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PRINCIPAL INVESTIGATOR(S): Doctor Charles W. Daniel

CONTRACTING ORGANIZATION: University of California
Santa Cruz, California 95064

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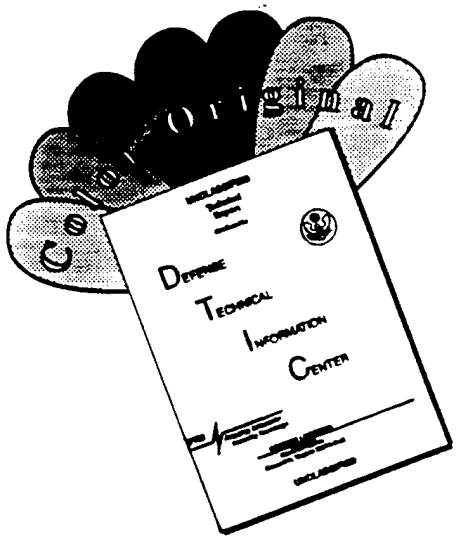

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We have made good progress in investigating the expression of homeobox-containing genes in the mouse mammary gland. We used PCR to amplify conserved sequences from cDNA of mouse mammary gland. Representatives of each of the four *Hox* clusters were expressed, as well as others outside of these major groupings. Several characteristics of homeobox gene expression suggest that they play a significant role. (1) Their expression is developmentally regulated, and is associated with morphogenesis but not milk secretion. (2) Certain homeobox genes are influenced by ovarian hormones. (3) Gene expression is both stromal and epithelial, as shown by *in situ* hybridization. In all cases, expression was associated with regions of epithelium-stroma interactions, and stromal transcription was strictly dependent upon the presence of contiguous mammary epithelium. (4) Misexpression of certain homeobox genes was found in both preneoplastic and malignant tissues, the pattern suggesting a role in later, rather than earlier stages of malignant progression. Experiments to expand our knowledge of the functional role of homeogenes in the mouse mammary gland are in progress, as are studies on homeogene expression in normal and malignant human breast tissues.

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TABLE OF CONTENTS

INTRODUCTION

The problem addressed by this grant, now entering its second year of funding, is to obtain a better and ultimately more clinically useful understanding of the genetic mechanisms underlying development of the normal breast and of the initiation, progression, and spread of breast cancer. The reasoning behind this question is as follows. The breast is a target organ for a variety of hormones. These, together with growth/differentiation factors, regulate the activities of the mammary cell. Unfortunately, this does not take us very far in understanding the biology of this interesting organ. Consider simply that other organs are also regulated by these same signaling molecules, but develop by quite a different pattern. The mammary gland itself varies enormously between species, between individuals, and of course in malignancy. How can this variation be accounted for, when all the signals are the same? There must exist additional layers of genetic regulation that interpret these signals and give rise to particular patterns of development, or to neoplasias. How do we search for these developmental regulatory genes? In organisms such as *Drosophila*, where detailed genetic analysis is possible, mutations provide clues that have led geneticists to identify gene families that act as master regulators of cell fate, determining for example, whether a wing or an antenna will develop at a particular location. The discovery of these regulators has had an enormous impact on thinking in biology.

One such group of genes is the homeotic family, which act as transcription factors, switching on or off groups of genes that specify the details of developmental processes. One of the triumphs of molecular biology has been the discovery that these "homeobox" genes are not limited to the fly, but are ubiquitously distributed and remarkably conserved. In the mammals, including the human, not only do they occur in the genome, but their numbers and types are greatly amplified. It is now well established in the mouse that these genes are essential for determining many aspects of early embryogenesis, and even more recently, it seems that they may be active in the development of tissues and organs, and that their malfunctions may contribute to cancer.

Do homeobox genes influence the breast, and if so, could they contribute to cancer? This is the question underlying our research, and during the first year we have discovered that many of these homeobox genes are active during the growth and development of both the mouse mammary gland and human breast. Their patterns of expression are frequently altered in cancer, some overexpressed, others underexpressed or not expressed at all. Because these genes serves such central regulatory roles, these discoveries create optimism that new insights into mammary development will be a product of these studies, and that an entirely new class of mammary oncogenes or tumor suppresser genes may exist.

Our approach is to use the mouse mammary tumor model system for those observations and experiments that describe the patterns of expression of these genes. In addition, the mouse model, with its capability of "reverse genetics," provides the potential to explore the role of homeotic genes in mammary development and in the etiology of cancer. These experiments cannot be done in humans, of course, but we are simultaneously carrying out expression studies on normal and malignant breast samples and comparing them with findings obtained with the mouse.

The following pages provided details of these experiments and we think will prove exciting. The task of proving that these genes act in a truly causal manner to influence development and malignancy will not be accomplished in a few months, or even years. Nevertheless, our data already identify an entirely new and totally unexplored area of research.

BODY OF NARRATIVE

During the first year of support we have brought in new personnel, established cooperative agreements with suppliers of tissues, and made gratifying progress in the specific aims outlined in the original proposal. Two papers have been published (Friedmann et al., 1994; Friedmann and Daniel., 1994), another is submitted for publication, and a fourth is in preparation. We are pleased that this research has already attracted widespread attention, as judged by requests for reprints, applications for postdoctoral positions, and invitations to present papers at national and international meetings. As I have hoped, it seems likely that several other laboratories will become engaged in homeogene research in the breast, and progress will accelerate.

All of our data indicate that homeobox genes are expressed in the development of mammary glands in both mice and humans. More important, the spatial and temporal pattern of this expression indicates a regulatory role for this interesting family of transcription factors, in which altered expression patterns may contribute to various morphotypes, and to cancer initiation or progression. Although we do not as yet have data on gene transfer studies that may pin down the functional role of homeogenes in the breast, the expression data continue to indicated that this project, which originally may have been considered somewhat speculative, has uncovered a new level of genetic regulation of breast growth, morphogenesis, and function, and that at least some of this large family of genes may be shown to constitute a new class of mammary oncogenes.

Our progress in the four areas identified as research objectives is summarized below:

Objective 1. *Do expression patterns suggest a role for homeobox genes in mammary growth and development in the mouse? What is the pattern of homeobox gene expression in precancerous tissues and in malignancies?*

Temporal patterns of homeogene expression in the mouse mammary gland. Although it is beyond the scope of this project to identify all expressed homeogenes, we have confirmed and extended the findings originally reported as preliminary results. Using RT-PCR to identify expressed sequences from highly conserved regions of the homeobox itself, and also using gene-specific probes provided by various investigators, we have now identified expression in *Hox* genes from each of the major clusters. Genes showing significant levels of expression are shown in Table 1. It seems likely that many more Hox genes (perhaps all) will ultimately be found to be expressed in one or more developmental stages of the mammary gland developmental cycle, or if not, in preneoplastic or malignant tissues. This speculation is based on the fact that in no case have we tested a clone that was completely silent.

All homeobox genes examined by northern analysis have shown some degree of developmental regulation, strongly suggesting a functional role in growth and morphogenesis. The most typical pattern shows significant levels of expression in the ductal phase of development, when the parenchyma is invading the mammary stroma to form the distinctively patterned mammary tree. In most cases, expression declines in late pregnancy, when functional differentiation is occurring, and during lactation. The latter is

open to some degree of interpretation, since the large number of milk protein transcripts may dilute the less abundant regulatory RNAs, as shown by the L7 loading controls. Examples are shown in Fig. 1.

TABLE 1. Summary of homeobox gene expression in mouse mammary gland [1, 2] (Northern Hybridization)

	5 weeks old	8-9 weeks old	16 weeks old	5-8 days pregnant	15-18 days pregnant	lactating[3]	ovariectomized at 5 weeks	ovariectomized at 12 weeks	tumor D1a	tumor D2a	tumor D2d	myc tumor
Hoxd-3	–	–	–	–	–	–	–	–	+	–	–	–
Hoxd-4	++	++	++	++	+	–	+++	++	–	–	–	–
Hoxd-8	+++	+++	+++	+++	+	+	+++	+++	–	–	–	–
Hoxd-9	++	++	++	+++	+	+	++	++	–	–	–	–
Hoxd-10	+	+	++	+++	++	++	++	++	–	–	–	–
Hoxd-11	–	–	–	–	–	–	–	–	–	–	–	–
Hoxd-12	–	–	–	–	–	–	–	–	–	–	–	+++
Hoxc-6	++	++	++	+	–	–	+++	+++	–	–	–	–
Hoxc-8	++	++	++	+	–	–	+++	+++	–	–	–	–
Hoxb-6	++	++	++	+	–	–	++	++	–	–	–	–
Hoxb-7	++	++	++	+	–	–	++	++	–	–	–	–
Hoxa-1	–	–	–	–	–	–	–	–	+++	+	+++	–
En-1	++	++	++	++	–	–	+++	++	+	+	+	+
En-2	–	–	–	–	–	–	–	–	–	–	–	–
Msx-1	++	+++	+++	+++	++	+	+++	+++	++	+	+/–	++
Msx-2	+++	+++	+++	++	–	–	+	+	–	–	–	–

1. Levels are compared for each gene individually and cannot be compared between different genes. (–) no expression detected; (+/–) very low expression; (+) low expression; (++) moderate expression; (+++) abundant expression.
2. Levels are adjusted according to L7 loading control.
3. During lactation, high levels of transcripts for milk proteins may dilute other mRNAs (as can be seen for loading control in the figures in chapter 2). Homeogenes expression may therefore be expressed during lactation at higher levels than indicated.

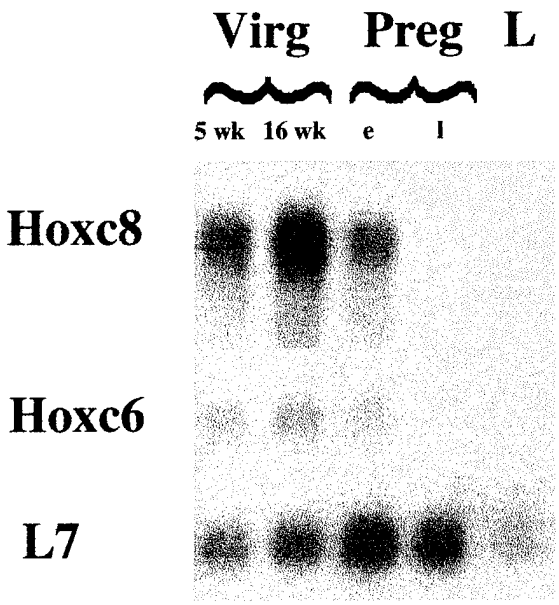

Fig. 1. Northern analysis of representative Hox genes in mouse mammary development excluding involution. Typically, expression declines during late pregnancy and is undetectable during lactation.

In addition to the numerous and widely expressed *Hox* genes, we have found certain of the divergent homeobox genes, located outside of the highly ordered *Hox* clusters, to be of exceptional interest. *Msx-1* and *Msx-2* are related to the *Drosophila melanogaster* muscle segment homeobox (*msh*) gene, that 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., 1991; MacKenzie et al., 1991; Monaghan, Davidson 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, Leeming et al., 1991; MacKenzie et al., 1992; Jowett et al., 1993), chick heart (Chan-Thomas et al., 1993) and chick craniofacial development (Nishikawa et al., 1994). The results suggested that the two genes play a role in epithelial-mesenchymal interactions in these developing organs.

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 (Fig. 2). *Msx-1*

(~2050 bp) and *Msx-2* transcripts (~1300 bp and ~2300 bp) were present in glands from virgin animals and glands from animals during early pregnancy (5-8 days post coitus) . Transcript levels of both genes decreased substantially in glands from animals in late stages of pregnancy (15-18 days post coitus). In lactating glands *Msx-2* expression was not detected, while *Msx-1* transcripts were seen at low levels. The transcript size of *Msx-1* in glands from lactating animals was somewhat smaller (~1.9 Kb) than the transcript size from other stages of the mammary cycle.

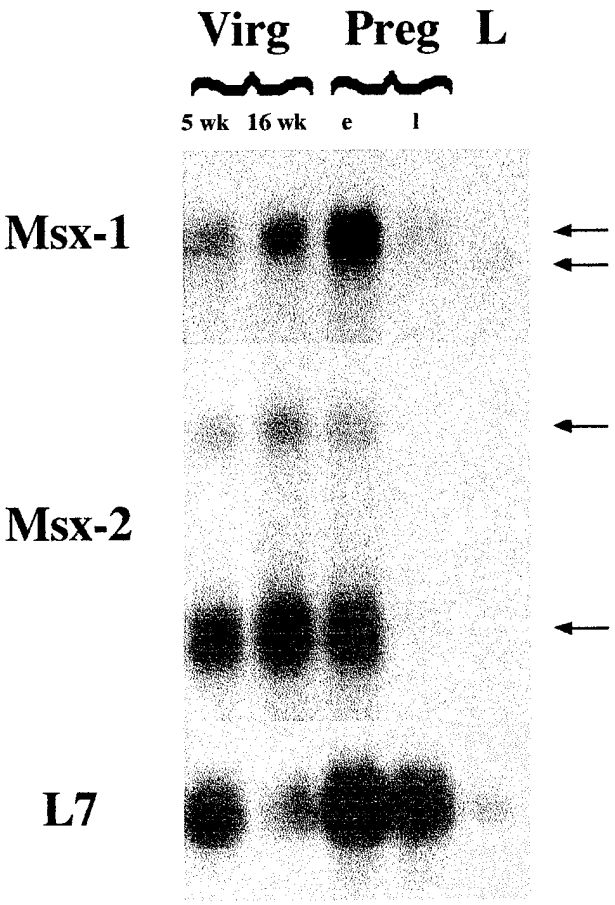

Fig 2. Msx expression in development, using poly (A)+ RNA in the northern blot. Note that Msx-2 displays two transcripts. In the lactating gland, the Msx-1 transcript is reduced in size.

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

gradually in days 4-8 and then reached a plateau. *These data* indicate a probable role for *Msx-1* and *Msx-2* in the dramatic and complex period of glandular remodeling that takes following weaning.

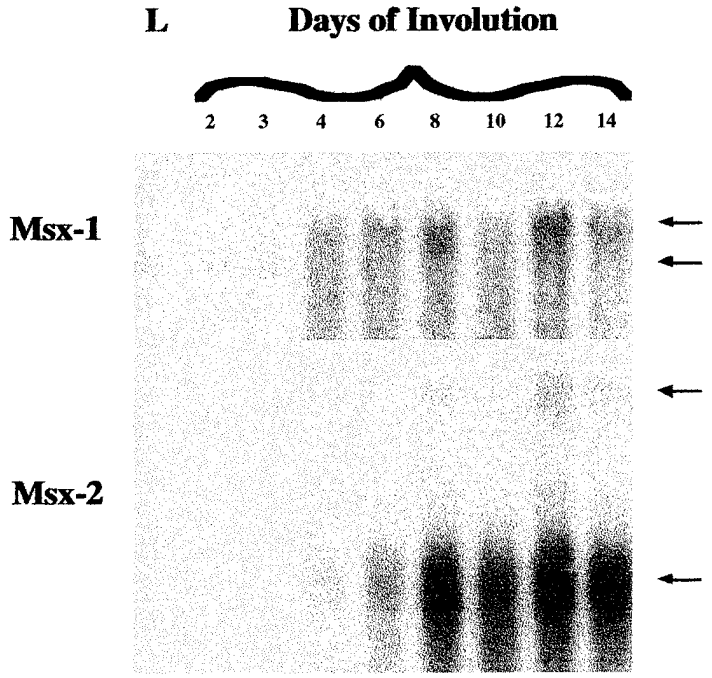

Fig. 3. Northern analysis of Msx-1 and Msx-2 in involuting mammary glands . Mice were lactating for 10 days before pups were removed, and the next day was considered the first day of involution.

Spatial patterns of homeogene expression in the mouse mammary gland.

As proposed, we have used *in situ* molecular hybridization using gene-specific probes on sections of mammary gland tissue. This was combined with a limited amount of immunohistochemistry (few reliable antibodies are available). Although we are pursuing these studies with several of the expressed *Hox* genes, our most complete data are from studies on *Msx-1* and *Msx-2* .

Fig. 4 shows *Msx-2* RNA localized to the periductal stromal cells, where these cells are forming extracellular matrix (ECM) in coordination with ductal growth. Fibrous ECM is a conspicuous feature around the ducts in the quiescent gland of the non pregnant mouse, and is thought to play an essential role in regulating branching and pattern formation. In glands from pregnant mice *Msx-2* is localized to stromal cells adjacent to mammary ducts and in most cases not around the developing secretory alveoli or at a distance from the gland.

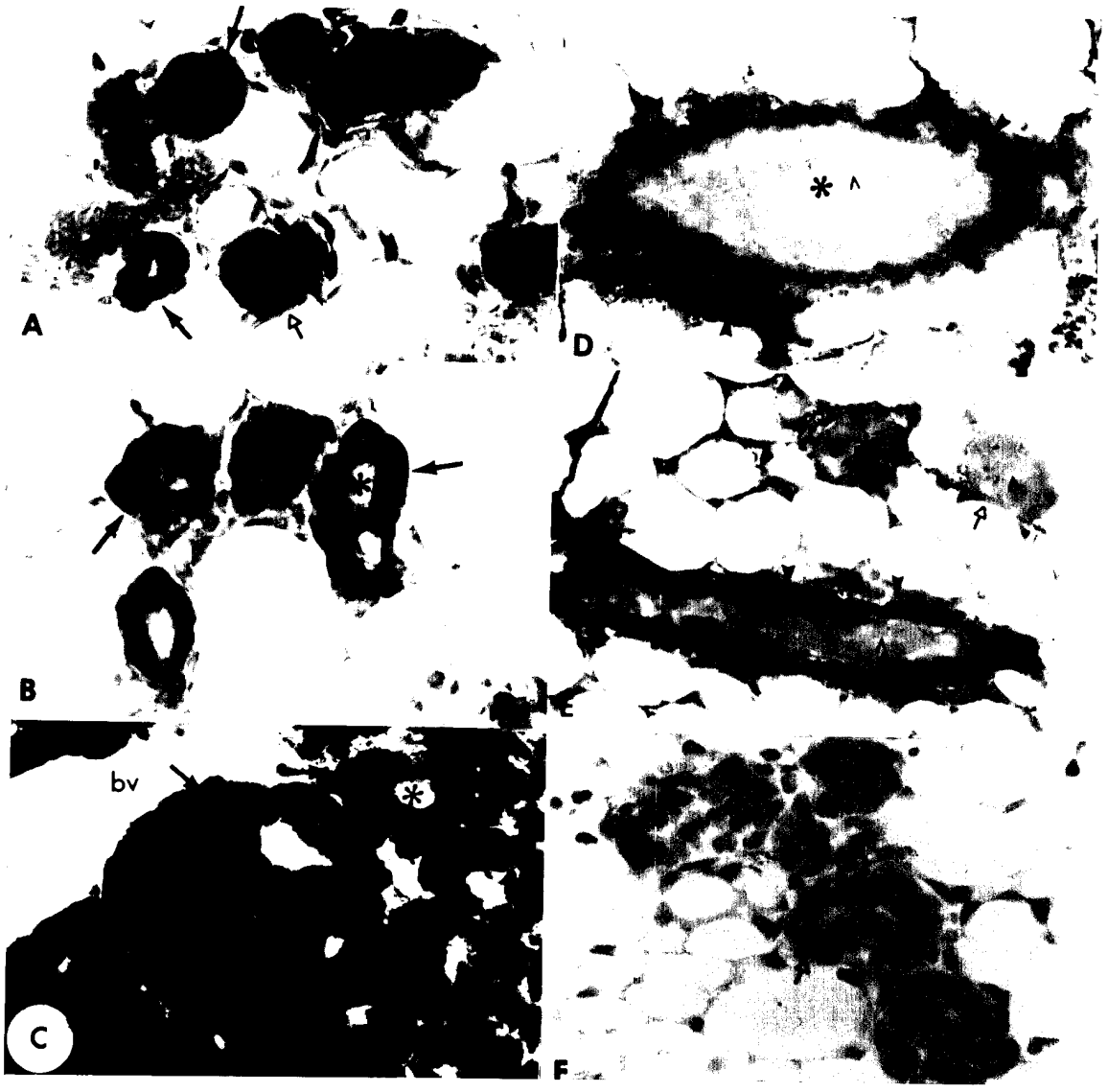

Fig. 6. Spatial expression of *Msx-1* and *Msx-2* mRNA in mammary gland. A. *Msx-2* expression in a gland of mature, virgin mouse. B. *Msx-2* expression in a gland of a pregnant mouse. C. *Msx-1* expression in mammary gland of a pregnant mouse. D. *Msx-1* sense control probe in a gland of a pregnant mouse. It is representative of the controls for other stages of gland development for *Msx-1* and *Msx-2* sense probes. Arrow heads point to *Msx-1*-positive epithelial cells. > points to *Msx-2*-negative epithelial cells. Arrows point to *Msx-2*-positive periductal stromal cells. Open arrows point to lobule-alveolar structures in pregnant glands. * indicate lumens. Bar, 15 μm.

Msx-1 and *Msx-2* are involved in epithelial-mesenchymal interactions in developing organs, including the mouse and chick limb buds (Davidson, Crawley et al., 1991; Robert, Lyons et al., 1991) and mouse tooth development (Jowett, Vainio et al., 1993). It is firmly established that epithelium-mesenchyme interactions are crucial to the development of the mammary gland
and are in large measure responsible for the process of pattern formation (Sakakura et al., 1976; Sakakura, 1987).

The localization of *Msx-1* and *Msx-2* transcripts at the epithelium-stroma interface by *in situ* hybridization (Fig. 4), suggested that these genes play a morphogenetic role in mammary development. 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 pre-pubertal 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. 5). *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* and clearly points towards a role for these genes in the realm of inductive tissue interactions.

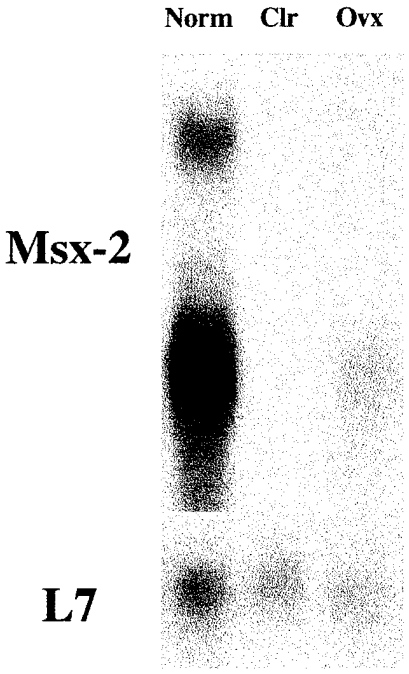

Fig. 5. RNA expression of *Msx-2* in gland-free fat pad. RNA was extracted from glands whose epithelial component had been removed in the three weeks old mice. Glands were collected 2 months after operation (lane 2). Expression is compared to levels in glands of epithelium-intact mature virgin mice (lane 1) and to glands of ovariectomized mice (lane 3).

Homeogene expression in precancerous tissue and in malignancies

The expression of several homeogenes has been tested in mouse mammary carcinomas and in the precancerous lesions (Hyperplastic Alveolar Nodules, or HAN) from which the

tumors were derived. The results indicate a highly variable pattern of expression, in which some genes are transcribed only in normal gland, others only in tumors, and a few in all stages. *Hoxd-4, Hoxd-8, Hoxd-9, Hoxd-10 and Hoxd-11* were not expressed in any of the tumors (not shown). *Hoxd-3* showed weak expression in tumor D1a (not shown) and *Hoxd-12* showed expression in a tumor derived from cells overexpressing the myc protooncogene.

In contrast, *Hoxa-1,* which was not expressed in any of the stages of normal development, is expressed in all three tumors derived from the HAN tissue lines. Msx-1 showed substantial expression in all tumors, whereas Msx-2 was not detected in any (Figure 6).

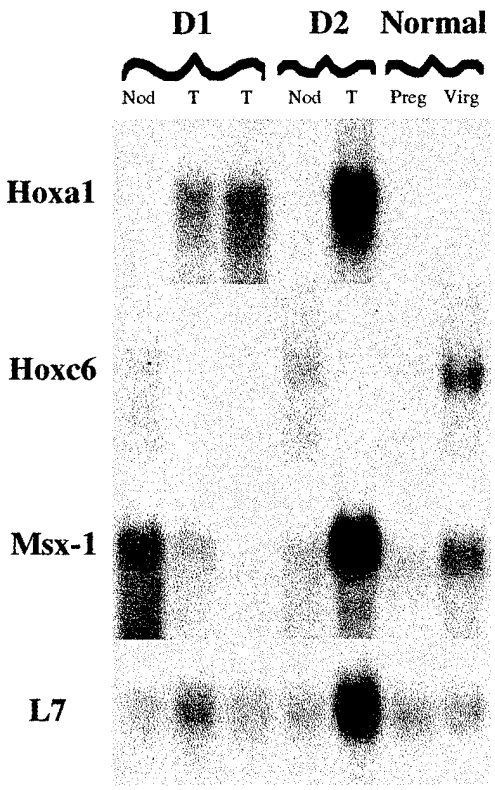

Fig 6. *Hoxa-*, *Hoxc-6* and *Msx*-1 expression in preneoplastic and neoplastic mammary outgrowths. RNA was isolated from hyperplastic alveolar nodules D1 and D2 (18) that were serially transplanted in Balb/C gland-free fat pads, and from spontaneous carcinomas (T) that arose from these nodules and were subsequently transplanted. Normal mammary gland RNA from 15-18 days pregnant animals and from mature virgin animals is shown in the two right-hand lanes. Each lane contains 20 micrograms of total RNA.

The function studies (Objective No. 4) will provide information as to whether the altered pattern of expression seen in neoplastic tissues is causally related to malignant progression. Even without this information, the expression data are exciting. We note that in nearly every case examined, expression of homeobox genes is substantially altered in mammary cancers. In a few cases expression is up regulated. Perhaps more exciting, however, is the

observation that many homeobox genes, including most of the Hox4 cluster, are silent in tumors even though they are quite active in normal development. Since one of the well established functions of homeotic genes is to channel cells towards differentiation and growth cessation, it is quite possible that loss of functions of these differentiation factors could lead to loss of growth regulation. Thus, these homeogenes may be shown to be tumor suppressors, mutations in which could contribute to neoplastic progression.

Objective 2. *Using human tissues obtained from mastectomy and reduction mammoplasty patients, we can ask if homeobox genes are expressed in human breast tissues. Are breast cancers associated with altered levels of expression?*

Tissues. In research with primary human tissues, success or failure may revolve on the cooperation between the surgeons and research personnel. This is particularly true when, as in the present case, success depends upon the tissue being specially treated by immediate freezing in liquid N_2 or by immersion in ice-cold paraformaldehyde fixative. We are fortunate in having the cooperation of surgeons who are committed to this research and who follow our protocols with care. We are collecting tissue on a regular basis, and adequate numbers of samples will not present a problem. We have established fruitful relationships with the surgical staff at Dominican Hospital, who are now routinely supplying us with normal and malignant breast tissues from mastectomy patients, and with normal tissues from biopsy specimens.

Our only difficulty with regard to tissue acquisition is finding that normal gland from post-menopausal women does not contain useful quantities of parenchyma. Therefore, we are using biopsy specimens from younger women, tissue from pre-menopausal mastectomy patients, and perhaps from the occasional reduction mammoplasty patient or accident victim.

Amplification and identification of expressed homeogenes.

As described in Methods, we have prepared cDNA from tissue samples and amplified fragments using degenerate primers to conserved homeobox sequences. These fragments have been cloned and we have begun sequencing. So far, we have identified the following expressed homeogenes either by direct sequencing or by hybridization to existing sequence-specific probes:

HOXA4, A7, B4, B7, C6 and **D10**.

Thus, at least one member of each of the four major HOX clusters is represented, and we anticipate that there will be many more. In addition, the expression of one homeobox gene outside of the clusters, **HOX7 (MSX1)** has also been identified. Again, there will almost certainly be others. Examples of our early data are given below.

Levels of Expression.

Because of the high sensitivity of PCR, it is important to establish that the levels of mRNA expression are comparable to that seen in other species and in the mammary

glands of mice. Figure 7 shows a northern blot of total RNA from three patients that was hybridized to a radiolabeled probe to HOX D10 (HOX 4.5).

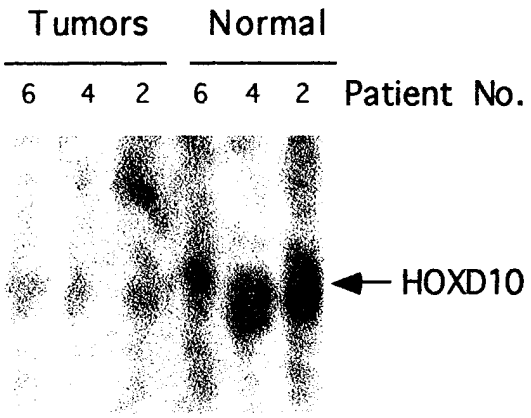

Fig. 7. Northern blot hybridized to a radiolabeled probe to HOX D10. Note that for each patient, the tumor tissue showed less signal that the corresponding normal samples. The blotchy appearance is because this blot was exposed in the PhosphoImager, which creates artifacts. When exposed on film a clean image will be produced.

Hybridization to this sample of total RNA indicates a fairly robust level of HOXD10 expression and shows reduced expression in each of the three tumors, as compared with the corresponding normal tissue. As mentioned previously, inadequate expression of HOX genes may delay or prevent differentiation, keeping the cells in a proliferative phase and contributing to malignant progression.

4. Spatial localization.

Although we have not yet carried out hybridization *in situ* with human breast tissues, immunohistochemistry using an antibody to HOXB7 (BabCo, Berkeley) has produced clear nuclear localization in epithelium (Fig. 8) of a malignancy classified as an alveolar carcinoma.

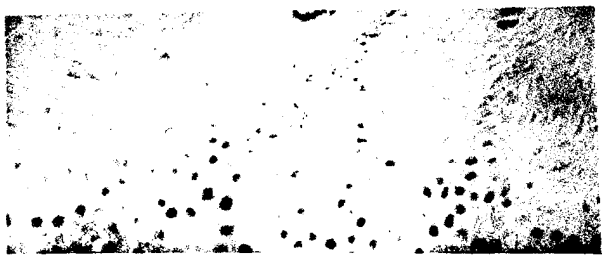

Fig 8. Immunohistochemistry showing localization of HOXB7 in a subset of epithelial nuclei of a breast carcinoma. Darkened nuclei indicate immunoreactive HOXB7. Controls incubated without primary antibody were negative (not shown).

In summary, our preliminary data show that several HOX genes and at least one homeobox gene outside of the four major HOX clusters are expressed in both the normal and malignant breast. As we have shown, the tools are immediately available to carry out the proposed studies that will provide detailed data on the particular genes expressed, their relative levels of expression in normal and malignant breast tissues, and their spatial localization within tissues.

Objective 3. *Are homeobox genes regulated by mammogenic hormones or growth factors? By retinoic acid?*

The breast is the quintessential example of an endocrine target organ, in which each developmental stage, from early budding, through growth and branching of the ductal tree, milk production, and finally involution, is regulated by a complex and continually changing hormonal mileau. It is therefore reasonable to suppose that genes regulating growth, patterning, and function will be under regulation of one or more of the mammogenic hormones. Indeed, if regulatory gene expression were unaffected by levels of reproductive hormones, one would be doubtful that their expression is causally linked to mammary development or function.

***Msx-2* expression in glands from ovariectomized animals and after estrogen replacement**

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

The expression level of *Msx-1* in glands from ovariectomized mice was similar to that in glands from intact controls (Fig. 9). 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, suggesting that *Msx-2* RNA level is up-regulated by ovarian secretions.

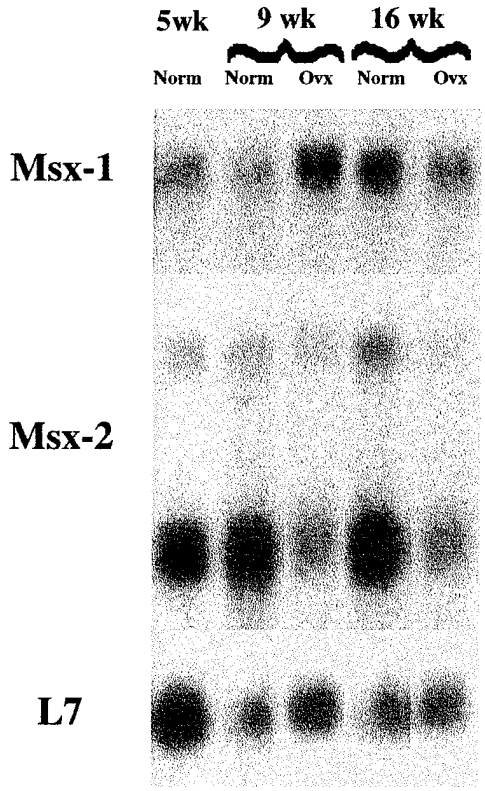

Fig. 9 RNA expression of *Msx-1* and *Msx-2* in the mammary gland in response to ovariectomy. Poly $(A)^+$ RNAs were extracted from glands of 5-week-old endocrine-intact mice (lane 1); 8-9 week-old endocrine-intact mice (lane 2); 9-week-old mice that were ovariectomized at 5 weeks of age (lane 3); 16 week-old endocrine-intact mice (lane 4); 16 week-old mice that were ovariectomized at 12 weeks of age (lane 5). Northern analysis was performed as described in "Materials and Methods". Each lane contains 5 μg of poly $(A)^+$ RNA.

To further test the important question of endocrine regulation, *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 returned to levels found in glands from intact animals (Fig. 10).

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

to the untreated glands (Fig. 10). These results confirm the hypothesis that *Msx-2* RNA is up-regulated by ovarian secretions, of which estrogen is an active agent.

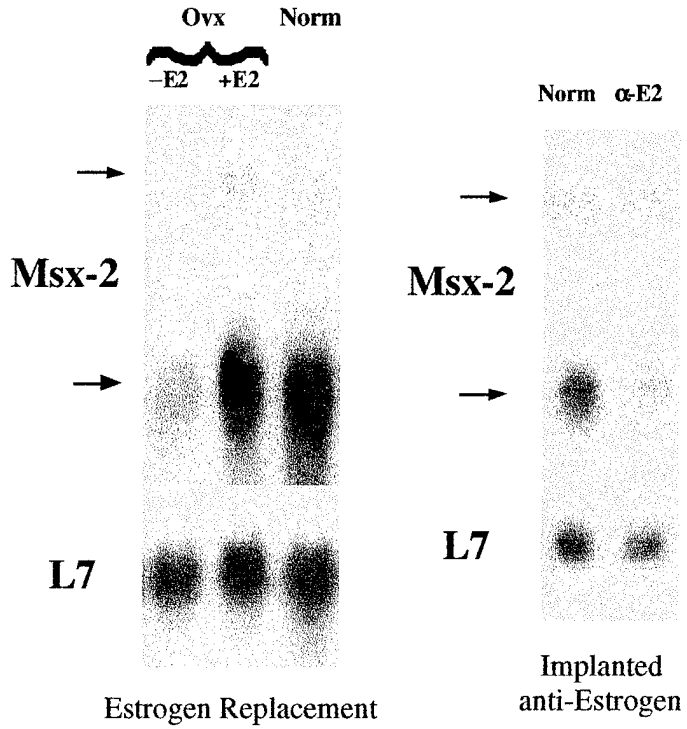

Fig. 10. Left. Expression of *Msx-2* RNA in the mammary gland in response to estrogen replacement in ovariectomized mice. RNA was extracted from mammary glands of mice that were ovariectomized at the age of 5 weeks, let mature for 6 more weeks, when estrogen was added subcutaneously. Glands were collected 4 days later . RNA levels are compared to RNA from glands of 6 weeks endocrine-intact animals and to glands from mice that were ovariectomized at 5 weeks of age and let mature for additional 6 weeks prior to glands collection. Each lane contains 20 µg total RNA. **Right**. RNA expression of *Msx-2* in the mammary gland in response to implanted antiestrogen. RNA was extracted from glands of pubescent mice that were implanted with 100 µg/gland of antiestrogen ICI 164,384 for 4 days (lane 2) or from untreated contralateral glands (lane 1). Each lane contains 20 µg total RNA. Solid arrows point to *Msx-2* transcripts.

Objective 4. *Does misexpression of homeobox genes in situ influence the incidence of precancerous and cancerous lesions?*

Functional analysis of expressed homeogenes is being carried out *"ex vivo"* by overexpressing homeogenes in mouse mammary gland and examining the resulting phenotype *in situ.* Preneoplansic and neoplastic changes, as well as alterations to normal growth patterns will be related to expression. In the case of the mouse mamary gland a unique variation on these techniques is available. Mammary epithelium cultivated as a monolayer can be transfected *in vitro,* where selection can be used to enrich the cultures for cells carrying introduced genes for antibiotic resistance. These cells are injected into a

gland-free fat pad where they are capable of regenerating mammary gland. Even small variations in growth rate, pattern, or functional activity of these regenerated glands can be readily recognized by experienced observers. Areas of interest can be selected and transplanted into other fat pads, creating tissue lines of genetic variants that can be tested for incidence of preneoplastic and neoplastic changes.

We are using the pNO4 retroviral vector to overexpress the various homeobox-containing genes in mouse mammary glands. The vector is derived from the Murine Sarcoma Virus (MSV) and contains the *neo* gene (which confers resistance to the antibiotic G418) as a selectable marker for transfection. pNO4 also contains a Cytomegalovirus (CMV) promoter which drives constitutive expression of the cloned gene of interest.

Thusfar, we have made three expression constructs in the pNO4 retroviral vector. The first is a reporter construct containing the coding region for the jellyfish green flourescent protein (GFP) to be used as a positive control for transfection and as a visible marker for identification of transfected cells *in vivo*. The other two constructs contain the coding regions of the mouse *HoxA1 (Hox 1.6)* and the human *Msx-1* genes, respectively. We have successfully transfected these constructs into the PA317 retrovirus packaging cell line for the production of replication-defective virus and are in the process of isolating stable cell lines that produce high-titre virus.

These experiments require extensive gearing-up, but are going well and we expect our first successful transfections in a few weeks. Phenotypic variants resulting from misexpression of homeogenes will provide the first experimental evidence for a morphogenetic role for homeogenes. Our extensive experience with the mouse mammary system will permit us to identify even subtle variations in pattern and functions. Tissue lines developed from homeogene-associated dysplasias will be developed to determine stability of the phenotype, and their neoplastic potential.

CONCLUSIONS

Although this work is in its early stages, a number of conclusions can be drawn.

- In the mouse, many homeobox genes are expressed, both within the Hox clusters and outside. In many cases the expression is quite robust.
- Expression is developmentally regulated, being most conspicuous during ductal development and involution, suggesting a role in growth and morphogenesis. Gene activity appears not to be associated with functional differentiation or secretion.
- In at least some cases, gene expression is influenced by mammogenic hormones, indicating again the probability of an important functional role.
- Transcription of homeobox genes is always associated with the epithelium-stroma interface, pointing towards a role in inductive tissue interactions.
- In the mouse, homeogene expression may in some cases be activated in tumors, and in other cases repressed. At this point we cannot know whether these genes may act as tumor suppressors, as oncogenes, both, or neither. But they do appear to be involved.
- In humans, we have also shown expression of several homeotic genes in normal and malignant breast tissue. This work is in a comparatively early state compared to that of the mouse, but is progressing well.

The principle change in the list of objectives presented in the original proposal is one of emphasis. At the original time of writing, we had barely begun preliminary studies on the human breast. Now, with evidence of substantial levels of transcription of several of these genes, an increased effort on the human part of the proposal is appropriate. It is my hope to add an additional Postdoctoral Researcher to accelerate progress.

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APPENDIX

[CANCER RESEARCH 54, 5981–5985, November 15, 1994]

Hox Genes in Normal and Neoplastic Mouse Mammary Gland[1]

Yael Friedmann,[2] Carla A. Daniel, Phyllis Strickland and Charles W. Daniel

Department of Biology, Sinsheimer Laboratories, University of California, Santa Cruz, California 95064

ABSTRACT

Homeobox-containing genes regulate embryonic developmental programs and are expressed in certain adult tissues and cancers. There has been no report of expression in the breast. We amplified homeobox complementary DNA from mouse mammary gland and found expression of members from each of the four major *Hox* gene clusters. The regulation of expression of two *Hox* genes was examined in greater depth. *Hoxc-6* transcripts were present in the glands of pubescent and mature mice and decreased during pregnancy. Levels were increased substantially following ovariectomy, indicating possible negative regulation by steroid hormones. *Hox* expression was studied in mammary adenocarcinomas and in transplant lines of the benign, precancerous tissues from which the cancers arose. *Hoxc-6* was expressed at low levels in the precancerous tissue but was not expressed in cancers. In contrast, *Hoxa-1* was expressed only in cancers, not in normal gland or in precancerous mammary tissues, suggesting that *Hox* genes may play a role in a late stage in the stepwise development of mammary malignancies.

INTRODUCTION

Homeobox-containing genes (homeogenes) comprise a large collection of developmental regulators that have aroused interest due to their role in specifying developmental pathways in embryos. Mutations in certain of these genes may result in incorrect specification of body parts in organisms ranging from invertebrates to mammals (1–3). Recently, interest in possible roles for homeobox genes in adult animals has been sparked by the observation that a number of homeobox genes are transcribed by more mature tissues, including kidney (4) and hemopoietic cell lineages (5). Of particular interest is the observation that certain cancers display altered homeobox gene expression (6–8); misexpression of homeobox genes can transform cells *in vitro* and produce tumors in mice on transplantation (9, 10). Thus, homeobox-containing genes may constitute a new class of proto-oncogenes (11).

All homeobox genes have a common structural characteristic of a box motif of about 183 bases, coding the 61 amino acid homeodomain. Thirty-eight murine genes containing an Antennapedia-like homeobox have been characterized and comprise the four *Hox* gene clusters. Recent work carried out with *Drosophila* genes encoding comparable homeodomain sequences suggests that homeoproteins can bind to DNA by a helix-turn-helix structure and thus probably play a regulatory function by influencing the expression of downstream genes (12–14). Although it is likely that these homeoproteins are involved in important developmental processes in vertebrates, the full extent of their influence, particularly in adults, remains unclear. One way to gain insight into this question is to examine the pattern of homeogene expression under normal and altered conditions, thus identifying some of the levels at which these genes may exert effects.

The mouse mammary gland represents the most fully developed system for the analysis of the normal and neoplastic breast, making it an attractive model for studying the regulation of *Hox* gene expression. The mammary gland is unique in that most of its growth and morphogenesis takes place in the subadult or adult mammal, where it is experimentally accessible. During puberty, a ductal tree is generated by epithelial growth and branching, resulting from embryonic-like inductive interactions between mammary epithelium and mammary stroma (15). The differentiative pathway of ectodermal epithelial cells depends on their position within the growth buds; they may become cells destined for ductal walls, milk synthesis, and secretion, or contractile myoepithelium (16).

The development of mouse mammary tumors is a multistage process that occurs through definable stages: normal; preneoplastic; and neoplastic. These cell populations have biologically distinct growth properties. Normal mouse mammary cells have a finite life span *in vivo* and after five or six serial transplant generations show a diminished growth potential (17). The preneoplastic stage is represented by the HAN[3] (18, 19), which represents a benign, immortalized cell population that is morphologically similar to differentiated alveolar cells normally found in glands of pregnant mice but does not require lactogenic hormones for maintenance. HANs can be grown indefinitely *in vivo* as tissue lines when serially transplanted into mammary gland-free fat pads. Because HANs have been shown to exhibit a far greater probability for tumor formation than the normal alveoli found in pregnant mice (20, 19), they have been termed "preneoplastic" and may represent an obligatory intermediate in the conversion to malignancy. The availability of large amounts of premalignant tissue and of tumors derived from it permits the simultaneous investigation of different stages of tumor progression within a well defined cell population.

The mammary gland appears to be a candidate target for homeobox gene action. It represents a variety of developmental interactions and pathway decisions as it undergoes its complex developmental cycle of branching morphogenesis, functional differentiation, secretion, and involution, in which master regulatory genes are likely to play a role. The stepwise progression from normal mammary gland to malignancy is more fully documented and is experimentally more accessible than other animal tumors.

In this paper we report expression of *Hox* genes in the mammary gland and show it to be independently regulated among the genes examined and developmental stage specific. We further demonstrate that expression of two *Hox* genes in the mammary gland responds to hormonal secretions from the ovaries and is altered in cancer, suggesting a role for homeogenes in mammary gland development and neoplasia.

MATERIALS AND METHODS

Animals. C57BL/crl and BALB/c mice were used for collection of the inguinal mammary glands in all RNA preparations, with the exception of the HANs and the tumors which were derived from them, which are carried only in BALB/c mice. Virgin mice were chosen randomly from multiple cages to minimize the chances of selecting animals in a particular stage of estrus. Thoracic glands of virgin and ovariectomized C57BL/crl mice and HANs carried in inguinal glands of BALB/c mice were used for whole mount histology. Tumors carried in inguinal glands of BALB/c mice were used for section histology. Stage of estrus was determined by vaginal smears.

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[1] This work was supported by PHS Grant HD 27845.

[2] To whom requests for reprints should be addressed.

[3] The abbreviations used are: HAN, hyperplastic alveolar nodules; poly(A)$^+$ RNA, polyadenylated RNA; PCR, polymerase chain reaction.

Surgery. Ovariectomy was carried out at about 5 weeks of age (animal weight, 16–17 g). 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 necessary blood vessels and the nipple area as described (20).

Transplantation. A small piece of HAN or tumor tissue from a donor animal was cut out and inserted into the cleared fat pad of host mice. Tissue lines were left at least 2 months before the animals were used for tissue harvest or as donors for extending the lines.

RNA Preparation and Northern Hybridization. Inguinal mammary glands were frozen in liquid nitrogen immediately after removal, and total RNA was prepared by the guanidine isothiocynate (4 M)-cesium chloride (5.7 M) method (21). Total RNA from the glands (number and age of animals used for each experiment is given in figure legends) was isolated. In several cases poly(A)$^+$ RNA was purified by oligodeoxythymidylic acid-cellulose chromatography as described (22). Five μg of poly(A)$^+$ enriched RNA or 20 μg of total RNA was electrophoresed in 1.0% agarose containing 2.3 M formaldehyde in 0.2 M morpholinopropanesulphonic acid-50 mM sodium acetate-5.0 mM EDTA, pH 7.0. RNA was transferred to a Nylon transfer membrane (0.45 μm Magna NT; Micron Separation, Inc.) by the established procedure of Maniatis *et al.* (23). Northern hybridizations were carried out under high stringency conditions using ^{32}P random primed labeled (1–10 $\times$ 10^9 cpm/ μg) murine *Hoxa-1* and *Hoxc-6* and human L7 cDNAs. *Hoxa-1* fragment is ~770 base pairs, derived from the 3′-trailer sequence and does not include the polyadenylic acid tail or the homeobox. The *Hoxc-6* fragment is ~270 base pairs, derived from coding sequence 5′ of the homeobox. Washes after hybridizations were in 0.1× saline-sodium phosphate-EDTA-0.1% sodium dodecyl sulfate at 65°C.

PCR Amplification, Cloning of Homeobox Sequences, and Sequence Analysis. RNA was extracted from glands from subadult and pregnant mice as described and used to make cDNA by reverse transcriptase, using oligodeoxythymidylic acid as a primer. cDNA was purified by centrifuging through Ultrafree-MC 30,000 NMWL filter unit (Millipore) and served as a template for PCR amplification using 40 μg/ml of degenerate oligonucleotide primers. The reaction conditions were initial denaturation at 94°C for 5 min, followed by 30 cycles of 95°C for 1 min, 52°C for 1 min, 72°C for 3 min, and a final incubation of 72°C for 20 min. The sequences of the degenerate primers were (International Union of Pure and Applied Chemistry code used): (I/L/V)YPWM, 5′-CGCGGATCCNTNTAYCCNTGGATG-3′; ELEKEF, 5′-CGCGGATCCGARYTNGARAARGARTT-3′; KIWFQN, 5′-CCCAAGCTTRTTYTGRAACCADATYTT-3′.

Similar sequences were used by Mackem and Mahon (24). PCR products were cloned into pGEM1 and sequenced using the dideoxy sequencing method of Sanger *et al.* (25)

Histology. For whole mount preparations, glands were fixed overnight in Tellyesnicky's fixative. They were defatted in three changes of acetone, hydrated through graded alcohols, stained for 2 h with hematoxylin, dehydrated through graded alcohols to xylene, and photographed. For histological sections, glands were treated as described for whole mount preparations, embedded in paraffin, sectioned at 5 μm, and mounted on slides. Sections were deparaffinized through three changes of xylene, hydrated through graded alcohols, stained with hematoxylin and eosin, dehydrated through graded alcohols to xylene, and coverslipped.

RESULTS

Detection of Candidate Homeobox Genes Expressed in the Mouse Mammary Gland. To identify homeobox genes that are expressed during mammary gland development, several degenerate oligonucleotide primers were constructed from highly conserved domains within and just upstream of the homeobox, and used to amplify cDNA from mammary glands of subadult and pregnant animals. The PCR products yielded predominantly DNA fragments of the expected size, which were cloned, and the DNA sequences of several individual clones were determined. Fig. 1 shows the deduced amino acid sequences for representatives of *Hox* genes from the four clusters [*Hoxa-7, Hoxb-6, Hoxc-6,* and *Hoxd-12*; in accordance with nomenclature suggested by Scott (26)]. Two *Hox* genes were chosen for further study, *Hoxc-6* and *Hoxa-1.*

Expression of *Hoxa-1* and *Hoxc-6* Is Independently Regulated and *Hoxc-6* Transcript Levels Are Developmentally Regulated. Total cellular RNA was extracted from mammary glands of mice at the onset of puberty (about 5 weeks after birth), 2 time points during adulthood of virgin animals (8–9 weeks and 16 weeks after birth), early pregnancy (5–8 days postcoitus), late pregnancy (16–18 days postcoitus), and lactation. The RNA was poly(A)$^+$ enriched and Northern blots of these RNAs were hybridized consecutively with *Hoxc-6, Hoxa-1,* and L7 probes. L7 RNA was used as a control for amount of RNA and its integrity. Fig. 2 shows the pattern of expression of the RNAs during mammary development. *Hoxc-6* transcripts (~1.8 kilobases; Fig. 2*A*) were readily detected in glands from pubescent and mature virgin animals. Transcript levels decreased in glands from animals that were in early stages of pregnancy and could not be detected in a later stage of pregnancy or during lactation (the same pattern was seen also in the BALB/c strain; data not shown). During lactation, high levels of transcripts for milk proteins may dilute other mRNAs, as can be seen for the L7 loading control. *Hoxc-6* may therefore be expressed during lactation at higher levels than indicated. *Hoxa-1* was not detected in any of these stages of development, even after prolonged autoradiographic exposure (Fig. 2*B*), nor was it detected in normal developmental stages of the BALB/c strain (data not shown). These different expression patterns indicate that the two *Hox* genes are independently regulated and that *Hoxc-6* transcript levels are developmentally regulated during the gland cycle, declining in pregnancy.

***Hoxc-6* Expression in Glands from Ovariectomized Animals and during the Estrus Cycle.** Mammary gland development is stimulated by the ovarian hormones estrogen and progesterone (27–29). These act directly on the mammary gland (30), probably by regulating growth factors (31), and may also act indirectly through mediation of pituitary secretions (32). In response to ovariectomy, mammary ducts become much smaller in diameter and simpler in pattern of branching (Fig. 3, *C* and *D*). If homeogenes have a significant role in the

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Hoxa-7:
        FYPWMRSSGPDSKRGRQAYTRYQTLELEKEFHFNRYLTRRRRIEIAHALCLTERQIKIWFQN
Hoxb-6:
LYPWMQRMNSCNSSSFGPSGRRGRQTYTRYQTLELEKEFHYNRYLTRRRRMEIAHALCLTERQIKIWFQN
Hoxc-6:
LYPWMQRMNSHSGVGYGADRRRGRQIYSRYQTLELEKEFHFNRYLTRRRRIEIANALCLTERQIKIWFQN
Hoxd-12:
                                ELEKEFLVNEFINRQKRKELSNSLNLSDQQVKIWFQN
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Fig. 1. Deduced amino acid sequences of homeobox domains from the four *Hox* clusters as amplified by PCR from cDNA from mammary glands of mature and pregnant mice. The primer-derived sequences are underlined.

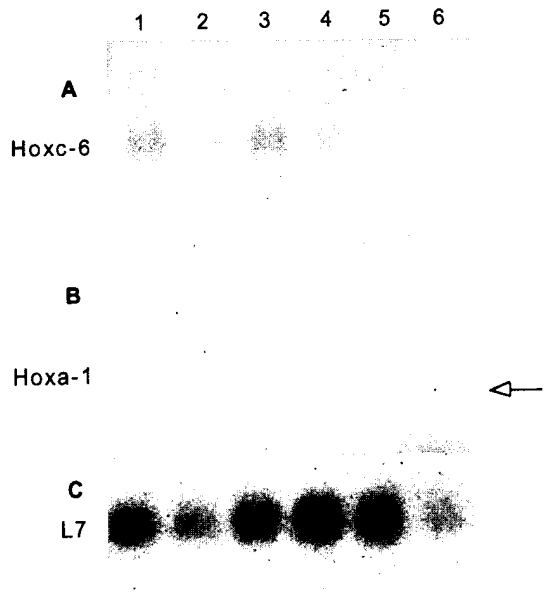

Fig. 2. RNA expression of *Hoxc-6* and *Hoxa-1* during different developmental stages of the mouse mammary gland. Poly(A)$^+$ RNAs were extracted from different stages of the mammary gland development. *Lanes 1–3*, 13–25 virgin mice were taken; *Lanes 4–5*, 5–7 timed-pregnant mice were taken; *Lane 6*, 3 lactating animals were taken. The Northern analysis was performed as described in "Materials and Methods." *Lane 1*, 5-week-old animals; *Lane 2*, 8–9-week-old animals; *Lane 3*, 16-week-old animals; *Lane 4*, 5–8 days pregnant; *Lane 5*, 15–18 days pregnant; *Lane 6*, 3–4 days lactating. Each lane contains 5 μg of poly(A)$^+$ RNA. *A*, *Hoxc-6*; *B*, *Hoxa-1*; *C*, L7 RNA. *Arrow*, ~2.5 kilobases, where *Hoxa-1* transcripts were detected in Fig. 4*B*.

mammary gland development, it is likely that a linkage between them and the mammogenic hormones that are required for growth, morphogenesis, and functional activity would be discovered.

To determine whether *Hoxc-6* transcript levels are regulated by ovarian secretions, we isolated RNA from glands of animals that had been ovariectomized at 5 weeks of age and allowed to mature for 4 weeks after ovariectomy, a time period that was determined adequate for the ovarian steroids to be depleted from the tissues. Fig. 3*A* shows the result of a Northern blot hybridization with a *Hoxc-6* probe, followed by L7 loading control (Fig. 3*B*). In glands from ovariectomized animals (Fig. 3, *Lane 1*) *Hoxc-6* transcript levels are substantially elevated compared to glands from intact virgin mice (Fig. 3, *Lanes 2* and *3*).

We also compared glands from animals that were in diestrus (low steroid) and early estrus (high steroid). *Hoxc-6* appeared to be expressed at slightly higher levels in glands from animals in diestrus (Fig. 3, *Lane 2*) than in glands from animals in early estrus (Fig. 3, *Lane 3*). The difference was small (13–22% as determined by densitometry measurements) but reproducible. *Hoxa-1* transcripts were not detected in glands from ovariectomized animals (not shown). These results suggest that *Hoxc-6* transcripts are down-regulated by ovarian secretions, the candidate hormones being estrogen, progesterone, and possibly prolactin, which is secreted by the pituitary and is regulated by estrogen (33).

***Hoxa-1* and *Hoxc-6* Expression in Preneoplastic and Neoplastic Mammary Glands.** Steroid hormones are implicated in breast cancer (34), and our observed regulation of *Hoxc-6* expression by the ovaries suggested a possible role for these genes in mammary cancer. The availability of precancerous tissues, and of several mammary carcinomas derived from them (Fig. 4, *D* and *E*), made it possible to examine *Hox* expression patterns in two stages of tumor progression.

Total cellular RNA was extracted from HAN lines D1 and D2 (18). We chose three tumors that arose spontaneously from these precancerous tissues. One tumor arose from nodule D1 and was designated D1a. Two tumors arose from nodule D2 and were designated D2a and D2d, respectively. Total cellular RNA was extracted from generation 1 of tumor D1a, generation 3 of tumor D2a, and generation 10 of tumor D2d. RNAs from HAN transplant lines and from their tumors were subjected to Northern blot hybridization analysis using *Hoxc-6*, *Hoxa-1*, and L7 probes, consecutively. *Hoxc-6* showed very low levels of expression in the two HANs and was not detected in any of the tumors, even after prolonged autoradiographic exposure (Fig. 4*A*). In contrast, *Hoxa-1* transcripts (~2.5 kilobases) were present in high levels in all three tumors but were not detected in glands from virgin or pregnant animals or in either of the HANs from which the tumors arose (Fig. 4*B*).

DISCUSSION

This paper describes the expression of several *Hox* gene members in the mouse mammary gland; it is likely that other *Hox* genes, as well as homeogenes outside the *Hox* clusters, are transcribed in one or more of the mammary developmental stages. In this initial report,

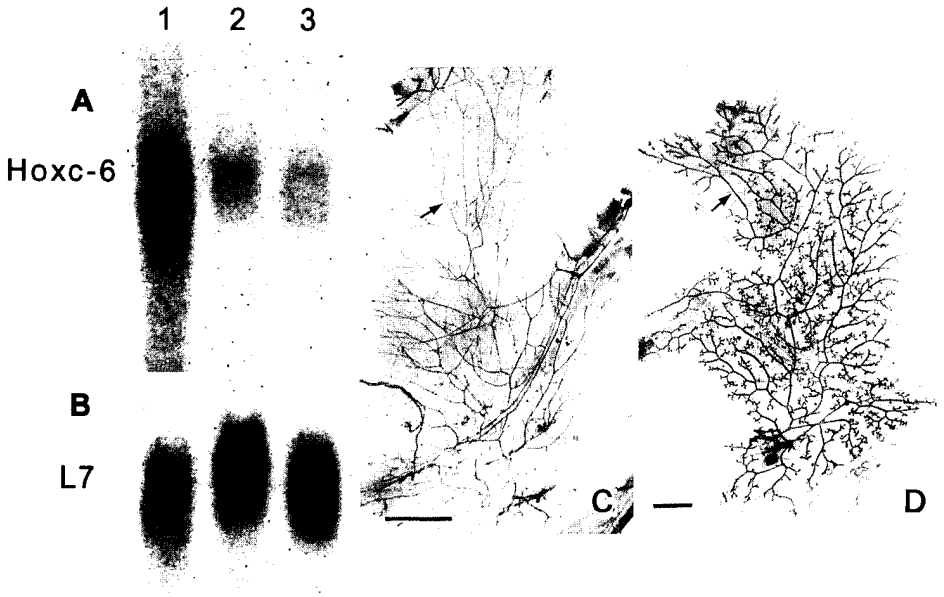

Fig. 3. RNA expression of *Hoxc-6* in the mammary gland in response to different levels of steroids. Northern analysis was performed as described in "Materials and Methods." *Lane 1*, mammary gland RNA from ovariectomized mice. Sixteen mice were ovariectomized at 5 week of age and allowed to mature to 9 weeks prior to glands collection. *Lanes 2–3*, the stage of estrus of 5-week-old mice was determined by vaginal smears; *Lane 2*, RNA from glands of 3 animals in diestrus (low steroid); *Lane 3*, RNA from glands of 6 animals in early estrus (high steroid). Each lane contains 20 μg of total RNA. *A*, *Hoxc-6*; *B*, L7; *C* and *D*, whole mount preparations of glands from an ovariectomized C57/Bl mouse (*C*) and an intact mature virgin C57/Bl mouse (*D*) *Arrows*, epithelial duct showing a decrease in ductal diameter and a simplification of ductal patterning in response to ovariectomy. *Bar*, 1 mm.

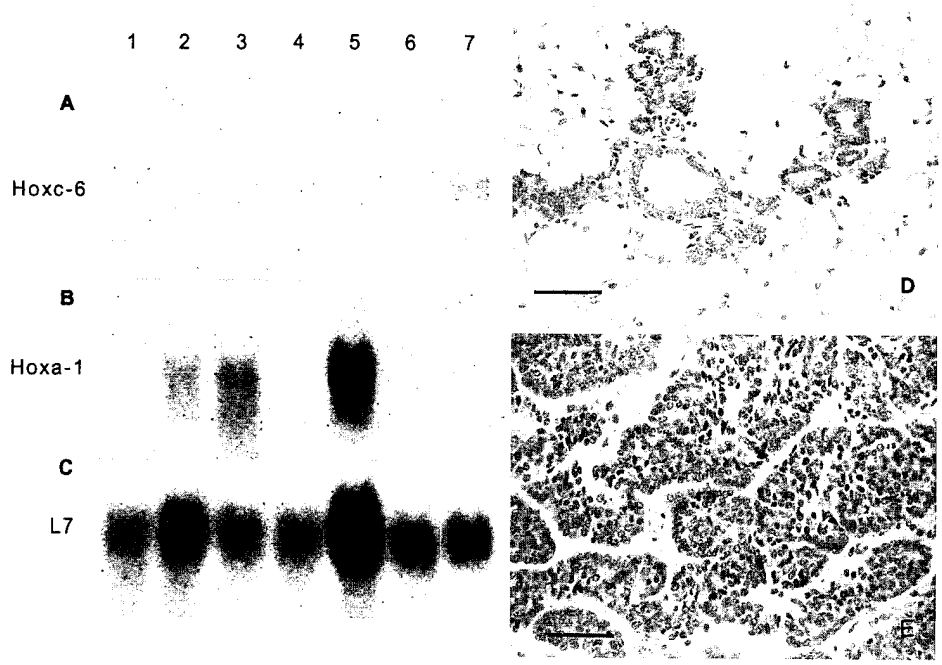

Fig. 4. *Hoxa-1* and *Hoxc-6* expression in preneoplastic and neoplastic mammary outgrowths. RNA was isolated from hyperplastic alveolar nodules (18) that were serially transplanted in BALB/c gland-free fat pads and from spontaneous carcinomas that arose from these nodules and were subsequently transplanted. *Lane 1*, HAN D2; *Lane 2*, tumor D2a; *Lane 3*, tumor D2d; *Lane 4*, HAN D1. *Lane 5*, tumor D1a; *Lane 6*, mammary gland RNA from 15–18 days pregnant animals; *Lane 7*, mammary gland RNA from mature virgin animals. Northern analysis was performed as described in "Materials and Methods." Each lane contains 20 μg of total RNA. (A) *Hoxc-6*. (*B*) *Hoxa-1*. (*C*) L7. (*D*) 5-μm section of HAN D1. (*E*) 5-μm section of tumor D1a. Like the C57/Bl strain, expression of *Hoxa-1* could not be detected in normal developmental stages of the BALB/c gland (data not shown). *Bars*, 45 μm.

rather than carry out a large scale survey of the 38 known *Hox* genes, we chose to examine two genes from different clusters, *Hoxa-1* and *Hoxc-6*, in sufficient depth to determine whether their expression patterns might suggest a functional role in mammary development or neoplasia. The criteria we chose were expression patterns that were: (*a*) regulated by developmental stage; (*b*) responsive to mammogenic hormones, or (*c*) altered in preneoplastic or neoplastic tissues.

Hoxa-1 is not expressed in the normal gland, while *Hoxc-6* is expressed during mammary development. Expression of *Hoxc-6* is related to the developmental stage of the gland, with declining transcript levels observed in response to pregnancy. The data show that homeogene expression is not simply uniform or constitutive. Although homeobox gene expression has not been reported previously in the mammary gland, *Wnt* proto-oncogene expression in the mammary gland has been documented (35). The *Drosophila* homologue of *Wnt-1* is the segment polarity gene *wingless*, which is known to regulate the homeobox-containing gene *engrailed* (36).

The mammary gland is an endocrine target organ of considerable complexity, in which hormones of reproduction drive the gland through its cycles of growth, secretory differentiation, lactation, and postweaning involution. Primary among these hormones are the ovarian steroids. Genes regulating tissue-specific responses to these hormones must be downstream of the primary steroid response elements, and it is reasonable to suppose that if *Hox* genes play a role in the mammary gland, their activity will be linked to this primary endocrine regulation. This was tested by examining glands from ovariectomized animals and glands from animals at different time points during the estrus cycle. *Hoxc-6* expression was greatly increased following ovariectomy. Transcript levels appeared to be slightly reduced during early estrus, a small but reproducible effect that supports the ovariectomy results by indicating an inverse relationship between ovarian steroids and *Hoxc-6* transcript levels.

At least two essential alterations occur during the development of mammary cancers in mice, the acquisition of immortality at the preneoplastic (HAN) stage, and the acquisition of cellular autonomy at the neoplastic stage (18). Like normal cells, preneoplastic HANs are growth-inhibited by the presence of nearby normal mammary epithelium and remain as discrete nodules. They are also unable to grow normally in ectopic sites, requiring mammary adipose tissue. Tumor cells, on the other hand, are able to overgrow normal mammary tissues and have escaped growth dependence on mammary stroma. Our results show that with respect to the expression of *Hoxc-6* and *Hoxa-1*, the preneoplastic stage of mammary tumor progression is similar to the normal mammary gland, whereas tumors derived from these tissues show elevated expression of *Hoxa-1* but not *Hoxc-6*.

Hox gene expression, in general, affects cell growth, differentiation, and fate (37). In vertebrate embryos, *Hox* genes are associated with maintenance of the proliferative state in the developing limb, while overexpression in cultured myoblasts induced the transformed phenotype (10). It has been shown that overexpression of *Hoxa-1* in NIH-3T3 or 208-F cells leads to transformation, enabling the cells to grow and form foci in soft agar (11). On injection of cells overexpressing *Hoxa-1* cells into athymic mice, the mice developed tumors as early as 12 days postinjection. More examples showing that deregulated homeogene expression leads to aberrant cell proliferation are recently available (38, 7). These and other correlative studies make the case that certain homeobox genes may constitute a new family of oncogenes (11). Our results are in accord with these data and extend the possible roles of homeobox genes to regulation of the mammary gland in the subadult and mature animal.

The perturbation of cell fate-determining switches may reasonably be expected to play a role in the acquisition of cell autonomy in the progression of cells to malignancy. Many of the known oncogenes are thought to alter early stages in the malignant progression, affecting the cell cycle or participating in the immortalization process. Our data show misexpression of *Hoxa-1* in malignant but not benign immortalized precancerous tissues, suggesting that *Hoxa-1* and perhaps other homeobox genes may play a role in the latter stage of tumor progression as cells acquire the ability to invade ectopic sites and establish dispersed metastatic colonies.

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EXPRESSION OF *HOX* GENES IN NORMAL AND NEOPLASTIC MOUSE MAMMARY GLAND

Yael Friedmann and Charles W. Daniel

Department of Biology,
Sinsheimer Laboratories,
University of California,
Santa Cruz, CA 95064, U.S.A.

INTRODUCTION

The striking phenomenon of homeosis, in which one body part is substituted for another, was described and named in *Drosophila* more than a century ago (Bateson, 1894). Homeobox genes have been intensively studied, particularly in recent years, as advances in understanding of the molecular genetic basis for homeosis captured the interest and imagination of developmental biologists. It is now clear that homeotic genes function as master regulators, determining the developmental pathway and cell fate of individual *Drosophila* segments.

Homeobox genes (homeogenes) are highly conserved and homologues are found throughout the animal kingdom (Bieberich *et al.*, 1991; Murtha *et al.*, 1991; Pendleton *et al.*, 1993). They comprise a large collection of developmental regulators whose common structural characteristic is a box motif of about 183 bases, coding for the 61 amino acid homeodomain that is the helix-turn-helix DNA binding region of the functional homeoprotein (Scott *et al.*, 1989). It is generally accepted that the homeoprotein functions as a transcription factor, regulating the expression of downstream genes (Andrew and Scott, 1992). *Drosophila* genes with homeotic potential are included in the Antennapedia and Bithorax complexes.

The closely related murine homologues, the *Hox*-C genes, occur in four clusters that, like their Dipteran homologues, are arranged along their respective chromosomes in a manner that is generally co-linear with their expression sequence (reviewed by Akam, 1989; Kessel and Gruss, 1990; McGinnis and Krumlauf, 1992). Most of the mammalian homeobox genes, including the 38 *Hox*-C in the mouse and human, were discovered by cross hybridization and only later, and with considerable difficulty, have the phenotype of a few of them been determined (McGinnis and Krumlauf, 1992). Because of the shortage

of developmental mutations in mammals, information on homeobox gene function has come from reverse genetics - either misexpression through the insertion of homeobox transgenes, or generation of the null phenotype through targeted gene disruption of the mouse germ line (reviewed by McGinnis and Krumlauf, 1992). Transgenic mice in which *Hox*-1.1 (Balling *et al.*, 1989) and *Hox*-4 (Wolgemuth *et al.*, 1989) were overexpressed, for example, were dominant lethals showing severe developmental defects. Misexpression of *Hox* 4.6 in the chick limb bud led to pattern alterations and apparent homeotic transformation of the digit (Morgan *et al.*, 1992).

Why might expression of homeobox genes be expected in the mammary gland, which is not considered a segmental structure? One argument is that the mammary gland is embryonic-like during its phase of ductal development following menarche, even though development occurs in the subadult animal (Sakakura *et al.*, 1979). In addition, there are numerous examples of homeobox gene expression in tissue development that are not considered segmental in nature (review by Sassoon, 1992). Particularly striking examples are the role of the *Hoxd* cluster in limb morphogenesis (Tabin, 1991; Morgan *et al.*, 1992; Tabin, 1992), and in the differentiation of haematopoietic cell lineages, in which many *Hox* genes are switched on or off in blocks, indicating coordinate regulation. Distinct patterns of *Hox* gene activation are found in different lineages, suggesting that these genes may play an important role in cell lineage determination during both normal and leukemic haematopoiesis (Magli *et al.*, 1991; Celetti *et al.*, 1993).

Thus, the mammary gland appears to be a candidate target for homeobox gene action. It represents a variety of developmental interactions and pathway decisions as it undergoes its complex developmental cycle of branching morphogenesis, functional differentiation, secretion, and involution, in which master regulatory genes are likely to play a role. In this chapter we report the expression of several *Hox* genes in the mouse mammary gland, and describe in more detail the activity of members of the *Hoxd* cluster.

RESULTS

Detection of candidate homeobox genes expressed in the mouse mammary gland

cDNA was made from mouse mammary gland RNA and was used to amplify homeoboxes using degenerate oligonucleotide primers from conserved domains within the homeobox. The PCR products were cloned, and the DNA sequences of several individual clones were determined. We found expression of genes from all four major clusters and chose the *Hoxd* cluster for further investigation.

Different *Hoxd* genes are independently regulated and regulation is related to developmental stage

Total cellular RNA was extracted at the onset of puberty (about five weeks after birth), two time points during adulthood of virgin animals (8-9 weeks after birth and 16 weeks after birth), early pregnancy (5-8 days post coitus), late pregnancy (16-18 days p.c.) and lactation. The RNA was poly A^+ enriched and northern blots of these RNAs were hybridized consecutively with *Hoxd*-3, *Hoxd*-4, *Hoxd*-8, *Hoxd*-9, *Hoxd*-10, *Hoxd*-11, *Hoxd*-12 and L7 probes. L7 RNA was used as a control for amount of RNA and its integrity. Figure 1 shows the pattern of expression of the RNAs of representatives of the *Hoxd* genes during mammary development. *Hoxd*-9 and *Hoxd*-4 transcripts were readily detected in glands from pubescent and mature virgins and animals in early stages of

pregnancy. Transcript levels decreased in glands from animals that were in late stages of pregnancy and could hardly be detected during lactation. During lactation, high levels of transcripts for milk proteins may dilute other mRNAs, as can be seen for the L7 loading control. *Hoxd*-9 and *Hoxd*-4 may therefore be expressed during lactation at higher levels than indicated. *Hoxd*-12 was not detected in any of these stages of development. Table 1 summarizes the mRNA expression pattern of all the *Hoxd* genes that were examined. *Hoxd*-8 and *Hoxd*-10 pattern of expression in the normal gland was very similar to the one seen for *Hoxd*-9 and *Hoxd*-4, while *Hoxd*-3 and *Hoxd*-11 were not expressed in any of the normal stages of the mammary gland development (similar to *Hoxd*-12). These different

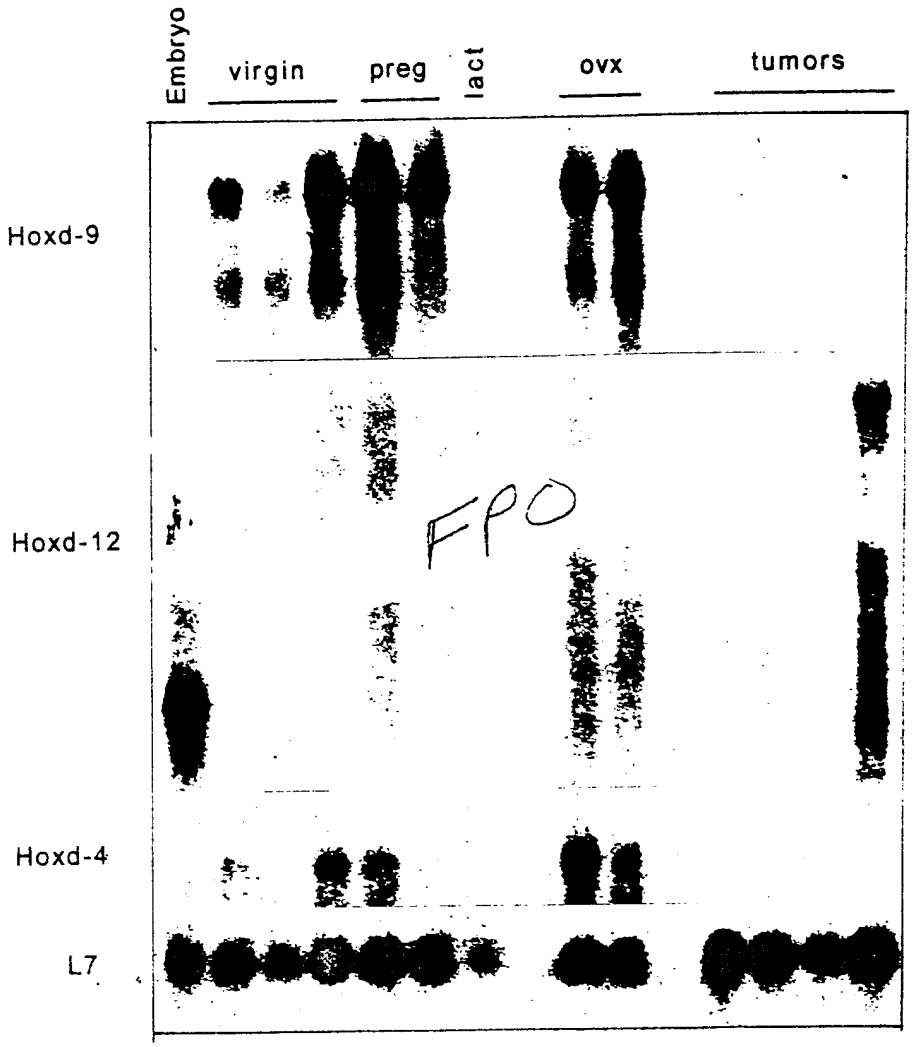

Figure 1. RNA expression of *Hoxd*-9, *Hoxd*-12 and *Hoxd*-4 at different developmental stages of the mouse mammary gland, in response to ovariectomy and in mammary tumors. Poly A^+ RNAs were extracted from different stages of mammary gland development, from glands of animals that were ovariectomized and from transformed mammary glands. RNA was subjected to northern blot analysis with random primed, ^{32}P-labeled cDNA probes for *Hoxd*-9, *Hoxd*-12 and *Hoxd*-4. None of the clones contains the homeobox sequence itself. A human ribosomal L7 probe was used as a control for the amount and integrity of the RNA. Each lane contains 5 μg poly A^+ enriched RNA. Lanes from left to right: 14 days old embryo, glands from five weeks old animals, glands from 8-9 weeks old animals, glands from 16 weeks old animals, glands from 5-8 days pregnant animals, glands from 15-18 days pregnant animals, glands from lactating animals, glands from animals that were ovariectomized at 5 weeks and allowed to mature for 4 more weeks, glands from animals that were ovariectomized at 12 weeks and allowed to mature for 4 more weeks, tumor D2a, tumor D2d, tumor D1a, ^{35}S Myc tumor.

expression patterns indicate that the *Hoxd* genes are independently regulated in the normal gland and that *Hoxd*-4, *Hoxd*-8, *Hoxd*-9 and *Hoxd*-10 transcript levels are somewhat developmentally regulated during the gland cycle.

Hoxd expression in glands from ovariectomized animals

Mammary gland development is stimulated by the ovarian hormones estrogen and progesterone (Topper and Freeman, 1980; Haslam, 1987; Silberstein *et al.*; 1994). These act directly on the mammary gland (Daniel *et al.*, 1987), probably by regulating growth factors (Dembinski and Shiu, 1987), and may also act indirectly, through mediation of pituitary secretions (Vonderhaar, 1987). The mammary ducts become much smaller in diameter and simpler in pattern of branching in response to ovariectomy, as can be seen in Figure 2 in a whole-mount preparation.

If homeogenes have a significant role in mammary gland development, it is likely that a linkage between them and the mammogenic hormones that are required for growth, morphogenesis, and functional activity would be discovered. To determine if *Hoxd* transcript levels are regulated by ovarian secretions, we isolated RNA from glands of animals that had been ovariectomized at 5 or 12 weeks of age and allowed to mature for 4 weeks after surgery, a time period that was determined adequate for the ovarian steroids to be depleted from the tissues. Figure 1 shows the result of representative northern blot hybridizations with *Hoxd*-9, *Hoxd*-4 and *Hoxd*-12 probes, followed by L7 as a loading control.

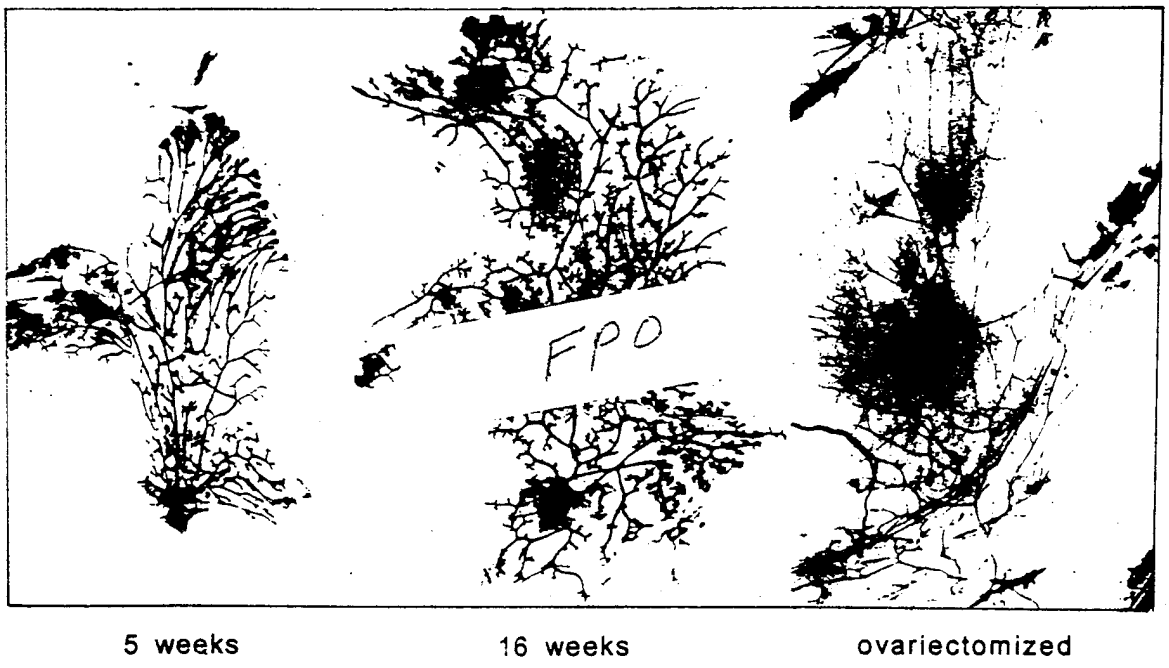

Figure 2. Whole mount preparations of glands from 5 weeks old animals, 16 weeks old animals and a gland from a mouse that was ovariectomized at 5 weeks of age and allowed to mature for 4 more weeks. Arrow points to an epithelial duct, showing a decrease in ductal diameter and a simplification of ductal patterning in response to ovariectomy. All pictures were taken at the same magnification.

Genes except for *Hoxd*-4 show the same level of expression in glands from ovariectomized animals as their intact counterparts (glands from subadult and mature virgin animals). *Hoxd*-4, on the other hand, shows a higher level of expression in glands from animals that

	5 weeks	8-9 weeks	16 weeks	early pregnant	late pregnant	lactating[3]	ovariectomized at 5 weeks	ovariectomized at 12 weeks	tumor D2a	tumor D2d	tumor D1a	Myc tumor
Hoxd-3	-	-	-	-	-	-	-	-	-	-	+	-
Hoxd-4	++	++	++	++	+	-	++++	++	-	-	-	-
Hoxd-8	+++	+++	+++	+++	+	+	+++	+++	-	-	-	-
Hoxd-9	++	++	++	+++	+	+	++	++	-	-	-	-
Hoxd-10	+	+	++	+++	++	++	++	++	-	-	-	-
Hoxd-11	-	-	-	-	-	-	-	-	-	-	-	-
Hoxd-12	-	-	-	-	-	-	-	-	-	-	-	+++

1. Levels are compared for each gene separately and cannot be compared between different genes.
2. Levels are adjusted according to L7 loading control.
3. During lactation, high levels of transcripts for milk proteins may dilute other mRNAs, (as can be seen for L7 loading control in figure 1). Hox expression may therefore be expressed during lactation at higher levels than indicated.

were ovariectomized at 5 weeks of age and allowed to mature for 4 more weeks than in any normal stage of development. Glands of animals that were ovariectomized when the animal was cycling (12 weeks old when ovariectomized and 16 weeks old when glands were taken) do not show the same effect.

These results suggest that *Hoxd*-4 transcripts are down-regulated by ovarian secretions, at least during some stages of the mammary gland cycle; the candidate hormones are estrogen, progesterone and possibly prolactin, which is estrogen-regulated (Lyons, 1958).

Hoxd expression in neoplastic mammary glands

Total cellular RNA was extracted from three tumors that arose spontaneously from hyperplastic alveolar nodule lines (Medina, 1973) and from a tumor induced by misexpression of the *myc* proto-oncogene in cultured mammary epithelium, followed by transplantation into mammary gland-free fat pads (Bradbury *et al.*, 1991). RNA was poly A^+ enriched and was subjected to a northern blot hybridization analysis using the previously described *Hoxd* probes. As can be seen from Figure 1 and Table 1, two *Hoxd* genes (*Hoxd*-3 and *Hoxd*-12) were expressed in one of the mammary tumors. These two genes were not expressed in any of the normal developmental stages of the mammary gland. *Hoxd*-12 transcript size in the tumor ($\sim$8.4 kb) is different from its size in the embryo ($\sim$2.7 kb). *Hoxd*-4, *d*-8, *d*-9 and *d*-10 which showed expression during the normal development of the mammary gland, were not expressed in any of the mammary tumors that were tested.

DISCUSSION

Our initial exploratory studies were aimed at determining whether homeobox genes were expressed in the mammary gland in enough numbers and enough abundance to warrant

further investigation. We amplified homeobox sequences from mammary gland cDNA that, after sequence analysis, was found to contain members from each of the four *Hox* clusters. In order to obtain semi-quantitative data we used sequence-specific probes to non-conserved regions 5' to the homeobox. In most cases we were able to obtain a clearly identifiable band on northern blots without enriching for poly A^+ (not shown), indicating a reasonably abundant level of transcripts.

In this initial investigation, rather than carry out a large scale survey of the 38 known *Hox* genes and numerous other non-clustered homeobox genes, we chose to examine in more detail the expression of members of the *Hoxd* group. This cluster is of particular interest because of its role in patterning of the vertebrate limb. Beginning with a nondescript cluster of undifferentiated cells, the developing chick limb displays progressively increasing cellular diversity. Using a combination of classical transplantation technology, activation of *Hox* genes by retinoic acid, and molecular techniques, the role of *Hoxa* and *Hoxd* clusters in limb morphogenesis has been elegantly documented (Tabin, 1991; Morgan *et al.*, 1992; Tabin, 1992). Thus, *Hoxd* well illustrates an extensive role for homeobox genes in vertebrate development.

In the normal mammary gland, four out of seven members of the *Hoxd* cluster examined displayed some degree of expression. Differences between individual genes (*Hoxd*-4 vs. *Hoxd*-11, for example) indicate differential regulation. Declining transcript levels in late pregnancy observed in *Hoxd*-4, *Hoxd*-8, *Hoxd*-9 and *Hoxd*-10 indicate a degree of developmental regulation. Steady-state mRNA was also reduced during lactation, but dilution by milk protein mRNA makes this conclusion tentative. In any case, the data show that homeogene expression is not simply uniform or constitutive, and in some cases is related to mammary stage.

Two of the three *Hoxd* genes that were not expressed in the normal gland showed expression in mammary tumors, indicating an association between the altered expression of *Hoxd* genes and mammary cancer. Such a relationship with other tumors and homeobox genes has been documented. In human leukemia, altered regulation of developmental homeobox control has been convincingly demonstrated (Celetti *et al.*, 1993). Also, other cancers display altered expression of homeobox genes (Kennedy *et al.*, 1991; Celetti *et al.*, 1993; De Vita *et al.*, 1993), and misexpression of homeobox genes can transform cells *in vitro* and produce tumors in mice upon transplantation (Perkins *et al.*, 1990; Song *et al.*, 1992).

Hormones of reproduction drive the mammary gland through its cycles of growth, secretory differentiation, lactation, and post-weaning involution. Primary among these hormones are the ovarian steroids, for which receptors exist in both the mammary epithelium and its contiguous stroma (Haslam and Shyamala, 1981; Daniel *et al.*, 1987), and a direct mammogenic role for estradiol has been demonstrated (Silberstein *et al.*, 1994; Haslam, 1988). Genes regulating tissue-specific responses to these hormones should be downstream of the primary steroid response elements, and it is reasonable to suppose that if *Hox* genes play a role in the mammary gland, their expression will be linked to this primary endocrine regulation.

We tested this proposition by ovariectomizing animals and comparing their levels of *Hox* expression with endocrine-intact controls. Expression of *Hoxd*-4 was substantially increased in mice ovariectomized at five weeks, but not at 12 weeks. This suggests that *Hoxd*-4 transcripts may be down-regulated by ovarian steroids in certain stages of development. The age difference in response may indicate that *Hoxd*-4 is regulated by ovarian steroids during the period of active ductal growth but not in the adult, where the gland is relatively quiescent. A small increase in expression during early pregnancy is consistent with this interpretation.

The functional role of homeobox genes in the mammary gland, if any, is of course unknown at this point, but several possibilities come to mind that are consistent with the observed phenotype of these genes in systems that are more amenable to genetic analysis. It is expected that the expression of many homeobox genes will occur in the mammary stroma. Homeobox action in these mesodermal tissues could be reflected in altered production or turnover of mammary-associated matrix components, or in the differentiation of mesenchyme-like cells into fibroblasts. Homeobox genes may also regulate the fate of epithelial cells in the end bud as cells are channeled into various differentiated populations in the subtending ducts, a possibility consistent with the known action of homeotic genes in determining cell fate. A particularly intriguing possibility is that homeobox genes might influence expression of cell-cell or cell-matrix adhesion molecules, thereby directly regulating morphogenesis and pattern formation.

Our results indicate that several *Hox* genes are expressed in the mouse mammary gland. It seems likely that many homeobox genes, both within and outside of the major clusters, will be found to be expressed at some level in one or more stages of the mammary cycle. Working out the functional implications of this gene activity will be a challenging but potentially fruitful task.

SUMMARY

There has been no report of expression of homeobox-containing genes in the breast. We amplified homeobox cDNA from mouse mammary gland and found expression of members from each of the four major *Hox* gene clusters. Northern analysis showed expression of these at significant levels. In order to determine if expression was restricted to only a few representatives, a survey was carried out on the *Hoxd* cluster using sequence-specific cloned probes for northern analysis. Expression in the normal mammary gland was seen in four out of the seven members of the *Hoxd* genes examined; highest levels were found in glands from immature and mature virgin animals and in glands of early pregnant animals, with decreasing transcripts detected during later stages of pregnancy and lactation. Three *Hoxd* genes were not expressed in the normal gland, indicating that the individual genes are independently regulated. In order to determine if expression is linked to mammogenic hormones, *Hox* expression was studied in ovariectomized animals. Most of the *Hoxd* genes were not significantly modulated by reduction in ovarian steroids, but *Hoxd*-4 expression was increased significantly in glands from animals that were ovariectomized at a young age, indicating possible negative regulation by steroid hormones. Looking at mammary tumors, we found two *Hoxd* genes to be expressed, each in a different tumor. Our findings indicate that several but not all *Hoxd* genes are expressed in the normal gland and some are expressed in mammary tumors and not in the normal gland. Expression is differentially regulated and is related to developmental stage. A relationship between mammogenic steroid hormones and *Hox* expression suggests that these genes may play a role in mammary development or function.

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Expression and Functional Role of E- and P-Cadherins in Mouse Mammary Ductal Morphogenesis and Growth

CHARLES W. DANIEL, PHYLLIS STRICKLAND, AND YAEL FRIEDMANN

Department of Biology, Sinsheimer Laboratories, University of California, Santa Cruz, California 95064

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Mammary ducts, and the highly mitotic terminal end buds from which they are derived, consist of two layers of ectodermally derived epithelium, forming a tube-within-a-tube structure. We investigated the role of Ca^{2+}-dependent cell–cell adhesion molecules in maintaining the integrity of these layers. Immunostaining showed abundant E-cadherin on the lateral membranes of end bud body cells and ductal lumenal cells, but no P-cadherin. The basally located cap cells and their differentiated descendants, the ductal myoepithelial cells, displayed only P-cadherin. We investigated the functional significance of this pattern of cadherin expression *in situ* by surgically implanting small, slow-release plastic implants releasing function-blocking antibodies. End buds exposed to a monoclonal antibody to E-cadherin showed disruption of the body epithelium, with epithelial cells floating freely in the lumen. Epithelial DNA synthesis, which is normally very high in these growth buds, abruptly declined. That this reduction in growth was not due to cell damage was shown by spontaneous reaggregation of the cells into a normal epithelium, with resumption of DNA synthesis, when the blocking antibody was depleted. A monoclonal antibody to P-cadherin had no effect on the lumenal layer but partially disrupted the basally located cap cell layer. These data indicate that spatially selective expression of E- and P-cadherins is required for mammary tissue integrity, which is in turn a prerequisite for normal rates of DNA synthesis.

INTRODUCTION

The mammary gland differs from most other organs in that growth and patterning occur in the juvenile, and in rodents the gland is readily accessible for observation and experiment. Even though development of the mammary ductal tree takes place in the sub-adult, it is embryonic-like with respect to active branching morphogenesis and the continuing inductive interactions between the epithelium and its contiguous stroma (Sakakura *et al.*, 1979; Cunha *et al.*, 1992). Mammary end buds, which represent the growth points for ductal morphogenesis, are of special interest because by their turning, branching, and alterations in growth rate, patterning of the ductal tree is achieved. The end bud is the structure that interprets morphogenetic signals from surrounding stroma. Within the end buds, epithelial cells are channeled into spatially segregated subpopulations of the ducts, where they differentiate and become mitotically inactive. Because of this apical growth habit, a longitudinal section through a terminal end bud and its duct provides both a spatial and a temporal picture of histogenesis, in which the rapidly dividing and unspecialized cap cells of the end bud differentiate into myoepithelium of the subtending ducts. Body cells of the end bud are a distinct, multilayered epithelium that becomes the single-layered lumenal tissue of the mammary duct.

The cadherin multigene family of Ca^{2+}-dependent cell–cell adhesion molecules is generally regarded as having a fundamental morphoregulatory role in the embryo and as participating in stabilization of more differentiated tissues (Geiger and Ayalon, 1992). Cadherins undergo spatiotemporal changes in expression during embryonic development, and E- and P-cadherins, in association with cell–substratum adhesion factors, are particularly implicated in the patterning of epithelial sheets and tubes (Takeichi, 1988). We studied the expression and functional activity of cadherins in mammary end buds and their subtending ducts to determine if these calcium-dependent adhesion systems are responsible, at least in part, for the sharp separation between basal and lumenal cell populations, which is a conspicuous feature of mammary ductal morphogenesis.

MATERIALS AND METHODS

Animals and Tissues

The thoracic No. 2 and No. 3 mammary glands of $5\frac{1}{2}$-week-old female C57/B1 mice were used in all experiments.

Immunolocalization

Antibodies ECCD-1 for blocking mouse E-cadherin, ECCD-2 for immunostaining mouse E-cadherin, and

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PCD-1 for both blocking and immunostaining mouse P-cadherin were a gift from M. Takeichi. Mammary glands were removed from anesthetized mice at the indicated times. Flattened glands were quick frozen on dry ice and stored at −80°C. Cryosections (5 μm) were thaw-mounted on silanized slides, postfixed in 1:1 acetone/methanol at −20°C for 10 min, air dried, and stored at −20°C. Slides were defrosted, washed in PBS, and successively treated with 0.2% glycine in PBS 2×5 min, H_2O_2/methanol for 30 min, and 5% milk in PBS for 30 min, with PBS washes between each step. Antibody ECCD-2 diluted 1:500 and PCD-1 diluted 1:20 in milk/PBS were applied to slides that were then coverslipped, sealed with rubber cement, and incubated at room temperature overnight. The slides were then washed in PBS/1% goat serum and the biotinylated goat anti-rat secondary antibody (Amersham) diluted 1:200 was applied for 1 hr. Slides were washed in PBS, and Vectastain ABC reagent (Vector Labs), regular or elite, respectively, was applied for the time specified, followed by washing in PBS. The peroxidase substrate was applied for 10 min and sections were lightly counterstained with hematoxylin, dehydrated, and coverslipped.

For whole-gland immunostaining we employed a modified version of the procedures used on embryos (Dent *et al.*, 1989; Le Motte *et al.*, 1989). Glands were removed, flattened onto strips of stiff paper, and fixed overnight in 4:1 methanol:DMSO at 4°C. Tissue was bleached for 5 hr in 4:1:1 methanol:DMSO:30% H_2O_2 at room temperature and stored at −20°C in 100% methanol from overnight to several weeks. Tissue was hydrated to PBS through a graded series of methanols, washed in two 1-hr changes of PBSMT (PBS, 5% milk, 0.1% Triton X-100), and incubated at 4°C overnight in primary antibody diluted in PBSMT; 1/100 ECCD2 and 1/20 PCD. Tissue was washed in PBSMT 2× for 1 hr at 4°C, and 3× for 1 hr at room temperature followed by secondary antibody HRP (horseradish peroxidase) goat anti-rat (Chemicon) diluted 1/300 in PBSMT and incubated overnight at 4°C. Tissue was incubated in primary antibody for 20 min in PBT (0.01% Triton X-100 in PBS) followed by 10 min in substrate without H_2O_2 and 10–15 min in substrate with H_2O_2. The reaction was stopped with a 5-min wash in PBT and tissue was dehydrated through a graded series of ethanols, cleared in xylene, and stored in methyl salicylate for examination. Some samples were subsequently embedded in paraffin and sectioned at 6 μm to determine antibody localization more precisely.

Antibody Neutralization of Cadherins in Vivo

Blocking antibodies were lyophilized and incorporated into EVAc (DuPont) plastic pellets as described, using bovine serum albumen as carrier (Silberstein and Daniel, 1982). Implants containing 200–300 μg of lyphotate were inserted into fat pads in front of the advancing end buds and left for 12, 24, 30, 48, and 72 hr. To monitor the cells engaged in DNA synthesis, animals were injected with 100 μci [^{3}H]thymidine (78 Curies/m*M*) or Brdu (5′-bromo-2′-deoxyuridine) (0.01 cc/g body wt of 25 μg/ml PBS) 1 hr before glands were taken and fixed in Tellysesniczkys fixative (10% formalin, 5% acetic acid, 70% EtOH). Glands were processed for whole-mount examination followed by paraffin embedding and sectioning at 5 μm. Effects on histoarchitecture and DNA synthesis were evaluated relative to contralateral control glands that had been implanted with equivalent amounts of rat IgG or nonblocking antibody to E-cadherin, ECCD-2.

RESULTS

Spatial Expression of E- and P-Cadherins in Ductal Morphogenesis

Mammary glands were immunostained and examined for E- and P-cadherins both in whole-mount preparations and in histological sections. Figure 1a shows a mammary ductal tree terminating in highly mitotic end buds that are actively penetrating the surrounding adipose stroma. When whole glands were immunostained with ECCD-2 to E-cadherin, the multilayered mass of epithelial body cells was deeply stained, whereas the basal layer of cap cells was not (Fig. 1b). In histological sections E-cadherin was conspicuously displayed on cell membranes of body cells (Fig. 2a and 2c). These multilayered body cells displayed E-cadherin on all cell surfaces except the membranes directly exposed to the lumen (Figs. 2c and 2e). Cap cells and their descendants, the myoepithelial layer of the subtending mammary ducts, did not display detectable E-cadherin.

P-cadherin, in contrast, was localized in the basally located cap cells of end buds (Figs. 1c, 2b, and 2d) and in small clusters of cells within the lumenal compartment (Fig. 2d). These may represent groups of cap cells that have detached from the basal lamina and migrated into the interior, as reported using microcinematography (Williams and Daniel, 1983). It is postulated that these detached patches of cap cells are visualized because they temporarily retain membrane-associated P-cadherin.

In differentiated mammary ducts the same sharp distinction between the distribution of E- and P-cadherins was observed. In lumenal tissue, which in these ducts is usually one or two cells in thickness, staining for membrane E-cadherin was more intense than in the end buds (Fig. 2e). Membrane surfaces facing the lumen were again unstained, as were the myoepithelial cells. The basally located, longitudinally aligned myoepithelial

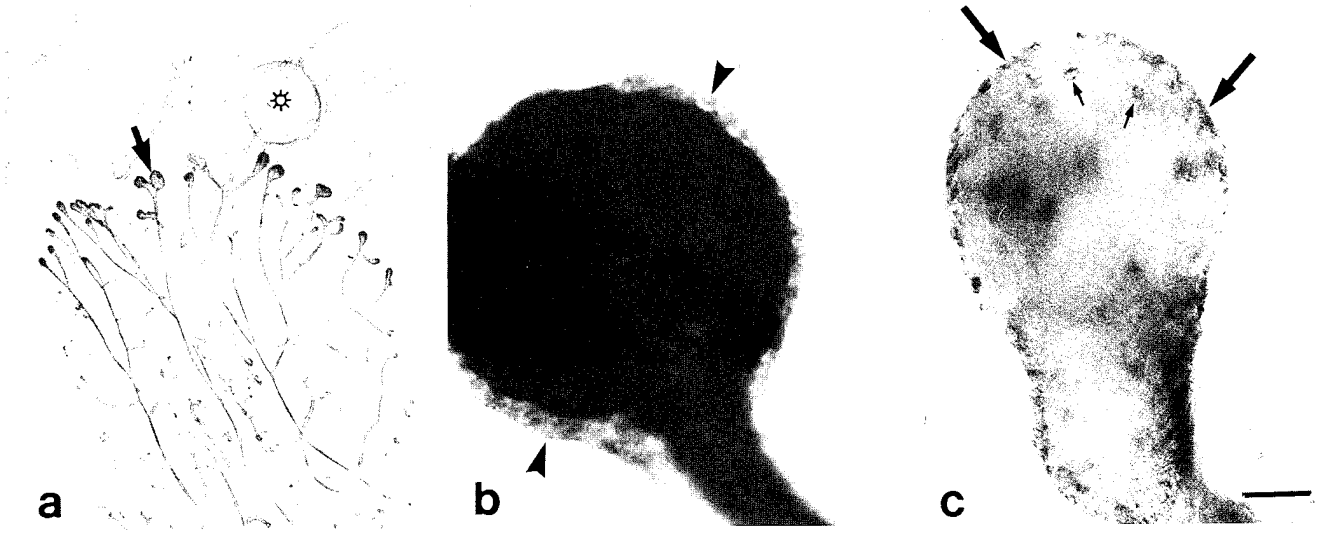

FIG. 1. Whole-mount preparations of a No. 3 thoracic mammary gland taken from 5-week-old virgin mice. (a) Hematoxylin-stained gland showing a branching system of mammary ducts terminating in enlarged end buds (arrow). An EVAc pellet has been implanted into the fatty stroma ahead of the advancing end buds (*). (b) An end bud and a portion of its subtending duct immunostained for E-cadherin with antibody ECCD-2. The multilayered body epithelium is darkly stained, obscuring the end bud lumen. The basal layer of cap cells (arrowheads) is unstained. (c) An end bud and a portion of its subtending duct immunostained for P-cadherin with antibody PCD-1. The cap cells are stained (large arrows), whereas the interior epithelium shows only occasional patches of P-cadherin immunostaining (small arrows). Bar, 1.6 mm in a, 28 mm in b, and 25 mm in c.

cells, like their progenitor cap cells of the end bud, were decorated with P-cadherin in a punctate distribution, presumably reflecting the distribution of adherans junctions (Fig. 2f).

Blocking Antibodies Applied in Situ

Slow-release plastic implant technology presents an opportunity to test the effects of blocking antibody directly in the mammary gland. Miniature pellets made from DuPont's ELVAX (EVAc) are nondenaturing to proteins, are capable of sustained release, and do not initiate an inflammatory reaction in the host (Silberstein and Daniel, 1987). EVAc implants have found use in investigations on the effects of exogenous materials acting locally at precisely determined locations within the mammary gland (Silberstein and Daniel, 1982; Daniel *et al.*, 1989).

Slow-release pellets were implanted directly ahead of the advancing end buds in 5-week female mice, in which the diffusion pattern could influence both the buds and the subtending ducts (Fig. 1a). Antibody ECCD-1 to E-cadherin produced dramatic effects in 12 hr in the end bud (Fig. 3b). Multilayered cells of the lumenal compartment, which had previously shown specific staining with antibody to E-cadherin, loosened and were found freely floating in the lumen. The cap cells of the end buds and the myoepithelial cells of the subtending ducts remained associated with each other and attached to the basal lamina, where they formed a sack enclosing the freely floating lumenal cells. In order to determine whether these effects of blocking antibodies were reversible, we examined the treated glands at later time points. At 72 hr, when the antibody had been largely depleted (Silberstein and Daniel, 1982), the end buds had regained their characteristic structure, apparently due to reassembly of the disaggregated lumenal cells (Fig. 3c).

The blocking antibody PCD-1 to P-cadherin had comparable but less dramatic effects on the cap cell population (Fig. 4). At 30 hr of treatment the majority of cap cells in end buds remained in place, but clusters of loosened cells were found between the basal layer and the lumenal population (Figs. 4b and 4c). These clusters appeared to represent regions in which the cap cell layer of the end buds had been more than one cell in thickness and were therefore susceptible to disaggregation by antibody. In areas where the cap cells were monolayered and each cell was attached to the basal lamina, antibody to P-cadherin was unable to dissociate the tissue. At 78 hr pockets of dissociated cells had disappeared and histoarchitecture was normal (Fig. 4d).

In subtending ducts, treatment with the E-cadherin blocking antibody ECCD-1 caused partial disassembly of the lumenal tissue by 12 hr, with epithelial cells floating freely in the lumen (Fig. 3e) while other cells remained attached to the basal lamina (Fig. 3e, arrow), presumably through integrin-mediated adhe-

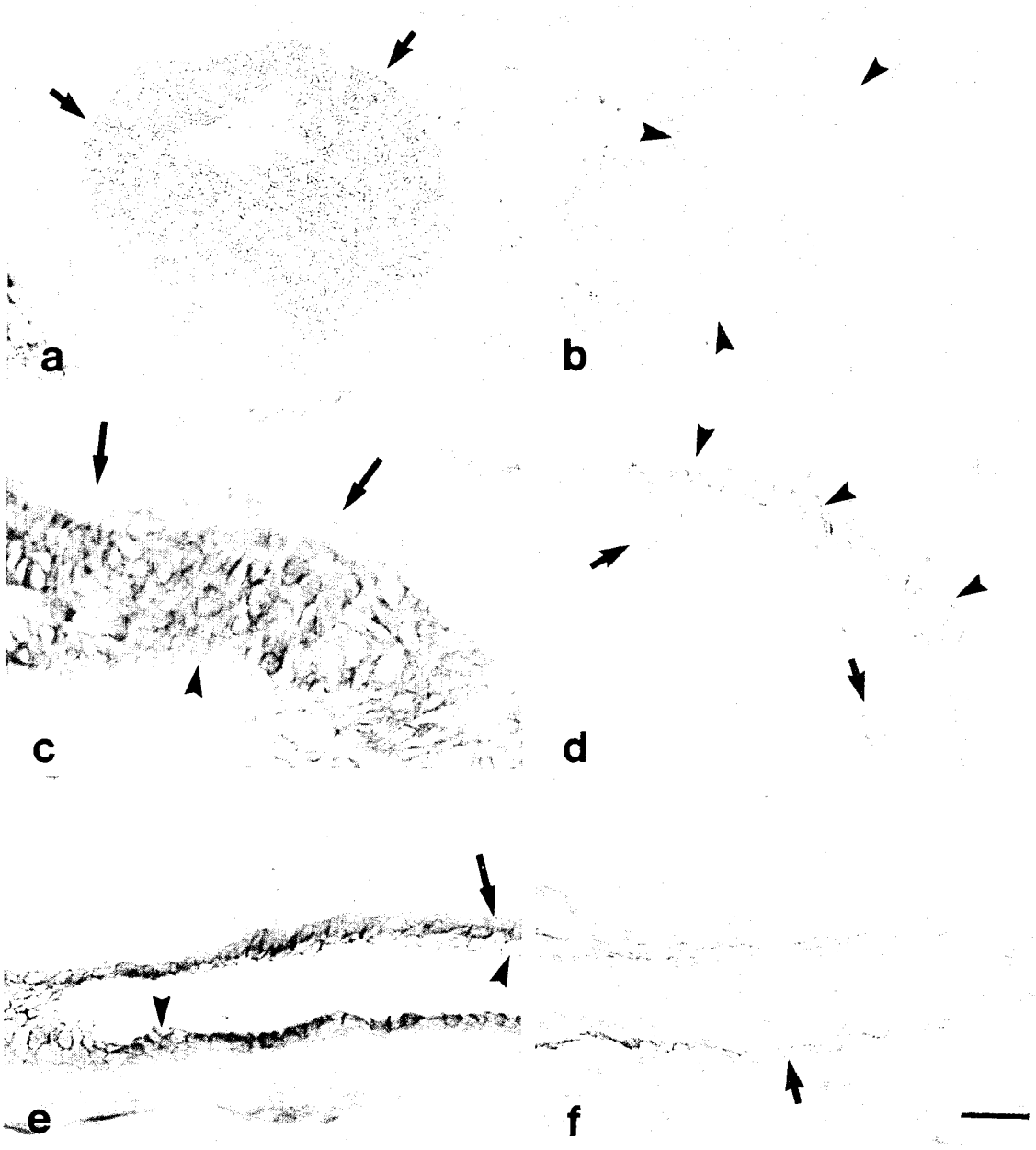

FIG. 2. Immunostaining patterns of E-cadherin (a,c, and e) and P-cadherin (b,d, and f) in histological sections of mammary end buds and ducts. E-cadherin is found on membranes of interior epithelium except for those cells directly facing the lumen of end buds and ducts (c and e, arrowheads). Basally located cap cells do not display E-cadherin (a and c, arrows) nor do myoepithelial cells along the mammary ducts (e, arrow). P-cadherin is located in the cap cells (b and d, arrowheads) and their differentiated descendants, the myoepithelial cells along the duct (f, arrow). Small clusters of epithelial cells staining for P-cadherin are seen in the multilayered interior epithelium (d, arrows). Bars, 72 μm in a and b, 36 μm in c through f.

sions to the basal lamina or perhaps adhesions of unknown type to myoepithelial cells. Like the end bud, normal tissue morphology was restored at 72 hr (Fig. 3f). Treatment with the blocking antibody PCD-1 to P-cadherin had no obvious effect (not shown), probably because the myoepithelium is entirely monolayered and each cell is anchored to the basal lamina (Williams and Daniel, 1983).

Effects of Blocking Antibodies on DNA Synthesis in Situ

Prior to the removal of glands for the blocking experiments described above, mice were injected with either [^{3}H]thymidine for autoradiography or bromodeoxyuridine for immunolocalization of cells engaged in DNA synthesis. Both methods yielded equivalent results. In 12 hr, the DNA synthetic index (percentage of cells in S-phase)

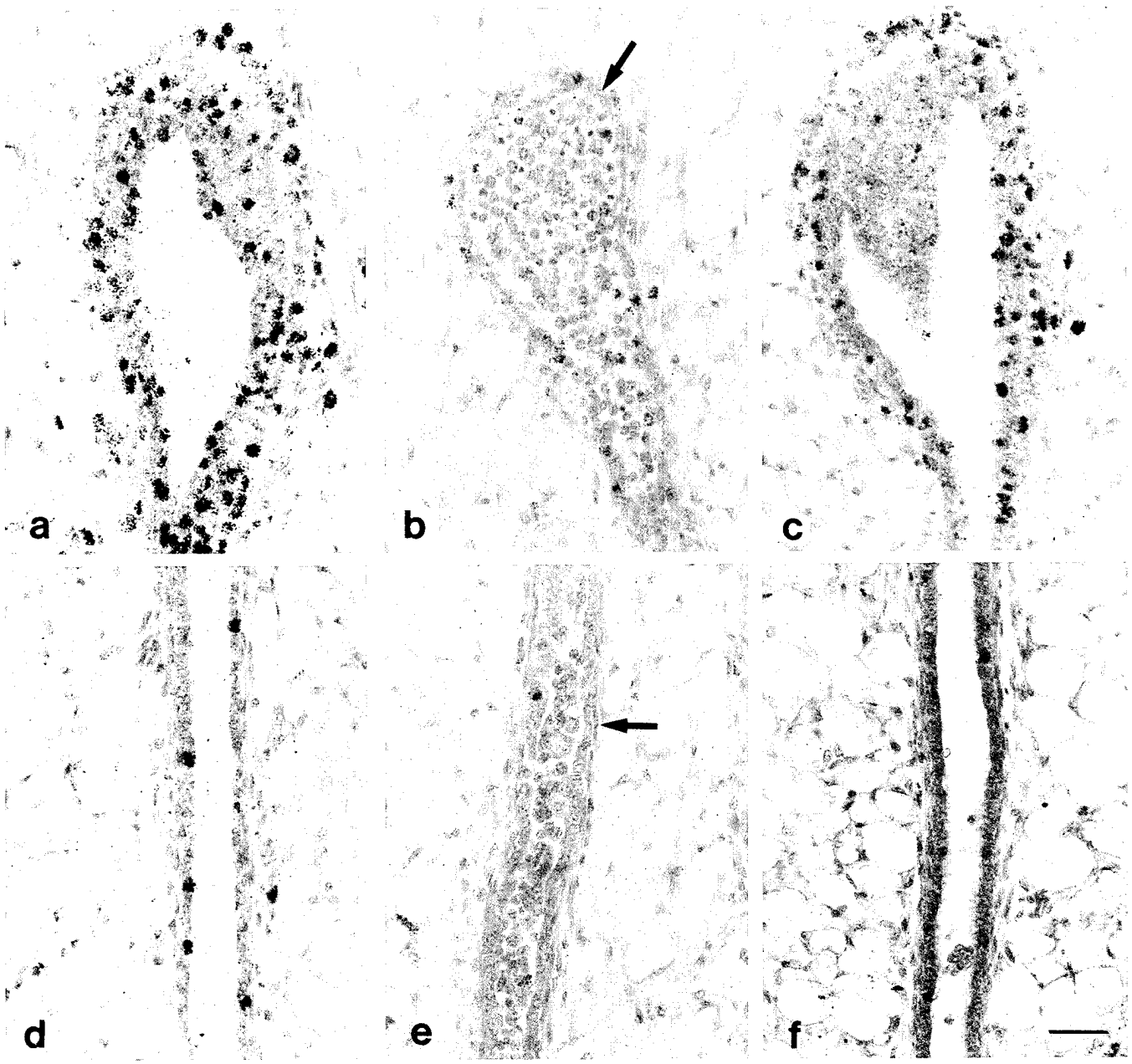

FIG. 3. Blocking antibody ECCD-1 applied to mammary end buds *in situ.* In these autoradiographs cells engaged in DNA synthesis display black silver grains over their nuclei. (a and d) Normal tissue architecture at Time 0. At 12 hr the lumenal epithelium of end buds was dissociated, with loosened cells filling the lumen (b). DNA synthetic cells were reduced in number at 12 hr compared to Time 0 (a) and 72 hr (c). In ducts, the lumenal epithelium was also dissociated at 12 hr (e). At 72 hr normal tissue architecture was restored (f). Cap and myoepithelial cells were relatively unaffected (b and e, arrows). Bar, 70 μm.

in end buds treated with ECCD-1, antibody to E-cadherin fell to 7% compared to 18% in control end buds in the same animal treated with nonblocking antibody to E-cadherin (Figs. 3a, 3b, and 5a). DNA synthesis remained reduced until 72 hr after treatment, when normal growth levels of DNA synthesis resumed, coincident with the reassembly of normal tissue structure from disaggregated cells (Fig. 3c). DNA synthesis was largely unaffected in cap cells (Fig. 5b). Blocking antibody PCD-1 to P-cadherin caused only small changes in DNA synthesis of lumenal cells (Fig. 5c), but cap cell DNA synthesis was reduced and remained so through 78 hr (Fig. 5d).

DISCUSSION

The essential morphoregulatory role of specific adhesion factors in tissue reconstruction and histogenesis

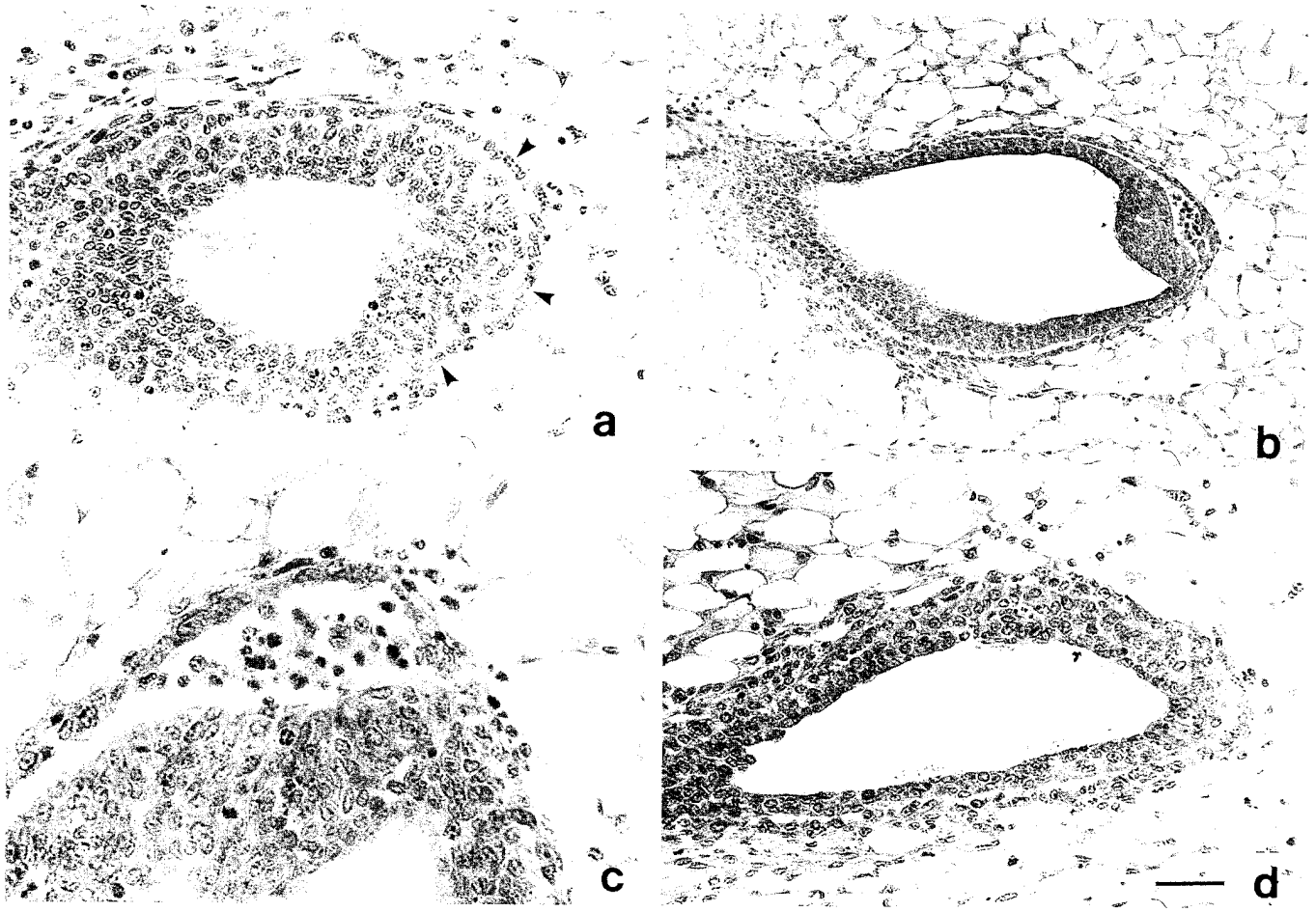

FIG. 4. Blocking antibody PCD-1 to P-cadherin applied to mammary end buds *in situ*. The well-organized cap cell layer at Time 0 of exposure is shown in a (arrowheads). At 30 hr (b and c) patches of cap cells are loosened and appear to be floating in the space between cap and lumenal cell layers. At 78 hr normal architecture was restored (d). Ducts treated with PCD-1 appeared to be unaffected by treatment (not shown). Bar, 25 μm in a and d, 50 μm in b, and 17 μm in c.

has become a central paradigm of development (Edelman *et al.*, 1990). The cadherins represent a multigene family of calcium-dependent adhesion molecules that mediate many homophilic cell–cell adhesive interactions in the initial formation of epithelial aggregates and in their subsequent growth, migration, folding, and bending during morphogenesis (Takeichi, 1991; Geiger and Ayalon, 1992). Together with cell–extracellular matrix adhesive interactions, cadherins trigger morphogenetic processes leading eventually to the assembly of organs, in which these adhesive systems may continue to play an essential role in the stabilization and maintenance of differentiated tissues through specialized adherens-type cell adhesions.

The mouse mammary gland represents a well-developed model for the study of organogenesis and is unique in that its development occurs mainly in the subadult animal where large size and ready accessibility confer experimental advantages over organs that develop mainly *in utero*. During puberty the rapidly growing terminal end buds penetrate the fatty stroma, creating an arborizing system of mitotically inactive, differentiated epithelial tubes from which secretory alveoli arise during pregnancy. The role of adhesive interactions during ductal branching morphogenesis has not been reported, although E-cadherin expression has been observed in alveolar tissues in the mouse (Streuli *et al.*, 1991) and human (Oka *et al.*, 1993) and in cell lines derived from human breast tissues (Bracke *et al.*, 1993; D'souza and Taylor-Papadimitriou, 1994). Attachment of mammary epithelial cells to the basal lamina through integrin receptors is also known to play an essential role in alveolar morphogenesis and secretory differentiation (Streuli, 1993).

The aim of the present experiments was to investigate the expression of E- and P-cadherins and, using function-blocking antibodies, their functional role in segregating subpopulations of epithelial cells in the end buds

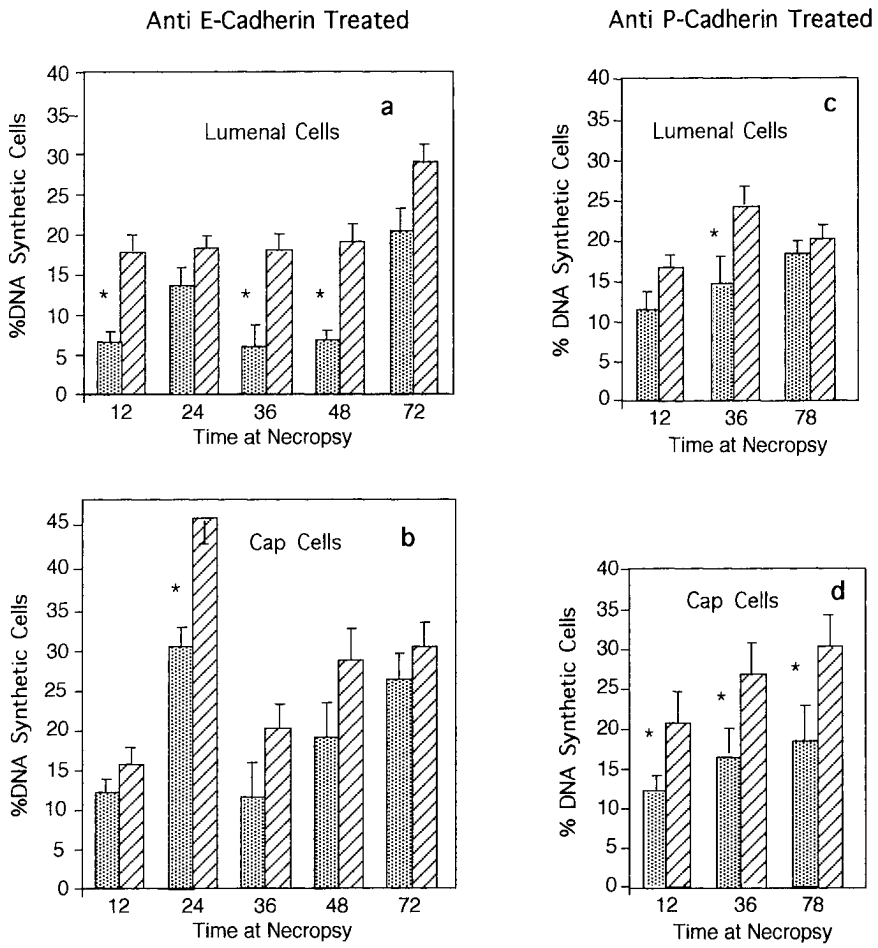

FIG. 5. Effects of blocking antibodies on DNA synthesis *in situ*. Shaded bars, glands implanted with function-blocking antibody; hatched bars, glands with control implants consisting of nonblocking antibody ECCD-2 to E-cadherin (a and b) or nonimmune rat IgG (c and d). Asterisks indicate that experimental tissues differed from the contralateral control tissues at a 0.95 confidence level or better. Vertical bars indicate standard errors. (a) End bud lumenal cells exposed to implant releasing antibody ECCD-1 to E-cadherin. (b) End bud cap cells exposed to implant releasing antibody ECCD-1 to E-cadherin. (c) End bud lumenal cells exposed to implant releasing antibody PCD-1 to P-cadherin. (d) End bud cap cells exposed to implant releasing antibody PCD-1 to P-cadherin.

as they proliferate and are channeled into the lumenal and myoepithelial compartments of their subtending ducts. A second aim was to examine the possible effects of altered adhesion on epithelial DNA synthesis. Longitudinal sections of elongating ducts showed bulbous, multilayered end buds consisting of two readily identifiable and clearly separated cell populations, the multilayered body (lumenal) cell compartment and the basally located monolayer of cap cells. Immunostaining revealed a discrete, nonoverlapping pattern in which cap cells displayed moderate levels of membrane-associated P-cadherin, whereas E-cadherin was present on all body cell membranes with the exception of those directly bordering the lumen. In the more differentiated tissues of the subtending duct this discrete pattern was maintained; myoepithelium, derived from cap cells, displayed P-cadherin, whereas lumenal cells showed strong immunostaining for E-cadherin, suggesting that the segregation of these tissue layers may arise from selective expression of these and perhaps other adhesion factors.

Cap cells and their myoepithelial derivatives appear to be topologically continuous with the basal layer of the epidermis (Daniel and Silberstein, 1987), although a direct developmental relationship has not been described. During development the mouse epidermis also expresses cadherins, though in a less discrete spatial distribution, with E-cadherin expressed in both the basal and the intermediate layers, and P-cadherin only in the basal layer (Nose and Takeichi, 1986; Hirai *et al.*, 1989b). This diffuse pattern of E-cadherin distribution may function in permitting cells derived from the basal layer to move into intermediate layers for differentiation into keratinocytes. In the mammary gland, where cadherin distribution is apparently nonoverlapping, cadherins may be

associated with the spatially discrete segregation of basal and lumenal cell layers.

We observed occasional clusters of cells in the lumenal compartment of the end bud staining anomolously for P-cadherin (Figs. 1c and 2d). These are probably identical to cell clusters shown by histology and by time-lapse microcinematography to dislodge from the cap layer and sink into the lumenal compartment (Williams and Daniel, 1983). It is postulated that these migratory cap cells acquire E-cadherin, enabling them to migrate between tissue layers while continuing for a time to display P-cadherin. It is not possible to verify the acquisition of E-cadherin by these cells because their membranes are closely contiguous with those of surrounding body cells. The nature of these migratory cells is unknown, but it is interesting to speculate that, because the cap cells are the least differentiated of identifiable mammary cell types, they may represent the source of mammary stem cells that are dispersed throughout the mammary tree (Faulkin and DeOme, 1960) and which have been tentatively identified as clear-staining cells (Smith and Medina, 1988).

Using slow-release plastic implants to expose selected regions of the gland *in situ* to blocking antibodies, we found that antibody to E-cadherin induced disruption of the multilayered body epithelium of the end bud, resulting in cells freely floating in the space contained within the basal lamina and its adherent basal cells (Fig. 3b). This result indicates that E-cadherin is required and could be sufficient for integrity of the lumenal tissue. Interestingly, reassociation of these loosened cells occurred after depletion of the antibody (Silberstein and Daniel, 1982), restoring normal tissue architecture (Fig. 3c). This reaggregation indicates both the reversible nature of the antibody treatment and the ability of these disaggregated cells to reconstruct histotypic structures, an ability usually associated with embryonic tissues (Townes and Holtfretter, 1955; Moscona, 1961). Exposure of mammary ducts to blocking antibody to E-cadherin also resulted in disaggregation of lumenal epithelium and the appearance of freely floating cells, but in this case many of the basally located cells appeared to retain associations with either the basally located myoepithelium or the basal lamina itself (Fig. 3e).

Treatment with antibody to P-cadherin had no effect on lumenal cells but produced partial disruption of the basally located cap cell layer (Figs. 4b and 4c). Here pockets of disaggregated cells were found next to cap cells that appeared to be normally arranged, presumably adhering to the basal lamina by integrins (Streuli, 1993). Occasional patches of disaggregated cells may represent areas in which the cap cells had become multilayered, losing their attachment to the basal lamina and becoming susceptible to loosening by antibody.

Experiments with cells transfected with cDNAs encoding different cadherins have demonstrated that forced expression of specific adhesive factors can give rise to aggregation and highly selective segregation (Nose *et al.*, 1988). In our experiments, the histotypical sorting of mammary cells *in situ* is conceptually consistent with this, but adds the dimension of tissue reconstruction in an adult rather than an embryonic organ.

An interesting consequence of exposure to blocking antibodies was the approximately $2\frac{1}{2}$-fold decline in DNA synthesis observed in end bud lumenal cells following tissue disaggregation (Figs. 3b and 5a). Declines as much as 5-fold were observed in other experiments (not shown). This decline was sustained through a gradual and progressive reaggregation, but was reversed following tissue reconstruction at 72 hr when DNA synthesis returned to normal levels (Fig. 3c). This decline in DNA synthesis was specific and was not observed in glands implanted with control pellets containing either rat IgG or nonblocking ECCD-2 (Fig. 5a). The observed association of a decline in DNA labeling index with disaggregation of epithelial cells suggests that these nontransformed mammary epithelial cells require normal cell–cell associations to permit passage through the cell cycle. The opposite is true with transformed cells, and there exist numerous reports of increased growth and malignant potential associated with decreased cadherin expression (Shiozaki *et al.*, 1991; Oka *et al.*, 1992; Gamallo *et al.*, 1993; Moll *et al.*, 1993; Oka *et al.*, 1993; Rasbridge *et al.*, 1993).

We conclude that in the normal gland, regulation of DNA synthesis and cell division requires at least some degree of tissue-level structure, for which cadherin-mediated adhesions are required. Because loss of tissue structure is associated with inhibition of DNA synthesis, a lack of cadherin-mediated cell adhesions cannot, in itself, account for loss of growth regulation in malignancies.

In lung epithelial morphogenesis studied in explant cultures, partial disruption of tissue structure was observed in response to antibodies to E- and P-cadherins, which appeared to be reversible when antibodies were removed (Hirai *et al.*, 1989a). Although DNA synthesis was not studied, growth of the explanted epithelial tissues appeared to occur normally in the presence of antibodies. The same blocking antibodies were shown to partially disrupt morphogenesis in organ cultures of embryonic mouse skin, in which, interestingly, secondary effects on dermal condensation were observed (Hirai *et al.*, 1989a).

In the present study, precise spatial distribution of cadherins is associated with the maintenance of the "tube-within-a-tube" architecture that is required both for the proper channeling of presumptive tissues into

their proper location within the duct and for the normally high rates of DNA synthesis and cell division required for growth. Together with cell–substrate adhesions, the cadherin-based adhesion systems play a central role in mammary growth and patterning of epithelial ducts in the mammary gland.

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