5 µg poly A^+ RNA using cDNA Synthesis System Plus (Amersham, Cleveland, OH), following the manufacturer's instructions. One-tenth of the cDNA obtained was heated for 10 min at 95°C and then combined with a set of degenerate oligonucleotide primers at a concentration of 1 µM each, 10 µl of 10× PCR buffer II (Perkin Elmer), 2 mM $MgCl_2$, 200 µM each dNTP, and 2.5 units of Taq DNA polymerase (Perkin Elmer) in a total volume of 100 µl and amplified by PCR. The reaction was carried out for 35 cycles with the following profile: 95°C, 1 min, 40°C, 1 min, and 70°C, 30 s.

The following degenerate primers were used (the code is used according to the International Union of Pure and Applied Chemistry): ELEKEF: 5′-CGCGGATCCGARYTNGARAARGARTT-3′ or 5′-GGAATTCCGARCTNGARAARGARTT-3′; KIWFQN: 5′-CCCAAGCTTR-TTYTGRAACCA-DATYTT-3′; WFQNRR: 5′-CGGGATCCCGNC-GRTTYTGRAACCA-3′.

Similar sequences were used to amplify *Hox* genes by Friedmann et al. [1994], Levine and Schechter [1993], Frohman et al. [1990], and others. Restriction endonuclease sites were added at the 5′ ends of the primers to facilitate cloning of PCR products.

Cloning and Identification of PCR Products

The PCR product (~130 bp) was digested with the corresponding restriction endonucleases (*Bam*HI, *Eco*RI, *Hin*dIII; from NEB Biolabs, Beverly, MA), size purified from an ethidium bromide-agarose gel with QIAEX kit (Qiagen, Chatsworth, CA) and ligated into digested-vector pBluescript KS II (Stratagene, La Jolla, CA). Miniprep DNA was prepared from individual colonies, checked for the presence of the 130-bp insert, and sequenced using the Sequenase version 2, DNA Sequencing Kit (U.S. Biochemicals, Cleveland, OH) according to the manufacturer's instructions. Sequences were compared with nucleotide databanks employing various programs of the GCG (Genetics Computer Group, Madison, WI) sequence analysis software package.

Northern Blot Analysis

Aliquots of 20–25 µg total RNA purified as described above were resolved on 1% agarose/2.3 M formaldehyde gels by electrophoresis. RNA was then transferred by capillary blot onto nylon membranes (Hybond-N, Amersham or Magna NT, MSI, Westboro, MA) as indicated by Maniatis et al. [1989]. Hybridization of the membranes was carried out in 50% formamide, 5× Denhardt's reagent, 4× SSPE, 20 mM KPO_4 (pH 6.5), 1% sodium dodecyl sulfate (SDS) and 10% dextran sulfate. *Hoxa-1* and *Hoxb-7* mouse cDNA fragments were labeled with ^{32}P-dCTP by random primed (REDIPRIME Kit from Amersham). *Hoxa-1* fragment was ~770 bp, derived from the 3′ untranslated region (a gift from Dr. Chris Wright, Vanderbilt School of Medicine, Nashville, TN). *Hoxb-7* fragment was ~550 bp, derived from coding sequence 3′ of the homeobox plus 3′ untranslated region. This fragment was generated by PCR using the following primers: direct primer 5′-AACCTCAGGACCGGGAACCACCG-3′ and reverse primer 5′-GAGGCTCGTGAATAGGACCTAG-3′. None of these probes includes either the homeobox or the polyadenylic tail. Final post-hybridization washes were done in 0.1× SSPE–0.1% SDS at 65°C. The filters underwent subsequent hybridization with a probe for 28S ribosomal RNA, for internal standardization. Bands on autoradiographs were scanned and analyzed by densitometry with Image 1.44 software from the National Institutes of Health (NIH).

Tissue Sections and In Situ Hybridization

Mammary glands from virgin and pregnant mice were fixed for 3 h in 4% paraformaldehyde/PBS, dehydrated through graded series of ethanol to xylene, and then embedded in paraffin wax; 7-µm sections were cut and mounted on siliconized glass slides. In situ hybridization was performed as described by Friedmann and Daniel [1996]. A ~640 bp fragment corresponding to 269-bp 5′ noncoding plus 390 bp coding sequence of *Hoxb-7* cDNA (lacking the homeobox) was amplified by PCR and cloned into the *Eco*RI site of pBluescript KSII multiple cloning site (Stratagene). Two independent clones, carrying the fragment in the sense or antisense orientation with respect to the T7 promoter, were selected and used for the generation of *Hoxb-7* digoxigenin-labeled RNA probes. Transcription reactions were done with DIG RNA labeling Kit (Genius 4, Boheringer Mannheim) according to the manufacturer's instructions.

The primers used for PCR amplification were 1896: 5′-GGAATTCCCAAT-CCGCAGAGCTCG-3′; and 2535: 5′-GGAATTCCAAGTTACTCTCG-GCCGCCA-3′. Assigned numbers correspond to nucleotide position on the mRNA (accession number X06762). Restriction sites were added to facilitate cloning.

RESULTS

Homeobox Gene Expression in Cultured Mouse Mammary Cells

In our initial search for homeobox-containing genes in breast cells, we chose a functional but heterogeneous mouse mammary cell line, CID-9 [Schmidhauser et al., 1990, 1992], which is composed of both epithelial and stromal-like populations. This was because the expression of certain homeobox-containing genes (e.g., *Msx-1* and *Msx-2*) has been shown to depend on epithelial–mesenchymal interactions [Jowett et al., 1993; Pavlova et al., 1994; Friedmann and Daniel, 1996]. CID-9 cells undergo morphological and functional differentiation in response to a laminin-rich BM and lactogenic hormones. The process can be evaluated by assessing the formation of three-dimensional alveolar-like structures and the synthesis and secretion of milk proteins by a large fraction of the epithelial cells.

To identify which *Hox* genes may be expressed in CID-9 cells, degenerate oligonucleotide primers were used in PCR to amplify cDNA from cells that were either proliferating or undergoing differentiation. The primers were designed based on the amino acid sequence of two highly conserved regions within the homeodomain of different members of the *Hox* family. The same strategy has been used successfully in other systems such as mouse intestinal epithelium [James and Kazenwadel, 1991], goldfish retina [Levine et al., 1993], erythroleukemia cells [Takeshita et al., 1993], pancreatic cells [Rudnick et al., 1994], and mouse mammary gland [Friedmann et al., 1994], as well as in the identification of known and new members from other gene families, such as protein kinases [Blaschke et al., 1991] and pou-proteins [He et al., 1989].

Electrophoretic analysis of PCR products showed a band of the expected size (~130 bp) which was cloned, and several individual colonies were randomly chosen and sequenced. Using this approach, we were able to identify five known members of the *Hox* gene family expressed in mouse mammary cells in culture: *Hoxa-1*, *Hoxa-5*, *Hoxb-7*, *Hoxb-8*, and *Hoxb-9*, all belonging to clusters *a* and *b* respectively, according to the nomenclature suggested by Scott [1992] (Table I).

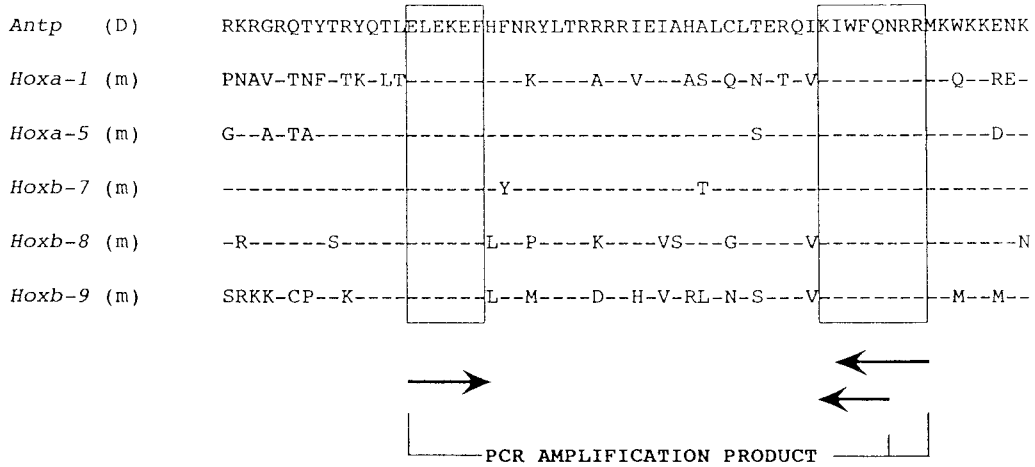
Hoxa-1 and *Hoxb-7* were chosen for further characterization for the reasons described. Total RNA was extracted from subconfluent cultures of CID-9 cells grown on tissue culture plastic and the expression of *Hoxa-1* and *Hoxb-7* was confirmed by Northern blots. We detected one *Hoxa-1* transcript of approximately 2.5 kb and two *Hoxb-7* transcripts of 1.6 and 1.4 kb (Fig. 1A). These are in agreement with the sizes described in previous reports [Friedmann et al., 1994; Meijlink et al., 1987].

Expression and Localization of *Hoxa-1* and *Hoxb-7* In Vivo

The expression of *Hoxa-1* in the mammary gland was studied previously by Friedmann et al. [1994]. Whereas no expression was detected in the normal gland and hyperplastic alveolar nodules (HANs), this gene was found to be expressed in mouse mammary adenocarcinomas generated from serially transplanted HANs. Thus, the expression of *Hoxa-1* in functionally normal cells in culture was surprising and suggested either the absence of regulatory suppressors or the presence of inducers in culture.

Expression of *Hoxb-7* in mammary tissue had not been studied previously. We carried out Northern blot analysis of different stages of mouse mammary gland development in normal mice. Total RNA was isolated from mammary glands of several mature virgin (70 days of age); midpregnant (13 days postcoitus); lactating (2, 5, and 9 days) and involuting (2, 5, and 9 days) animals. Only one transcript, of approximately

TABLE I. Alignment of Amino Acid Homeodomain Sequences Corresponding to *Drosophila* Anntenapedia Gene (D), and Mouse *Hox* Genes (m) Detected in Cultured Mammary Cells by RT-PCR*



*Boxed areas correspond to conserved regions among members of the *Hox* complex; arrows, degenerate oligonucleotide primers used in the amplification reactions.

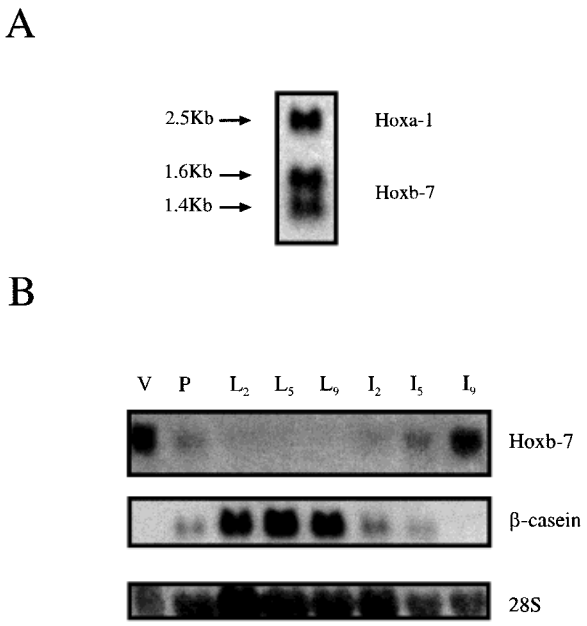


Fig. 1. Expression of *Hoxa-1* and *Hoxb-7* in culture and of *Hoxb-7* during mammary gland development. **A:** Northern blot analysis of total RNA from subconfluent monolayers of mouse mammary cells (CID-9) consecutively probed with ^{32}P-labeled *Hoxa-1* and *Hoxb-7* cDNA fragments. Arrows, observed transcripts. Approximate sizes are indicated (in kilobases) on the left side of the panel. **B:** Northern blot analysis of *Hoxb-7* mRNA in the mammary gland. Northern blot analysis of total RNA from glands of 70 days virgin (V) mice; 13 days pregnant (P); 2, 5, and 9 days lactating (L) mice; and, 2, 5, and 9 days involuting (I) mice. The same blot was sequentially hybridized with *Hoxb-7,* β-casein and 28S rRNA probes. Please note that the expression of *Hoxa-1* was shown previously to be absent in vivo [Friedman and Daniel, 1994]; see also Figure 5.

1.6 kb, was detected throughout development with higher expression in glands of virgin animals, decreasing during pregnancy, practically absent during lactation, but increasing again up to day 9 of involution by which time the gland had reached a resting state (Fig. 1B). Thus, the expression of *Hoxb-7* and full differentiation of the mammary gland, achieved during lactation, are inversely correlated.

To examine which cell type is responsible for the expression of *Hoxb-7* in this tissue, we performed in situ hybridization with antisense and sense *Hoxb-7* RNA probes. By this technique, *Hoxb-7* mRNA was detected in the epithelial compartment of the glands of virgin animals. Some stromal cells in the vicinity of the ducts in glands from virgin animals also appeared to express this gene, but it was at considerably lower levels than the epithelium (Fig. 2A). In the glands of pregnant animals, reduced expression of *Hoxb-7* was detected by Northern blot, but some of the ductal structures were shown to be strongly positive for *Hoxb-7* by the in situ technique (Fig. 2C). *Hoxb-7* expression was absent in the epithelial cells of the gland from lactating animals (not shown).

Expression of *Hoxa-1* and *Hoxb-7* in Mammary Cells in Culture Is Downregulated by an Exogenous BM

We had shown previously that cultivation of primary mammary epithelial cells on an exogenous BM matrix (EHS) (see under Materials and Methods) leads to induction of a near normal lactating phenotype [Aggeler et al., 1991]. We had also shown that two mammary cell lines CID-9 and SCp2 (see under Materials and Methods) turn on milk protein genes and undergo similar morphogenesis on such substrata. We therefore asked whether cultivation on EHS would also recapitulate the loss of these *Hox* genes observed in the lactating gland in vivo.

The two cell lines were cultured in the presence of lactogenic hormones either on EHS, where they acquire a three-dimensional organization, or directly on tissue culture plastic, where they form a flat, two-dimensional monolayer (Fig. 3A). Cells were harvested at different time points after plating, and total RNA was extracted and analyzed by Northern blot.

Hoxb-7 and *Hoxa-1* transcripts were downregulated in mammary cell lines plated on EHS (the same result was found in growth "factor-free" EHS). While this effect could be observed as early as 12 h after plating (not shown), it became more pronounced after 24 h and was sustained for approximately 72 h, the latest time point studied (Fig. 3B, B′, C, C′). Downregulation of these *Hox* genes preceded expression of milk protein genes in the cell lines (data not shown). This finding is interesting in a number of respects: it indicates that BM can downregulate both *Hoxb-7* and *Hoxa-1,* but it also indicates that either the BMs surrounding the epithelial cells of mammary glands from virgin and pregnant animals are different from the BM surrounding the lactating glands or that the epithelial cells have a different spacial relationship to the underlying BM (see under Discussion). The EHS matrix clearly mimics the latter. Furthermore, *Hoxa-1* appears to be subject to a less stringent regulation than *Hoxb-7* in vivo because it is suppressed in all normal epithelia at all stages of development.

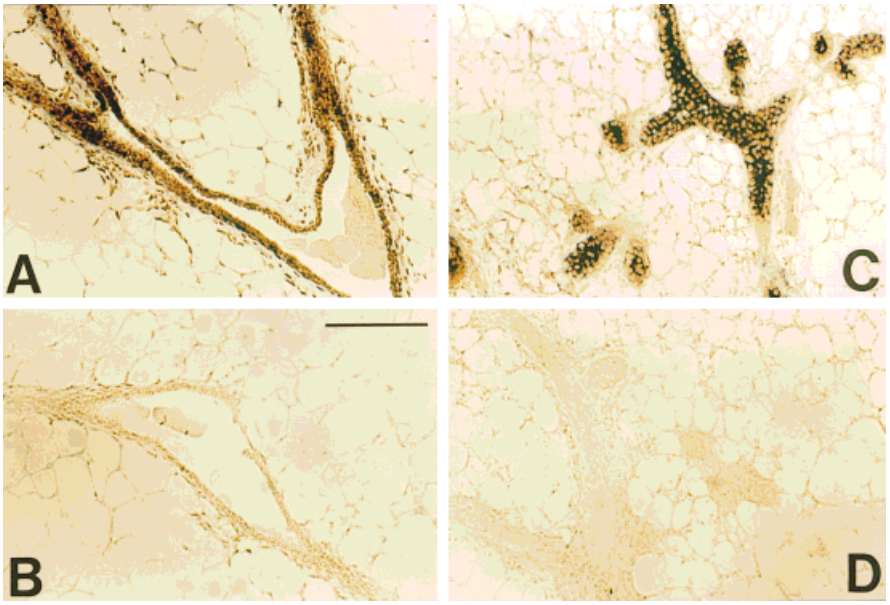


Fig. 2. Localization of *Hoxb-7* mRNA in the mammary gland by in situ hybridization. Sections of mammary glands from mature virgin (**A,B**) and early pregnant mice (**C,D**) were hybridized with *Hoxb-7* sense (B,D) or antisense (A,C) riboprobes. Please note that while some ducts were expressing *Hoxb-7* (C), the total expression was much lower in pregnant; lactating gland was negative (not shown). Scale bar = 100 μm.

Nevertheless, when EHS matrix was dried or cross-linked to confer a rigid structure [Chen and Bissell, 1989], neither *Hoxb-7* nor *Hoxa-1* was downregulated (not shown). This means that malleability of the substratum, and of the matrix molecules themselves, may be important in suppressing these *Hox* genes.

Signal Transduction by "Cell Shape" Versus Specific ECM Ligands

When mammary epithelial cells are placed on EHS, ECM-dependent changes in morphology precede ECM signaling through cell surface receptors, and the latter is dependent on the former [Roskelley et al., 1994]. Whereas EHS can provide both morphological and functional cues, culturing cells onto the nonadhesive substratum polyHEMA [Folkman and Moscona, 1978] induces only the "cell shape" response; that is, cells round up and cluster, but do not deposit, a functional endogenous BM, judged by the lack of β-casein expression and full differentiation [Roskelley et al., 1994] (Fig. 4A). Using the epithelial cell line SCp2, which is unable to deposit its own BM, we were able to separate the consequences of a change in cell shape and cytostructure (which would lead to altered signaling) from ECM biochemical signaling on *Hoxa-1* and *Hoxb-7* expression.

SCp2 cells were plated either on tissue culture plastic or on polyHEMA-coated dishes in the presence of lactogenic hormones. RNA was extracted at different times after plating and was analyzed by Northern blot.

Hoxa-1 transcript levels were markedly reduced in SCp2 cells plated on polyHEMA-coated dishes, compared to the level on tissue culture plastic. By contrast, *Hoxb-7* expression was not altered when the cells were clustered on polyHEMA (Fig. 4B). The pattern of expression of both genes persisted regardless of the length of culturing (from 24 to 72 h). This finding shows that inhibition of *Hoxa-1* expression depends primarily on epithelial cell shape modulated, in vivo, by the stroma and BM at any stage of the gland's development, whereas *Hoxb-7* requires additional signaling from the ECM. It is tempting to suggest that the regulation of expression of *Hoxa-1* depends on a putative "shape response element," whereas *Hoxb-7* gene expression relies on an ECM-response element [Schmidhauser et al., 1992; Roskelley et al., 1995].

Not surprisingly, this response to the microenvironment was abrogated in mammary tumor cells. A mammary tumor generated in transgenic animals [Sympson et al., 1995] expressed high levels of *Hoxa-1* (Fig. 5A), as re-

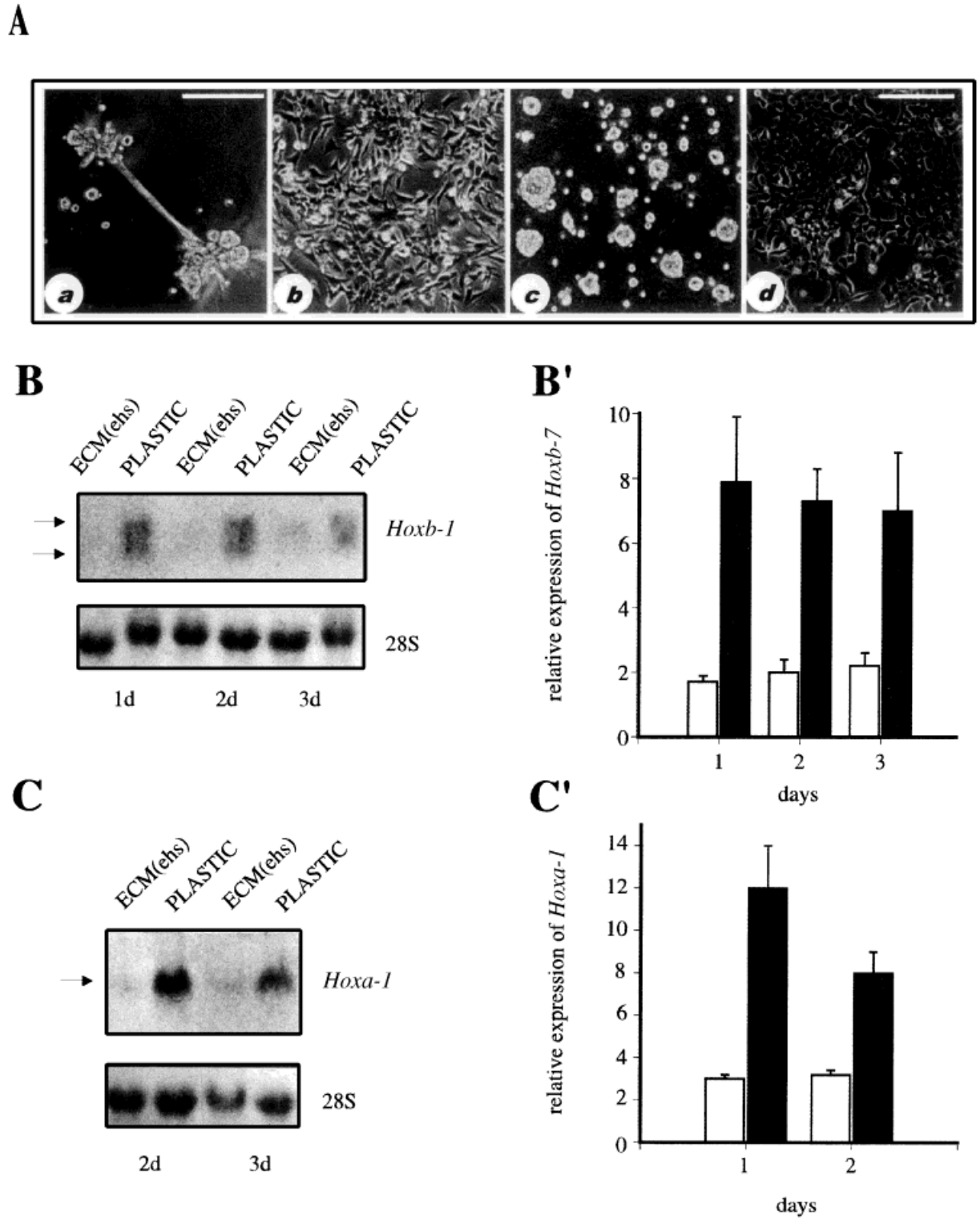


Fig. 3. Influence of an exogenous BM-like ECM on the expression of *Hoxa-1* and *Hoxb-7* in cultured mouse mammary cells. **A:** Morphology of CID-9 cells (a,b) and SCp2 cells (c,d) cultured for 2 days on a reconstituted BM (EHS) (a,c) or on tissue culture plastic (b,d). Scale bar = 200 μm. **B:** Northern blot of total RNA from cells cultured as described above for 3 days. Two *Hoxb-7* transcripts (B) and one *Hoxa-1* transcript **(C)** were identified (arrows). The same blots were stripped and rehybridized with a probe for 28S rRNA. Autoradiographs were scanned and analyzed densitometrically. *Hoxb-7* (**B**′) and *Hoxa-1* (**C**′) expression values were standardized against the corresponding value for 28S rRNA. Graphs show average values and standard deviations (s.d.) from at least three independent experiments. White bars, ECM/EHS; black bars, PLASTIC.

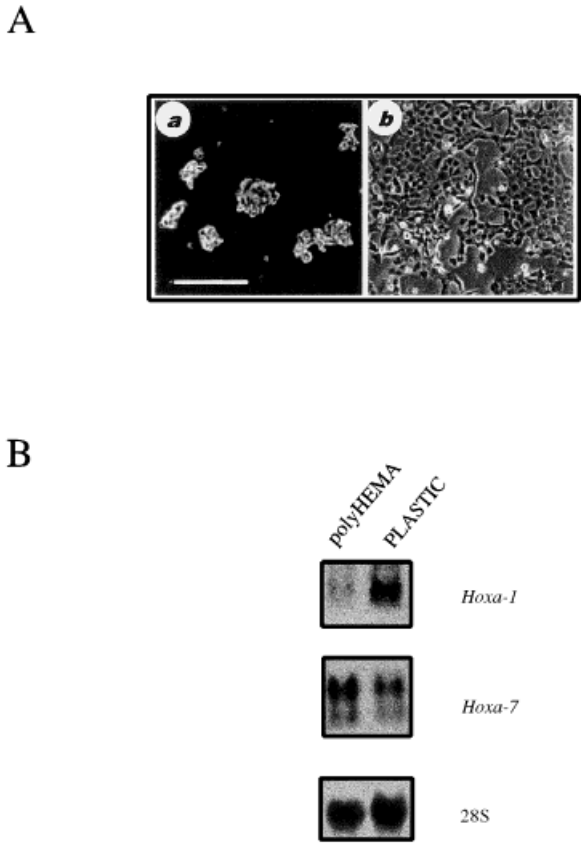


Fig. 4. Influence of cell shape on the expression of *Hoxa-1* and *Hoxb-7* in SCp2 cells. **A:** Morphology of mouse mammary epithelial cells (SCp2) cultured as nonadherent clusters on polyHEMA-coated dishes (a) or as a monolayer on tissue culture plastic (b). Scale bar = 200 µm. **B:** Northern blot of total RNA from cells cultured under conditions mentioned above. The same blot was probed consecutively for *Hoxa-1, Hoxb-7* and 28S rRNA.

ported for other mammary tumors by Friedmann et al. [1994]. A highly tumorigenic cell line (TCL-1) derived from this tumor [Lochter et al., 1997] was cultured on tissue culture plastic, on a reconstituted BM or on polyHEMA-coated dishes (Fig. 5B). RNA was extracted and analyzed by Northern blot. The expression level of *Hoxa-1* was slightly higher in TCL-1 cells than in the other two functional cell lines, whereas *Hoxb-7* was expressed at considerably lower levels in the tumor cell line (data not shown). *Hoxa-1* was downmodulated in CID-9 and SCp2 cells under these conditions (as we described above). Furthermore, its expression not only was not suppressed in TCL-1 cells cultured on an exogenous BM or on polyHEMA, but it was slightly increased compared to the level of expression on tissue culture plastic (Fig. 5C; cf. a and b with c).

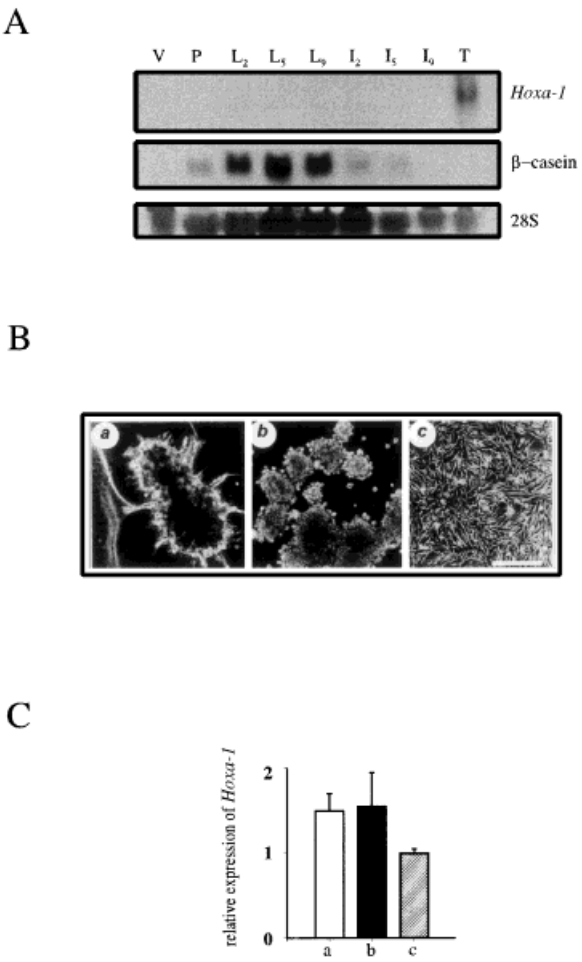


Fig. 5. Expression of *Hoxa-1* mRNA in normal mammary gland, in a mammary adenocarcinoma and in a derived mammary tumor cell line (TCL-1). **A:** Total RNA from glands of 70 days virgin (V); 13 days pregnant (P); 2, 5, and 9 days lactating (from left to right, L) and 2, 5, and 9 days involuting (from left to right, I) mice, as well as from a mammary tumor from stromelysin-1 transgenic mice (T) was analyzed by Northern blot. The same blot was sequentially hybridized with *Hoxa-1,* β-casein, and 28S rRNA probes. **B:** TCL-1 cells cultured on a reconstituted BM (a), on polyHEMA-coated dishes (b), or on tissue culture plastic (c). Scale bar = 200 µm. Total RNA from TCL-1 cells cultured on the above mentioned substrata was analyzed by Northern blot. Graph **C** shows relative expression values for *Hoxa-1* transcript standardized against the corresponding 28S rRNA band. The plotted values represent the average of three independent experiments, and s.d. are shown: a, EHS; b, polyHEMA; c, plastic.

Hoxa-1 Regulation and Cell Cycle Progression

Many nonmalignant cells, including primary mammary epithelial cells, CID-9 and SCp2 cell lines, when cultured on a BM gel or on polyHEMA, where cells are prevented from attachment and spreading, undergo growth arrest [Folkman and Moscona, 1978; Petersen et al.,

1992; Desprez et al., 1995]. By contrast, when these cells are allowed to spread on tissue culture plastic or on other substrata, they continue to proliferate for several days, even in the absence of serum [Boudreau et al., 1996]. If the observed downregulation of *Hoxa-1* in SCp2 cells on polyHEMA-coated dishes was related to an inhibition of cell-cycle progression, the absence of regulation in malignant cells could be due to an inability to suppress growth in these cells, even when they are grown on a reconstituted BM.

SCp2 cells were allowed to spread onto tissue culture plastic and then maintained for 24 h in the absence or presence of increasing concentrations of insulin (0–5 μg/ml), which was previously shown to be required for the growth of these cells. DNA synthesis was determined by BrdU incorporation (Fig. 6A). Despite a dramatic decrease (from 60% to $<$10%) in cell cycle progression observed in the absence of insulin (Fig. 6B), the expression of *Hoxa-1* was, in fact, slightly increased (Fig. 6C). This finding indicates that suppression of *Hoxa-1* expression in nonmalignant mammary epithelial cells cultured on polyHEMA is modulated by the change in cell shape rather than by a change in growth rate. The fact that mammary epithelial cells do not express *Hoxa-1* in either virgin or pregnancy states supports this conclusion. This finding also indicates that the inability of tumor cells to downregulate *Hoxa-1* as a result of clustering is not related to their loss of growth control, but to their inability to appropriately respond to the microenvironment [Petersen et al., 1992].

DISCUSSION

The mammary gland retains the plasticity to undergo repeated cycles of growth, morphogenesis and functional differentiation at the onset of each pregnancy. Furthermore, mammary epithelial cells are capable of recapitulating these morphogenic events when cultured under proper conditions [Barcellos-Hoff et al., 1989; Aggeler et al., 1991]. These observations lead to the idea that specific regulatory molecules must exist that are able to sense and integrate the variety of signals emanating from the cell's microenvironment (i.e., neighboring cells, ECM, growth factors, hormones) and process them in a coordinated manner so as to ensure the maintenance of cell fate and tissue identity. Homeobox-containing genes appear to be good candidates for the orchestration of this complex developmental phenomenon, in which form and function are dynamically intertwined.

Several homeobox-containing genes have been observed to display an altered pattern of expression in some malignancies when compared with the corresponding normal tissues [Cillo et al., 1992; De Vita et al., 1993]. Moreover, misregulation of certain homeotic genes can lead to cellular transformation in culture, as well as tumor formation in vivo [Aberdam et al., 1991; Song et al., 1992; Maulbecker and Gruss, 1993]. These observations suggest that in addition to their role in embryogenesis, homeobox-containing genes may play an important role both in controlling cell differentiation and in the multistep process of tumorigenesis.

Experimental evidence suggests that many of homeotic genes are involved in the regulation of morphoregulators, such as adhesion molecules. For example, *Hoxc-6*, *Hoxb-8*, and *Hoxb-9* can regulate N-CAM promoter activity in cotransfection experiments [F.S. Jones et al., 1992, 1993]. Overexpression of *Hoxd-3* alters the adhesive properties of a human erythroleukemia cell line (HEL), along with an increase in integrin αIIbβ3 [Taniguchi et al., 1995] and *Evx-1*, a homeobox-containing gene outside the *Hox* cluster, activates the tenascin-C (cytotactin) promoter [Jones et al., 1992].

What regulates the homeotic genes themselves is less understood. In the mouse mammary gland, *Hoxc-6*, *Msx-1*, and *Msx-2* expression are regulated by mammogenic hormones [Friedmann et al., 1994, 1996]. The latter further appear to play a role in stromal–epithelial interactions. However, it is difficult to decipher molecular mechanisms and interconnections between the regulatory pathways in vivo. We used a number of culture conditions developed in our laboratory to investigate broad signals that impinge on regulation of *Hoxa-1* and *Hoxb-7*, and which could not be studied easily in vivo. While a change in cell shape alone was sufficient to suppress *Hoxa-1* expression, it was not enough for *Hoxb-7* where the BM itself was also required for downregulation. This effect may be attributed to either a direct signal generated by a particular ECM ligand present in EHS preparations, ECM-dependent modulation of growth factors produced by the cells themselves [Streuli et al., 1993; Lin et al., 1995], formation of an endogenous BM as a result of the cultivation in

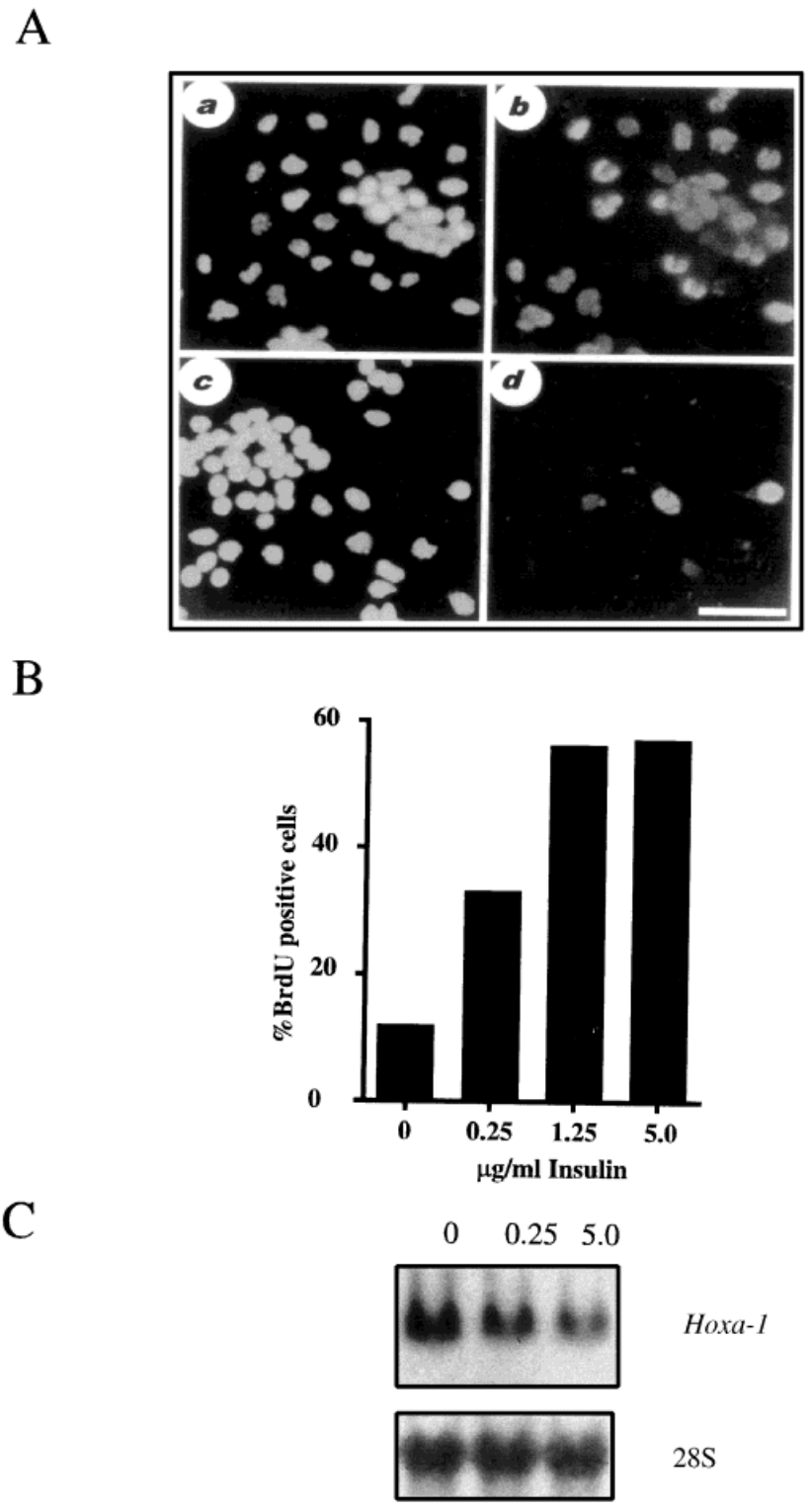


Fig. 6. Downregulation of *Hoxa-1* in SCp2 cells is not due to a decrease in the rate of cell-cycle progression. **A:** Cell proliferation assay. SCp2 cells cultured with 5 µg/ml of insulin (a,b) or in the absence of insulin (c,d). Nuclear DAPI staining (a,c) and detection of incorporated BrdU by indirect immunofluorescence (b,d). Scale bar = 50 µm. **B:** Percentage of BrdU-positive SCp2 cells cultured with increasing concentrations (0, 0.25, 1.25, 5.0 µg/ml) of insulin over 24 h. **C:** Northern blot analysis of *Hoxa-1* expression in SCp2 cells cultured as indicated above. Only those insulin concentrations that led to different cell proliferation indices are shown. The same blot was sequentially hybridized with *Hoxa-1* and 28S rRNA probes. Note that levels of *Hoxa-1* may be even lower in growing cells and not as the result of the absence of growth.

exogenous EHS [Petersen et al., 1992] or the acquisition of secretory epithelial polarity observed only when cells are plated onto this malleable substratum. As mentioned previously, dried EHS, which becomes rigid and promotes cell attachment and spreading [Chen and Bissell, 1989], did not suppress the expression of either *Hox* genes studied, supporting the conclusion that changes in cytostructure may be necessary.

It is interesting to note that the suppression of *Hoxa-1* and *Hoxb-7* in CID-9 and SCp2 mammary cells, mirrors the expression patterns of several milk proteins studied in our laboratory. The functional lactational phenotype is acquired by a hierarchy of ECM-dependent signals [Lin and Bissell, 1993; Roskelley et al., 1995]. A change in cell shape ("rounding"), in the absence of specific ECM signaling, leads to cessation of growth, reorganization of the cytoskeleton, changes in nuclear morphology and concomitant expression of the iron-binding protein lactoferrin [Roskelley et al., 1994, 1995]. This step is also sufficient to suppress *Hoxa-1* expression. A second level of complexity is required for the expression of β-casein. In this case, not only a cell shape change, but also biochemical signaling through β-integrin is necessary to induce β-casein expression and promoter activity [Streuli et al., 1991; Roskelley et al., 1994]. Additional morphogenic signals associated with cell–cell junctions, polarity, and three-dimensional organization are needed to turn on the expression of whey acidic protein (WAP) [Chen and Bissell, 1989; Lin et al., 1995]. The regulation of *Hoxb-7* appears to have characteristics in common with both β-casein and WAP. Although a BM overlay that induces β-casein expression [Roskelley et al., 1994] also modulates *Hoxb-7* on tissue culture plastic, the same overlay does not suppress *Hoxb-7* expression when applied to cells that were already preclustered on polyHEMA-coated dishes (data not shown). This finding suggests that a change in polarity may also be required for downregulation of this gene. Clustered cells on polyhema, although rounded and growth arrested, do not form hemidesmosomes and are not polar. Which mechanism is, in fact, involved is currently under investigation.

The pattern of expression of *Hoxb-7* in the mammary gland in vivo is intriguing. It is expressed in the epithelial cells of virgin animals, but transcript levels decrease appreciably through pregnancy and become virtually absent during lactation. As the gland involutes, expression increases until the gland is remodelled by day 9 of involution. It is therefore reasonable to suspect that *Hoxb-7* may indeed play a role in remodeling and in reestablishing ductal branching. The expression of this homeogene in the virgin gland at a time when ductal branching is occurring would be consistent with this hypothesis. The expression of *Hoxb-7* in the glands of both virgin and pregnant animals in vivo, despite the presence of a visible BM, may be attributable to differences in composition and structure of the ECM, the balance and the composition of integrins and/or other microenvironmental regulators. Also, it is important to note that the mammary gland is a double-layered tube in which myoepithelial cells are localized between the epithelium and the BM. It is known that the relation of the epithelial and myoepithelial cells changes as a function of developmental stage. Thus, one other possibility may be that in the gland of virgin and pregnant animals, the epithelium may be only in occasional contact with the BM, while in lactation the shape of the myoepithelial cell changes allowing a more direct contact between the epithelium and the BM [J.L. Jones et al. 1997; J.C. Jones et al. 1991; Emerman and Vogl 1986].

Our finding that *Hoxb-7* is suppressed in culture when a BM-like ECM is present further supports the "lactational" phenotype of these cells in culture [Aggeler et al., 1991]. Furthermore, the expression of *Hoxb-7* in cells plated on tissue culture plastic is consistent with the "involuting" phenotype of cells on this substratum. CID-9 and SCp2 cells on plastic apoptose when they reach confluence [Boudreau et al., 1995], have little or no milk protein expression but express tenascin [Jones et al., 1995] and *Hoxb-7* (this study).

Two different transcripts corresponding to *Hoxb-7* can be detected in mammary epithelial cells cultured on plastic, whereas only one is observed in normal mammary gland. Although different alternative-spliced variants have been reported for several *Hox* genes [Lopez, 1995], the absence of one isoform in the tissue and its presence in culture is intriguing and warrants further investigation in order to understand its biological significance as well as the molecular mechanisms that regulate its expression.

The fact that epithelial cells on tissue culture plastic express *Hoxa-1*, while the normal breast epithelium in vivo never does indicates that plastic substratum is an abnormal microenvironment for these cells. We know that cells on a flat or rigid substrata express and secrete many growth factors [Streuli et al., 1993; Lin et al., 1995] and inappropriate ECM components such as tenascin [P.L. Jones et al., 1995]. This finding also indicates that expression of *Hoxa-1* need not be limited to the malignant phenotype

only. SCp2 cells do not form tumors when injected into nude mice [S. Galosy, P.Y. Desprez, and M.J. Bissell, unpublished results) and downregulate *Hoxa-1* on polyHEMA-coated dishes and on a reconstituted BM. This regulated expression contrasts with the lack of regulation observed in tumor cells. *Hoxa-1* may thus be expressed in those tumors that have lost their ECM ("shape") responsiveness. This would be consistent with the observation that most human mammary carcinoma cell lines and primary cultures established from tumor biopsies were unresponsive to their microenvironment when grown within a BM [Petersen et al., 1992; Howlett et al., 1995; Weaver et al., 1995].

The availability of functional mammary epithelial cell lines and the existence of differences in the modulation of *Hoxa-1* and *Hoxb-7* could be used to further explore the nature of the regulatory pathways upstream and downstream of *Hox* genes in mammals as an alternative to genetic manipulations in the intact organism. These experiments are in progress.

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