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We are investigating a newly discovered family of genes, the T-box genes, that play critical roles in inductive interactions in the development of many organs. In the mouse, two of these genes, *Tbx2* and *Tbx3*, are expressed in the developing mammary glands. One is also expressed in adult glands and in mammary tumors. Our hypothesis is that *Tbx2* and *Tbx3* are required in the signalling pathway of normal mammary gland development, and could be involved in mammary carcinogenesis. Previously, we proposed *TBX3* as a candidate gene for the Ulnar-mammary Syndrome in humans, based on the observed expression patterns and the chromosomal location of the locus, and this has recently been proven to be the case. Mutations in this gene are responsible for the mammary hypoplasia characteristic of this syndrome. Our goals are to explore the roles of *Tbx2* and *Tbx3* in mammary development and carcinogenesis by producing mutations in these genes. In work to date we have detailed the expression of the genes in embryo and adult mammary glands and have begun to characterize the genes. The significance of this work is in elucidating normal mechanisms of mammary development and thereby aiding in the understanding of how growth controls can go wrong in cancer.

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FOREWORD

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INTRODUCTION

A vital missing link in understanding the ontogeny of breast cancer is the identification and investigation of genes controlling normal mammary gland development during embryonic organogenesis and during pregnancy and lactation. We are investigating a newly discovered family of putative transcription factors, called the T-box family, that is thought to play critical roles in controlling inductive interactions in the development of many organs, including the mammary glands.

The T-box gene family is defined by a region of homology to the DNA binding domain of the mouse *Brachyury*, or *T* locus gene product. Phylogenetic studies have demonstrated the ancient origin of this gene family. At least 16 different T-box genes have now been identified in the genomes of animals ranging from *C. elegans* to humans (1). In the mouse, we have identified 6 T-box genes that are expressed in highly specific patterns during early embryogenesis and organogenesis, in areas of classic embryonic inductive interactions (2). Two of these genes, *Tbx2* and *Tbx3*, are expressed in the developing mammary glands, and one of them is expressed in adult glands and in mammary tumors (our unpublished data). In the embryo, they are expressed reciprocally in the mesenchymal and epithelial components of the gland, suggesting they work in concert during the branching of the epithelial ducts.

Our hypothesis is that *Tbx2* and *Tbx3* are required in the signalling pathway of normal mammary gland development, and could also be involved in neoplasia. Previously, we identified *Tbx3* as a candidate gene for the Ulnar-mammary Syndrome in humans, based on the observed expression patterns and the chromosomal location of the locus (3). This has recently been proven to be the case (4), and mutations in this gene have been shown to be responsible for the mammary hypoplasia characteristic of this syndrome. Our goals are to explore the roles of *Tbx2* and *Tbx3* in mammary development and mammary carcinogenesis using a mutational approach.

BODY

Experimental Methods

Mammary glands were dissected from adult virgin, pregnant, lactating, and involuting female mice, and from a mammary tumor dissected from a MMTV-*neu* transgenic mouse. RNA was isolated (5) and used for Northern analysis. Hybridization of *Tbx2* and *Tbx3* probes was performed at 65°C. Embryos were dissected at various gestational ages and sexed by the gross morphology of the gonads or by staining for sex chromatin in the amnion. Embryos were then either subjected to whole mount *in situ* hybridization with probes specific for *Tbx2* and *Tbx3* (2), or the mammary gland primordia were dissected and cultured in DMEM medium supplemented with 10% fetal bovine serum in Transwell Clear culture chambers. Branching of mammary buds *in vitro* was assessed by observation with a dissecting microscope.

RESULTS

Expression of *Tbx2* and *Tbx3*

We have used standard Northern anlysis of mRNA isolated from mammary glands of normal adult mice and standard whole mount *in situ* hybridization analysis of embryonic mice at different days of gestation. Using these methods, we have detected *Tbx3* expression in virgin, pregnant, lactating and involuting mammary glands of adult mice (Figure 1), although *Tbx2* is not expressed in these adult tissues nor in the tumors (data not shown). At the onset of mammary development in the midgestation embryo, *Tbx2* is expressed in the mesodermal stroma or milk line, underlying the mammary epithelial buds, although this expression is not evident beyond the 12th day of gestation. *Tbx3*, on the other hand, is expressed in the epithelial buds of both male and female embryos through the 15th day of gestation (Table 1).

Gene Targeting

Because *Tbx2* and *Tbx3* are expressed in several embryonic tissues in addition to the mammary glands (2), mutations in either gene are likely to have multiple effects on embryogenesis. Although we are producing null mutations in both these genes as part of our general program, our aim for this project is to produce mammary-specific loss of function through conditional targeted mutagenesis using the Cre/*loxP* system. Toward this aim, we have been characterizing the *Tbx3* gene and its transcripts preparatory to making a targeting construct. Northern blot analysis of adult and embryonic mRNA indicates two predominant transcript sizes, 4.5 and 5.2 kb (6). Currently a number of genomic clones are being restriction mapped, aligned and subcloned (Figure 2). The largest of these is ~20 kb, spanning the T-box which codes for the DNA binding domain. A construct for mammary-specific, conditional gene targeting will be made from this genomic clone according to the strategy outlined in Figure 3).

Organ Cultures

Although not a part of our original proposal, we have begun culturing mammary gland primordia in order to develop a culture system in which to examine branching morphogenesis. Mammary buds and underlying stroma are dissected from midgestation embryos and cultured in the upper well of a culture chamber. We have been successful in growing these mammary primordia for two days, during which the epithelial buds undergo branching. This system will be used to study the effects of antisense oligonucleotides of the T-box genes and for recombination of tissues of different genotypes when they are available.

CONCLUSIONS

Work in other laboratories has now identified mutations in *Tbx3* as the causal factor in the Ulnar-Mammary Syndrome in humans, vindicating our interest in this gene as a controlling gene in the normal development of the mammary gland. We have made progress in identifying areas of expression of the genes in the developing and adult mammary glands. We have also made progress towards characterization of the gene in preparation for gene targeting which will be a major effort in the coming year.

References

1. V. E. Papaioannou, L. M. Silver, *BioEssays* **in press** (1998).
2. D. L. Chapman, et al., *Developmental Dynamics* **206**, 379-390 (1996).
3. V. E. Papaioannou, *Journal of Trends in Genetics* **13**, 212-213 (1997).
4. M. Bamshad, et al., *Nature Genetics* **16**, 311-315 (1997).
5. P. Chomczynski, N. Sacchi, *Analytical Biochemistry* **162**, 156-159 (1987).
6. R. J. Bollag, et al., *Nature Genetics* 7, 383-389 (1994).

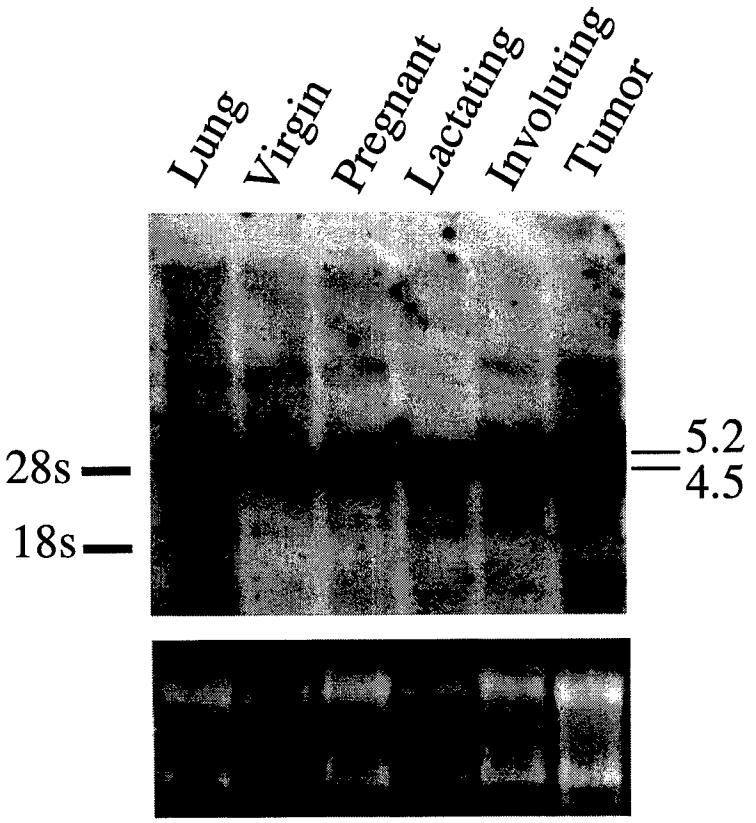

Figure 1. Northern blot analysis of *Tbx3* expression in mammary glands from adult virgin, pregnant, lactating, involuting mice and from mammary tumors dissected from an MMTV-neu mouse. *Tbx3* is expressed at a constant level in all tissues examined. 10mg total RNA was loaded in each lane. The positions of the 28s and 18s ribosomal bands are indicated and the ethidium bromide staining of the ribosomal bands is shown below to indicated differences in RNA loaded.

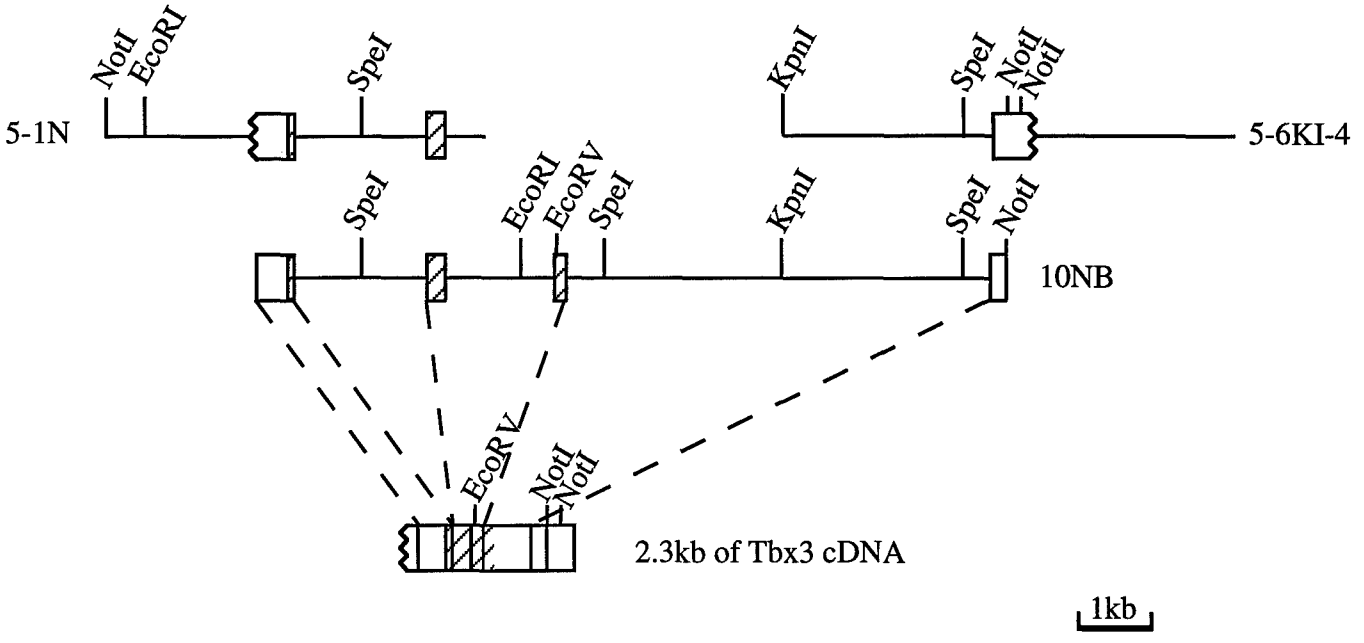

Figure 2. *Tbx3* genomic clones aligned with *Tbx3* cDNA. Three genomic clones, p5-1N, p5-6KI-4, and p10NB, were cloned into plasmid from three independent genomic phage clones and aligned by restriction mapping. Southern hybridization and DNA sequencing were used to map exons. Hatched region indicates the T-box.

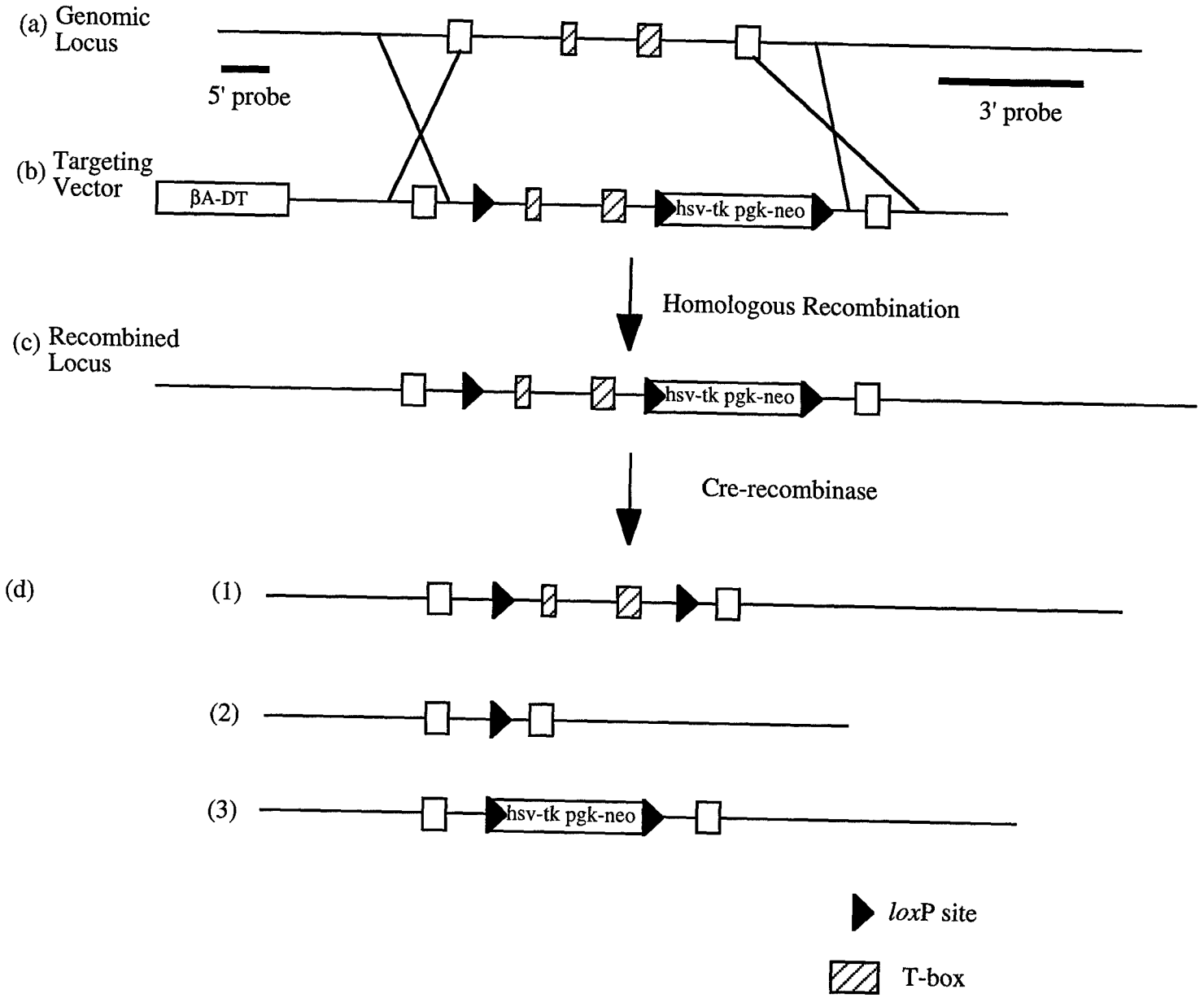

Figure 3. Mammary-specific conditional gene targeting strategy. Homologous recombination between the genomic locus (a) and the targeting vector (b) introduces three *lox*P sites into the recombined locus (c). Cre recombinase catalyzes recombination between the *lox*P sites to produce three possible products (d). The firest product leaves the gene intact, but flanks the T-box with *lox*P sites. The second product deletes the T-box. The third is selected against with gancyclovir. The *lox*P flanked T-box of the first product can be deleted in mammary glands in vivo by mating with a mouse carrying a *Cre* recombinase transgene under a mammary-specific promoter.

Table 1. Expression of *Tbx2* in stroma and *Tbx3* in epithelium of developing mammary glands in mouse midgestation embryos of both sexes.

		EXPRESSION	
Age (days post coitus)	Sex	*Tbx2* (stroma)	*Tbx3* (epithelium)
11.5	-	+	-
12.5	♀	-	
	♂	-	+
13.5	♀	-	+
	♂	-	+
14.5	♀	-	+
	♂	-	+
15.5	♀	n.d.	-
	♂	n.d.	-
16.5	♀	-	-
	♂	-	-