

The TALE Homeodomain Protein Pbx2 Is Not Essential for Development and Long-Term Survival

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Received 31 October 2003/Returned for modification 8 December 2003/Accepted 18 March 2004

Pbx2 is one of four mammalian genes that encode closely related TALE homeodomain proteins, which serve as DNA binding partners for a subset of Hox transcription factors. The expression and contributions of Pbx2 to mammalian development remain undefined, in contrast to the essential roles recently established for family members Pbx1 and Pbx3. Here we report that Pbx2 is widely expressed during embryonic development, particularly in neural and epithelial tissues during late gestation. Despite wide Pbx2 expression, mice homozygous mutant for Pbx2 are born at the expected Mendelian frequencies and exhibit no detectable abnormalities in development and organogenesis or reduction of long-term survival. The lack of an apparent phenotype in Pbx2^{-/-} mice likely reflects functional redundancy, since the Pbx2 protein is present at considerably lower levels than comparable isoforms of Pbx1 and/or Pbx3 in embryonic tissues. In postnatal bone marrow and thymus, however, Pbx2 is the predominant high-molecular-weight (MW)-isoform Pbx protein detectable by immunoblotting. Nevertheless, the absence of Pbx2 has no measurable effect on steady-state hematopoiesis or immune function in adult mice, suggesting possible compensation by low-MW-isoform Pbx proteins present in these tissues. We conclude that the roles of Pbx2 in murine embryonic development, organogenesis, hematopoiesis, immune responses, and long-term survival are not essential.

Pbx genes code for a family of highly conserved homeodomain proteins of the TALE (three-amino acid loop extension) class that participate in multiprotein complexes to regulate developmental gene expression (20). *Pbx1* was originally identified in human pre-B acute lymphoblastic leukemias as a result of its disruption by t(1;19) chromosomal translocations (13, 23). The highly related *Pbx2*, *Pbx3*, and *Pbx4* genes were subsequently identified on the basis of sequence conservation with *Pbx1* (22, 40). *Pbx* orthologs in *Caenorhabditis elegans* (Ceh-20), *Drosophila* (Exd), and zebra fish (*lazarus*) (5, 28, 29, 39) have also been characterized. In *Drosophila*, Exd acts as a cofactor to direct homeotic selector proteins to target genes (44), thus conferring higher specificity to Hox DNA binding in developmental programs (6, 27, 30). In zebra fish, *lazarus* has been shown to globally mediate *Hox* gene function, while orchestrating the corresponding segmentation of the hindbrain and pharyngeal arches (28, 41).

Biochemical studies have demonstrated that *Pbx* proteins interact with a subset of *Hox* proteins to enhance their DNA

binding affinities and specificities (6–9, 16, 19, 24–26, 37, 38). *Pbx* proteins also heterodimerize with the Meis/Prep subfamily of TALE-class homeodomain proteins (4, 8, 14) to form trimeric complexes with *Hox* proteins on appropriate DNA sites (3, 4, 12, 33) that regulate developmental gene expression. *Pbx* proteins are highly similar to each other and share extensive sequence identity within and flanking their DNA binding homeodomains. Additional isoforms of mammalian *Pbx* proteins arise from differential splicing of *Pbx* transcripts to yield high-molecular-weight (MW) (*Pbx1a*, *Pbx2*, *Pbx3a*, and *Pbx4*) and low-MW (*Pbx1b* and *Pbx3b*) forms of the respective proteins (22, 40). Although the DNA binding properties of *Pbx* proteins appear similar *in vitro*, the transcriptional effector properties of various isoforms can be distinguished on the basis of differential recruitment of transcriptional cofactors (2).

In contrast to their well-characterized roles as transcription factors, the differential contributions of *Pbx* proteins during mammalian development and organogenesis have not been fully determined. To assess their functional roles *in vivo* and their possible overlapping and unique contributions, we have generated and characterized mice deficient for each of the *Pbx1*, -2, and -3 proteins. Our previous studies demonstrated that *Pbx1* and *Pbx3* have unique, essential functions required for embryonic development and postnatal survival, respectively. *Pbx1*-deficient embryos die at gestational day 15 or 16 with severe hypoplasia or aplasia of multiple organs (15, 32, 34, 36), as well as homeotic transformation (36) and hematopoietic abnormalities (11). Mice deficient for *Pbx3* develop to

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term but die within a few hours of birth due to central respiratory failure (J. Rhee, A. Arata, L. Selleri, Y. Jacobs, S. Arata, H. Onimaru, and M. Cleary, submitted for publication). In this study, we demonstrated that, despite widespread embryonic expression, *Pbx2* is not an essential gene, whose loss does not affect normal development, organogenesis, fertility, hematopoiesis, or immune function, likely due to redundancy with the related *Pbx* family members.

MATERIALS AND METHODS

Targeted disruption of the *Pbx2* gene and generation of *Pbx2* knockout mice.

The *Pbx2* gene was mutated by deletion of a 316-bp SacI-XbaI genomic fragment comprising 248 bp covering the entire *Pbx2* exon 3, the largest 5' exon containing a nonunit number of codons, and 68 bp of *Pbx2* intron 3, to the XbaI site. In place of the removed 316-bp SacI-XbaI *Pbx2* genomic fragment, a 2-kb PGK-neo cassette (from the pNT vector) (36) was inserted into the unique SacI site of exon 3. A 7.9-kb segment of genomic DNA spanning the disrupted *Pbx2* exon 3 was then cloned into the targeting vector containing the herpes simplex virus thymidine kinase cassette (see Fig. 2). The targeting construct was linearized by NotI digestion and then electroporated into embryonic stem (ES) cell line GS (purchased from Genome Systems) and line W9.5 (18). Following positive/negative selection in G418 and ganciclovir (1), homologous recombinant clones were identified by Southern blot analysis using three different enzyme and probe combinations (5' and 3' external probes and a neo-specific internal probe). Of 182 informative clones, 18 yielded restriction digest patterns diagnostic for homologous recombination. Euploid clones were microinjected into C57BL/6J host blastocysts. Chimeric male mice from two independently derived ES clones (W9.5 # 68 and GS # 45) passed the targeted *Pbx2* allele through the germ line. Phenotypes were analyzed in mice derived from the third or fourth backcross generation on a C57BL/6 background and on a fully inbred 129/SvTer background. All observations and results obtained were identical in mice derived from either of the two independently targeted clones (W9.5 # 68 and GS # 45). Genotype analysis was performed on DNA extracted from tail biopsies of adult mice or from yolk sacs of embryonal conceptuses (gestational days 9 to 17 [E9-17]) dissected free of maternal tissues. Following digestion with EcoRI, DNAs were subjected to Southern blot analysis using the *Pbx2* 5' external probe.

Northern blot analysis. Total RNA was isolated from E17 embryos using an RNeasy kit (Qiagen, Valencia, Calif.). Poly(A)⁺ RNA was purified from total RNA with the Polyadenylated RNA kit (Ambion, Austin, Tex.). After transfer and before hybridization, the membrane was stained with a 0.04% methylene blue solution for 5 min to visualize the quality of RNA transfer and to precisely quantify the amounts of rRNAs transferred to each lane. After the membrane was destained in 0.2× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate)–1% sodium dodecyl sulfate (SDS), Northern blot analysis was conducted following standard protocols (22). The hybridization probe consisted of a murine *Pbx2* cDNA containing approximately 650 bp of 3' UTR (NCBI accession numbers MMAF20198 and AC AF020198).

Western blot analysis. Embryonic tissues (E17), thymocytes, or adult bone marrow cells were lysed in 2× SDS sample buffer following homogenization using established procedures (12). Proteins were subjected to SDS-polyacrylamide gel electrophoresis and immobilized on nitrocellulose filters following electrophoretic transfer. Filters were probed with a murine monoclonal antibody that recognizes all three *Pbx* high-MW isoforms (α -PbxL) or monoclonal antibodies specific for *Pbx1a* or *Pbx3a*. A *Pbx2* affinity-purified rabbit polyclonal antibody raised against a peptide mapping at the amino terminus of *Pbx2* was also used (Santa Cruz Biotechnology, Inc.). Immune complexes were detected by using a horseradish peroxidase-conjugated secondary antibody and an enhanced luminescence system (ECL; Amersham Pharmacia Biotech, Inc., Piscataway, N.J.).

Histology. For histological analysis, embryos were fixed in formalin and embedded in paraffin for sectioning using standard procedures (35). Sections of 5 μ m thickness were stained with hematoxylin and eosin, mounted in distrene plasticizer xylene, and photographed.

Skeletal preparations. Differential staining of cartilage and bone in whole mouse embryos (E16) and newborn mice was visualized with alcian blue and alizarin red (10, 21)

Whole-mount and section in situ hybridizations. Whole-mount in situ hybridizations were performed on embryos at E9.5 and E10.5 as previously described (43). In situ hybridizations were performed on frozen sections, generated from wild-type (wt) embryos at E13.5 and E15.5, as previously described (43). Single-

stranded sense and antisense riboprobes specific for the *Pbx2* cDNA were generated and hybridized to frozen sections containing all developing organs in order to establish *Pbx2* expression patterns in later stages of murine development. The *Pbx2* hybridization probe was composed of approximately 650 bp of 3' UTR as described above. Probes were labeled with digoxigenin, using standard procedures (42). Images were obtained using an Olympus BX41 compound light microscope and digital camera or an Olympus SZX12 dissection microscope.

Real-time quantitative RT-PCR. For reverse transcription-PCR (RT-PCR) total RNA was isolated from E17.5 tissues by using Trizol reagent (Gibco BRL) and treated with DNase Treatment and Removal Reagent (DNA-free; Ambion). RNA was reverse transcribed by using the High Capacity cDNA Archive kit (Applied Biosystems, Foster City, Calif.), using random primers for initiating cDNA synthesis. TaqMan real-time quantitative PCR assays were performed on a Perkin-Elmer ABI PRISM 7700 sequence detection system, using SDS version 1.9 software (Applied Biosystems). Specific primers and fluorogenic TaqMan probes were custom designed using the Assay by Design Service (Applied Biosystems). Primers, probes, and PCR conditions are available upon request.

Hematologic studies. Blood (50 to 100 μ l) from the tail vein was collected in an EDTA-coated tube, and automated blood counts were performed using a Coulter cytometer.

Immunological studies. Freshly isolated cells from bone marrow and thymus were stained for four-color analysis, and the fluorescence was analyzed by using a dual-laser FACS Vantage system (Becton Dickinson Immunocytometry Systems, Mountain View, Calif.) with a four-decade logarithmic amplifier. Dead cells were detected by staining with propidium iodide (1 μ g/ml) and gated out electronically. Residual erythrocytes were also gated out electronically. All antibodies were purchased from BD Pharmingen (San Diego, Calif.). Specificities of antibodies were as follows: phycoerythrin-conjugated RM4-5 (anti-CD4), biotinylated 53-6.7 (anti-CD8a), fluorescein isothiocyanate-conjugated S7 (anti-CD43), and allophycocyanin-conjugated RA3-6B2 (anti-B220).

Mice were immunized by intraperitoneal injection of 100 μ g of 2,4-dinitrophenol (DNP) conjugated to keyhole limpet hemocyanin (DNP-KLH) with complete Freund's adjuvant, as described previously (31). The titers of anti-DNP antibodies were measured by enzyme-linked immunosorbent assay using DNP-conjugated bovine albumin (DNP-albumin) as the capture antigen, alkaline phosphatase-conjugated rabbit anti-mouse immunoglobulin G (IgG) or IgM as the secondary antibody, and Sigma Fast *p*-nitrophenyl phosphate as the substrate for alkaline phosphatase. Absorbances (405 nm) of pre- and postimmune sera were compared to monitor immune responses against the hapten.

RESULTS AND DISCUSSION

Pbx2 is widely expressed during murine development.

Whole-mount in situ hybridization experiments at E9.5 and 10.5 of murine gestation showed widespread expression of *Pbx2* throughout the mouse embryo (data not shown). In situ hybridizations on frozen mouse embryonic sections at later days of gestation (E13.5 and 15.5) showed more restricted expression of *Pbx2*, which was nevertheless present in numerous distinct structures (Fig. 1). *Pbx2* expression was prominent in several neural structures at E15.5, including the telencephalon (particularly in the neocortex, including the ventricular and subventricular zones), the hippocampus, and the developing striatum and globus pallidus (Fig. 1B and C). *Pbx2* was also expressed in the mesencephalon, particularly the roof of the midbrain, the choroid plexus (Fig. 1B and D), the cranial ganglia (the trigeminal ganglion is depicted in Fig. 1F and G), and cervical dorsal root ganglia. High-level *Pbx2* expression was observed in the epidermal layer of the developing skin and the vibrissae; in the latter it was limited to the root sheath and was not observed in the surrounding mesenchyme (Fig. 1E).

At E15.5, expression of *Pbx2* was also particularly prominent in numerous epithelial structures, including those lining the nasal cavity, cochlear ducts, and tubotympanic recess and in the submandibular glands (Fig. 1G). Respiratory epithelium lining the segmental bronchi of the lung also contained *Pbx2*, whereas little to no expression was detected in the pulmonary

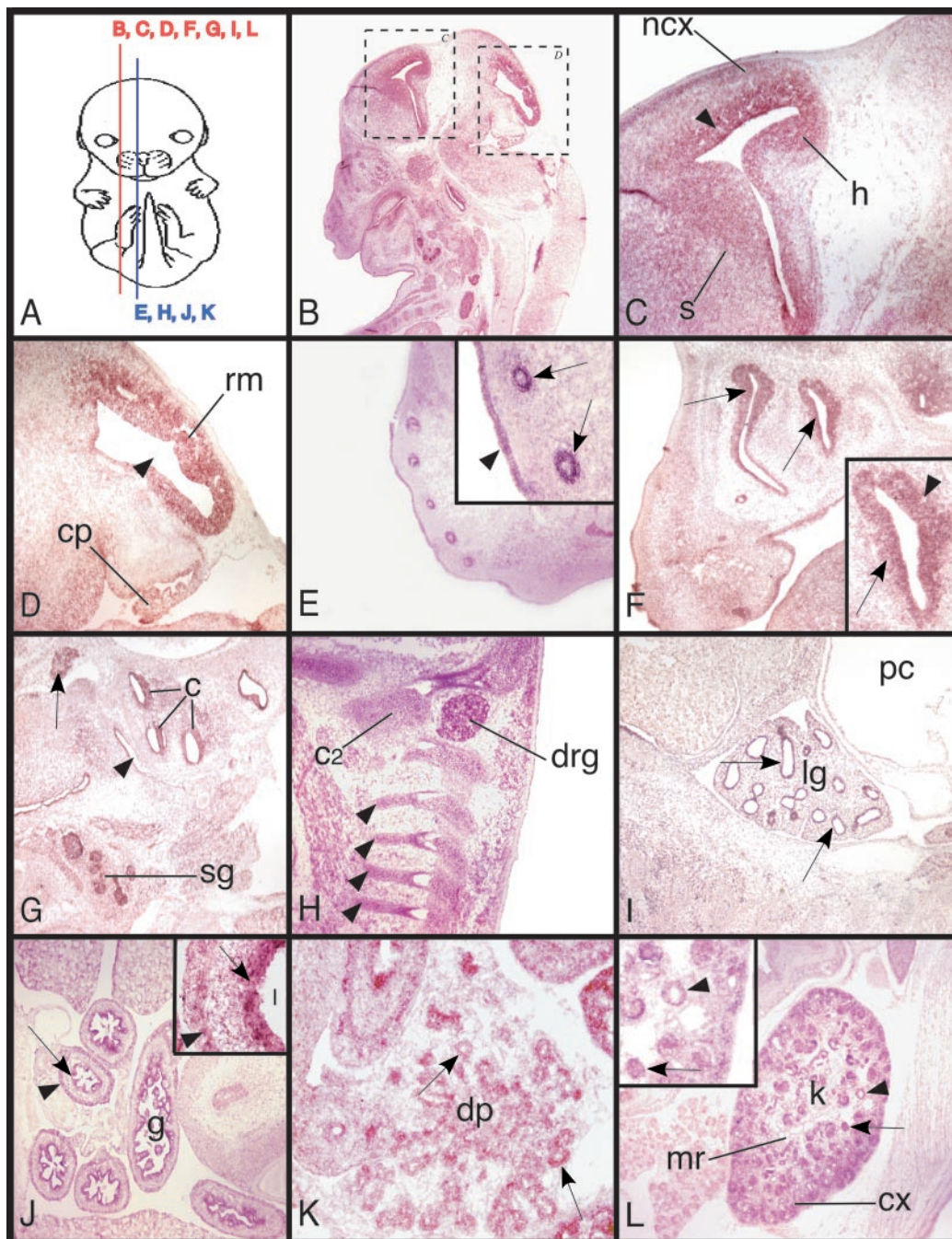


FIG. 1. *Pbx2* expression during mouse development. (A) In situ hybridizations were performed on frozen sagittal sections of E15.5 embryos (unless noted otherwise) as shown. (B to D) *Pbx2* is expressed in the telencephalon, specifically in the neocortex (ncx), including the ventricular and subventricular zones, the hippocampus (h), and the developing striatum (s) and globus pallidus. The black arrowhead in panel C indicates the lateral ventricle. (D) In the mesencephalon, *Pbx2* expression is particularly prominent in the roof of the midbrain (rm) and in the choroid plexus (cp). The black arrowhead indicates the mesencephalic vesicle. (E) *Pbx2* expression in the epidermal layer of the developing skin (as denoted by the black arrowhead in the inset) and in the developing vibrissae, where expression is limited to the root sheath (arrows) but is not evident in the surrounding mesenchyme. (F) *Pbx2* is highly expressed in the olfactory epithelium, as indicated by arrows. The arrowhead indicates the nasal cavity (inset). (G) *Pbx2* is expressed in the epithelium lining the cochlear ducts (c), in the trigeminal ganglion (arrow), in the epithelium of the tubotympanic recess (black arrowhead), and in the submandibular glands (sg). (H) *Pbx2* expression is marked in the second cervical dorsal root ganglion (drg) and in the chondrocytes of the body of the second cervical vertebra (C2). Black arrowheads depict spinal nerves and their roots. (I) *Pbx2* expression at E13.5 is predominant in the respiratory epithelium lining the segmental bronchi (arrows) of the lung (lg). Little to no expression of *Pbx2* is detected in the mesenchyme of the lung or in the mesothelial lining of the pericardial cavity (pc). (J) *Pbx2* is markedly expressed in distinct areas of the gut (g), specifically in the epithelium (arrow) covering the lumina (l, inset) and in the muscularis propria (black arrowhead). (K) *Pbx2* is expressed in the developing dorsal pancreas (dp), specifically in the epithelium of the pancreatic acini (arrows) and ducts. (L) *Pbx2* is expressed in the cortical (cx) and medullary (mr) kidney (k), in particular in the epithelium lining the collecting ducts (black arrowheads) and in the glomeruli (arrows). Little to no expression of *Pbx2* is detected in the nephrogenic mesenchyme. Image B was obtained using a dissection microscope and digital camera, images E to J and L were photographed using a light microscope at a power of $\times 4$. Image K and insets E, F, and L are at a magnification of $\times 10$, while the inset of image J is at a magnification of $\times 40$. All images were digitally processed.

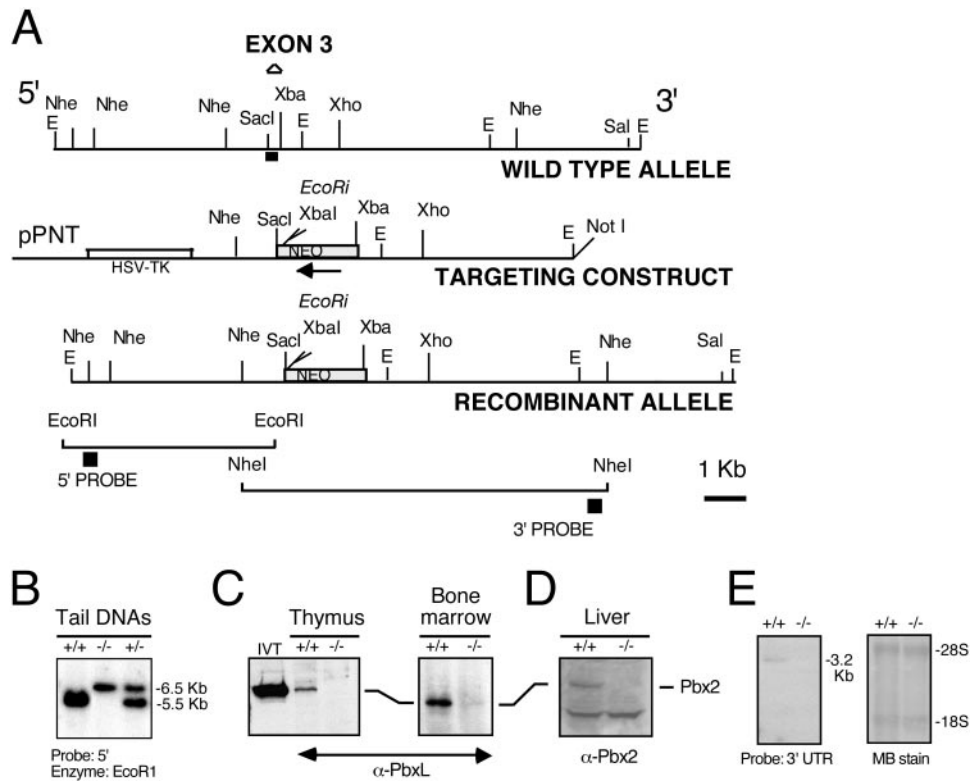


FIG. 2. Targeted inactivation of *Pbx2* and Southern, Northern, and Western analyses of wt and mutant mice. (A) Schematic representation of the mouse *Pbx2* genomic locus, targeting vector, and mutated allele following homologous recombination. Approximately 16 kb of the *Pbx2* locus flanking exon 3 (black box) are depicted along with mapped restriction sites. The targeting construct carries a PGK-neo cassette (shaded box) inserted into the unique SacI site of *Pbx2* exon 3, and the herpes simplex virus thymidine kinase gene (HSV-TK) (white box). The transcriptional orientations of the *Pbx2* arms of homology are opposite to that of the PGK-neo cassette. The 5' and 3' external probes used for Southern blot analyses are shown as solid black boxes below the mutated allele. Restriction enzyme sites: E, EcoRI; Nhe, NheI; Sal, SalI; Xba, XbaI; Xho, XhoI. An EcoRI site (italicized) was introduced to facilitate diagnostic analysis. (B) Southern blot analysis of *Pbx2* alleles. DNA from mouse tissues was analyzed with the probe and enzyme indicated beneath the panel. wt (5.5 kb) and mutant (6.5 kb) *Pbx2* alleles are indicated to the right of the panel. (C and D) Western blot analysis of *Pbx2* expression. Protein extracts were subjected to Western blot analysis using a monoclonal antibody specific for all Pbx long isoforms (α -PbxL) and a Pbx2-specific polyclonal antibody raised against a peptide mapping at the amino terminus of Pbx2 (α -Pbx2 in panel D). Smaller bands, of less than 50 kDa, are present in both wt and *Pbx2*^{-/-} livers and represent nonspecific cross-reactivity by this antibody (D). Genotypes determined by Southern blotting are listed at the top. The left lane of panel C contains in vitro translated Pbx2. (E) Northern blot analysis of *Pbx2* expression. Poly(A)⁺ RNAs from E17 embryos were hybridized with a probe specific for the *Pbx2* 3' UTR. (Left panel) No *Pbx2* transcripts are present in *Pbx2*^{-/-} embryos, in contrast to the 3.2-kb wt transcripts (as previously reported [22]) detected in *Pbx2*^{+/+} embryos. (Right panel) Methylene blue staining indicates equal amounts of 28S and 18S rRNAs in each lane.

mesenchyme or mesothelial lining of the pericardial cavity (Fig. 1I). Although not typically expressed in mesenchymal tissues, *Pbx2* was detected in vertebral chondrocytes (C2 is illustrated in Fig. 1H). *Pbx2* was expressed in distinct areas of the gut, including the epithelium and muscularis propria (Fig. 1J) and the developing dorsal pancreas, specifically in the epithelium of the acini and ducts (Fig. 1K). *Pbx2* was also markedly expressed in the cortical and medullary kidney, specifically in the epithelium lining the collecting ducts and in the glomeruli (Fig. 1L), whereas little to no expression of *Pbx2* was detected in the nephrogenic mesenchyme. Heart tissue was notably devoid of *Pbx2* expression. These in situ hybridization data are consistent with results of previous Northern blot and RT-PCR studies that detected *Pbx2* RNA in most embryonic and adult tissues (22, 40).

In sum, in early mouse development *Pbx2* expression is widespread, comparable to the ubiquitous *Pbx2* expression during the first hours of zebra fish development (41). Later in mouse

development, *Pbx2* expression becomes more restricted and mostly preponderant in epithelial and neural tissues, comparable to expression patterns of the *Drosophila* homolog Exd (29). Conversely, *Pbx2* is expressed at very low levels or is undetectable in the mesenchymal components of most tissues and organ systems, in contrast to *Pbx1*, which is highly expressed in mesenchymal and mesodermal cells (35).

***Pbx2* is not essential for fetal and postnatal survival.** Mice with targeted disruption of *Pbx2* were generated to determine whether the wide expression of *Pbx2* during murine embryogenesis reflected a major role for its encoded protein in development. A null allele of the endogenous mouse *Pbx2* gene was created by deletion of the entire third exon and insertion of a neomycin resistance gene through homologous recombination in ES cells (Fig. 2A). Exon 3 was chosen for targeting since: (i) it is the largest *Pbx2* 5' exon (other than exon 1), whose disruption allows a truncation of *Pbx2* immediately downstream of the initiating methionine to minimize possible expression of

a truncated protein with dominant-negative properties; (ii) it lacks a unit number of codons, thereby minimizing the generation of a protein product with an in-frame deletion retaining partial or complete function, due to splicing around the targeted exon; and (iii) it codes for a portion of Pbx2 that is highly conserved in humans, mice, and flies. Exon 1 was not targeted since no information was available regarding possible alternative initiation sites downstream of exon 1, within the first intron. Overall, the above-described strategy proved successful, as a complete knockout of *Pbx2* was obtained (Fig. 2).

The expected wt and mutated *Pbx2* alleles were observed by Southern blot analysis of DNA extracted from targeted ES cell lines (results not shown) and mouse tissues (Fig. 2B). Western blot analysis of postnatal thymocytes and bone marrow cells showed that homozygous mutant mice did not produce Pbx2 protein, the only high-MW-isoform Pbx protein detectable in these tissues (Fig. 2C). Furthermore, Western blot analysis of fetal livers at E16.5 with a Pbx2-specific antibody raised against a peptide mapping at the amino terminus of Pbx2 clearly demonstrated that homozygous mutant mice did not produce full-length (50 kDa) or truncated forms of Pbx2 (Fig. 2D). Northern blot analysis was performed to exclude the possibility that a mutant Pbx2 protein, not detectable by Western blot analysis, might be produced from an altered transcript encoded by the targeted *Pbx2* gene. This analysis demonstrated the absence of either full-length or truncated *Pbx2* transcripts in *Pbx2*^{-/-} embryos (Fig. 2E). *Pbx2*^{-/-} mice were viable, with binomial proportion analysis showing no deviations from a 2:1 ratio of heterozygote to wt or heterozygote to homozygous mutant (data not shown). The sizes and body weights of *Pbx2*^{-/-} mice were indistinguishable from those of their wt littermates (18.68 ± 1.25 g and 18.35 ± 1.34 g, respectively), as was their longevity and fertility (data not shown). The normal survival of *Pbx2*^{-/-} mice suggested that Pbx2 is not solely or primarily responsible for any processes that are critical for development or survival.

Pbx2 is not essential for organogenesis. Although lack of Pbx2 was compatible with life, this did not rule out possible subtle effects on organ structure. Major internal organs of *Pbx2*^{-/-} newborn mice and their wt littermates were examined grossly and by histology. No structural or morphological abnormalities of internal organs were observed in *Pbx2*^{-/-} mice (lung, liver, spleen, and kidney of wt and *Pbx2*^{-/-} mice are shown in Fig. 3). Furthermore, cartilage/skeletal preparations of *Pbx2*^{-/-} embryos and newborn mice showed neither gross nor subtle defects in cartilaginous and skeletal development (data not shown). These data demonstrate that lack of *Pbx2* expression has no detectable effect on the morphogenesis of organs in which it is normally expressed.

Pbx proteins are redundantly present in major organs during late embryogenesis. The absence of detectable phenotypes in *Pbx2*^{-/-} mice raised the possibility that other Pbx family proteins may compensate for the lack of Pbx2 function. This was assessed by determining the relative levels of Pbx2 proteins compared to the levels of the other highly related Pbx proteins. Immunoblotting experiments were performed with a monoclonal antibody (α-PbxL) that recognizes a Pbx2 epitope shared with the high-MW isoforms of Pbx1 and Pbx3 (Pbx1a and Pbx3a, respectively) but not present in the low-MW isoforms Pbx1b or Pbx3b (Pbx2 has no low-MW isoform). In most tis-

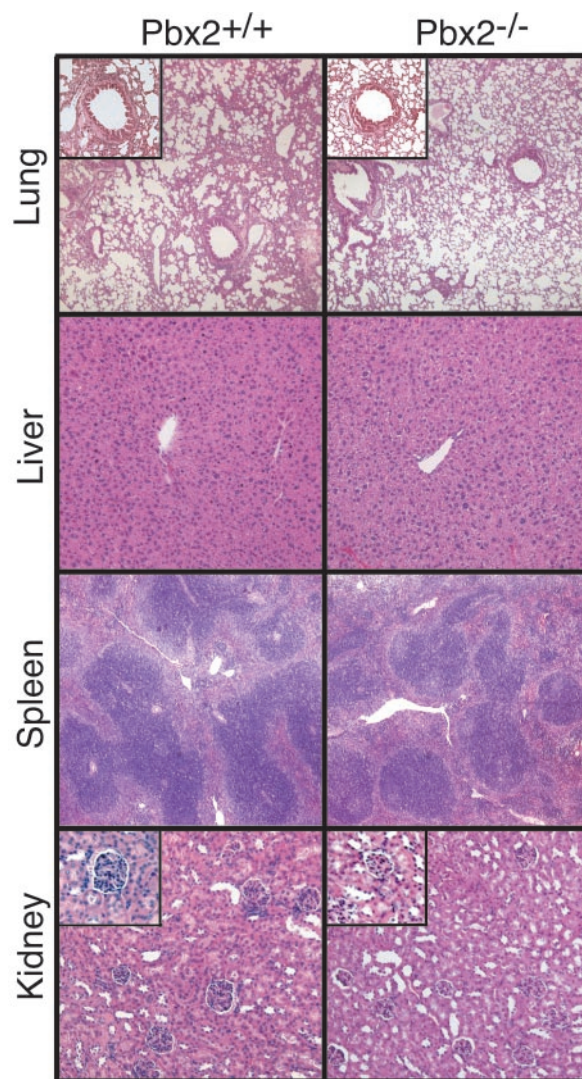


FIG. 3. Histological analysis of major organs of wt and *Pbx2*^{-/-} newborn mice. Formalin-fixed, paraffin-embedded tissue sections of various organs (indicated to the left) of adult wt and *Pbx2*^{-/-} littermates were stained with hematoxylin and eosin and photographed using a light microscope. Magnifications: lung, liver, and kidney, ×10; spleen, ×4; insets, ×20. All images were digitally processed.

sues examined from embryos at E17.5, there were no differences in the abundance of the high-MW-isoform Pbx proteins in *Pbx2*^{-/-} embryos as compared to wt embryos (Fig. 4A). A modest decrease was observed in fetal thymus, brain, and liver (Fig. 4A). The general lack of (or minimal) decrease in Pbx proteins detected with this antibody in tissues of *Pbx2*^{-/-} embryos suggested that Pbx2 is not the preponderant Pbx protein expressed in these tissues during embryogenesis. Furthermore, tissues and organs such as the developing brain, where *Pbx2* is highly expressed, as demonstrated by in situ hybridization (Fig. 1), were not affected either morphologically or functionally by its loss. Quantitative real-time RT-PCR showed that transcripts for other high-MW Pbx isoforms (Pbx1a and Pbx3a) were expressed in developing brain and other tissues (Fig. 4B and C), but there was no evidence of compensatory increases

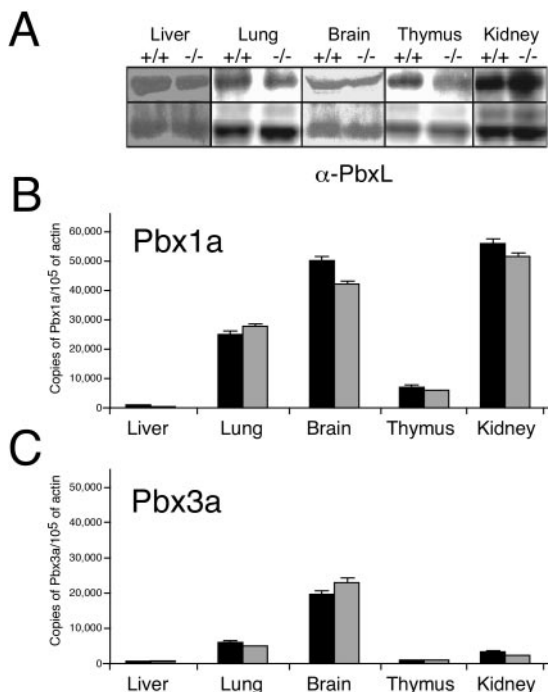


FIG. 4. Western blot and quantitative RT-PCR analysis of Pbx expression. (A) Protein extracts prepared from various tissues (indicated at the top) of E17.5 embryos were analyzed by Western blotting using a monoclonal antibody specific for all Pbx long-form proteins (α -PbxL). (B and C) Quantitative RT-PCR analysis of *Pbx1a* and *Pbx3a* expression. Total RNA was isolated from the indicated tissues of E17.5 wt (black bars) and *Pbx2*^{-/-} (grey bars) embryos. Bars (means \pm standard deviations) indicate numbers of transcripts normalized against 10^5 β -actin transcripts.

in their levels in *Pbx2*^{-/-} compared to wt embryos. Taken together, these observations suggested that normal organ development and survival of *Pbx2*^{-/-} mice is likely to result from functional compensation by other Pbx proteins. Interestingly, it has been reported that in zebra fish the *Pbx* genes are functionally equivalent in that each one, when overexpressed, can efficiently rescue the *lazarus/Pbx4* mutant phenotype (41).

Pbx2 is not essential for normal hematopoiesis. In contrast to fetal tissues, Pbx2 was observed to be the predominant high-MW-isoform Pbx protein expressed in postnatal bone marrow (Fig. 2C). This raised the possibility that Pbx2 may contribute to adult hematopoiesis. To determine whether the absence of Pbx2 had any noticeable effect on adult hematopoiesis, peripheral blood cell counts were determined at various ages of *Pbx2*^{-/-} and wt littermates. No statistically significant differences between *Pbx2*^{-/-} and wt mice were observed in hemoglobin content, erythrocyte indices, or leukocyte or platelet counts (Table 1 and data not shown). These data suggest that, despite the fact that Pbx2 is the predominant Pbx protein (high-MW isoform) expressed in adult bone marrow, its absence produces no measurable effect on the production of mature blood cells from immature progenitors. This is consistent with the finding that a large cohort of *Pbx2*^{-/-} mice achieved an age of 18 to 24 months without evidence of hematological disorders, including malignancy (data not shown).

TABLE 1. Hematological parameters of *Pbx2*-deficient mice

Age and genotype of mice and reference	Hemoglobin (mg/dl)	Platelet counts (10^3 /ml)	ANC ^a (10^3 /ml)	ALC ^b (10^3 /ml)
9–11 mo				
<i>Pbx2</i> ^{+/+}	13.6 \pm 1.9	1,167 \pm 239	817 \pm 469	7,266 \pm 3,697
<i>Pbx2</i> ^{-/-}	14.2 \pm 0.6	1,283 \pm 305	915 \pm 698	5,558 \pm 2,305
14–16 mo				
<i>Pbx2</i> ^{+/+}	14.3 \pm 0.3	1,342 \pm 654	1,035 \pm 798	8,986 \pm 1,48
<i>Pbx2</i> ^{-/-}	14.6 \pm 1.2	1,292 \pm 572	855 \pm 352	9,650 \pm 1,805
Normal range	13.7–16.4	675–1,338	825–2,604	3,685–7,812

^a ANC, absolute neutrophil count.

^b ALC, absolute lymphocyte count.

Pbx2 is not essential for lymphoid cell production or function. Pbx2 is also the predominant Pbx protein (high-MW isoform) expressed in the postnatal thymus (Fig. 2C). To investigate whether T-cell maturation might be affected by the absence of Pbx2, the relative proportions of lymphocyte subsets in wt versus *Pbx2*^{-/-} littermates were quantified by immunofluorescent staining and flow cytometry (fluorescence-activated cell sorter). Using standard B- and T-cell surface markers, no differences were detected in the absolute or relative numbers of B and T cells (Table 1 and Fig. 5). Moreover, when *Pbx2*^{-/-} mice were challenged with DNP conjugated to KLH they mounted humoral anti-DNP responses that were indistinguishable from those of wt littermates. These studies suggest that Pbx2 is not essential for normal lymphopoiesis and that lack of Pbx2 in the thymus does not detectably alter T-cell maturation. Since no overt autoimmunity manifestations (i.e., skin changes, weight loss) were observed in *Pbx2*^{-/-} mice, it seems likely that Pbx2 is also not required for tolerance induction.

Functional redundancy in the Pbx protein family. *Pbx2* null mice are viable and healthy with normal organ structure and hematopoietic and immune function, despite widespread *Pbx2* expression during embryogenesis. The lack of an obvious phenotype contrasts with the severe defects exhibited by mice deficient for Pbx1 or Pbx3. *Pbx1* null embryos do not survive past E16.5 and exhibit severe developmental defects in nearly every organ system (11, 15, 32, 34, 36) and homeotic transformation (36). Conversely, mice lacking Pbx3 survive to term but die within hours of birth due to an inadequate respiratory drive (Rhee et al., submitted). These phenotypes likely reflect a disruption of multiple developmental pathways subordinate to Hox and/or other homeodomain transcription factors that require a Pbx binding partner (17, 44). The lack of any obvious phenotype in the absence of Pbx2 indicates that its function as a DNA binding partner for Hox and/or other transcription factors is not essential.

Two models for mammalian Pbx protein function can be envisioned for conceptualizing the role of multiple Pbx isoforms and the divergent phenotypes induced by their absence. In a quantitative model, the various Pbx protein isoforms may be largely redundant, but with a requirement to maintain a critical threshold of Pbx protein concentration, regardless of

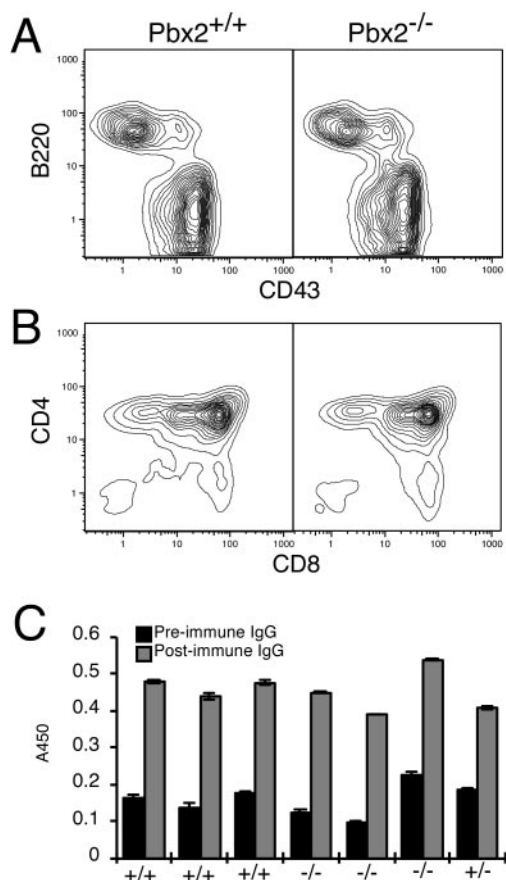


FIG. 5. Immunological studies of *Pbx2*^{-/-} mice. Fluorescence-activated cell sorter analyses of bone marrow cells (A) and thymocytes (B) of wt and *Pbx2*^{-/-} littermates demonstrated no detectable differences in lymphoid cell subsets. (C) IgG levels were comparable in the sera of wt and *Pbx2*^{-/-} littermates before and after immunization with the hapten DNP conjugated to KLH.

isotype, necessary for normal development. This scenario would be comparable to Pbx function in zebra fish, where the Pbx proteins are functionally equivalent (41) and is also supported by preliminary results indicating that *Pbx1*^{-/-}*Pbx2*^{+/+} compound mutant embryos die in utero at E13.5, earlier than *Pbx1*^{-/-} embryos, which succumb at E15/16 (T. Capellini and L. Selleri, unpublished observations). Since our immunoblot analyses indicated that Pbx2 does not seem to be the preponderant Pbx protein in most tissues during embryonic development, its absence would not substantially reduce the total Pbx protein levels below the necessary threshold, thus accounting for the lack of measurable phenotypes. Alternatively, in a qualitative model, various Pbx isoforms (high versus low MW) may display different functional properties, consistent with *in vitro* biochemical studies of their divergent transcriptional effector properties (2). However, our analyses indicate that *Pbx2* null mice lack detectable phenotypes attributable to deficiencies in tissues (thymus and adult bone marrow) that express Pbx2 as the only high-MW Pbx isoform protein. Since these tissues also express low-MW Pbx isoforms, such as Pbx1b, they may be able to functionally replace Pbx2. The alternative possibility that Pbx protein function is simply not required for postnatal he-

matopoietic and immune function has not been conclusively ruled out since *Pbx1*- and *Pbx3*-deficient mice die in utero or in the immediate postnatal period, respectively (Rhee et al., submitted; 36). Our results are most consistent with a quantitative model of mammalian Pbx protein function, but elucidation of the potential interrelationships and overlapping functions of Pbx family proteins in different developmental pathways will require detailed characterization of various organ systems in compound null mice.

ACKNOWLEDGMENTS

We thank Joseph Lipsick and Uta Francke for many helpful discussions; Michael Depew, Inma Cobos, and Stewart Anderson for comments on the manuscript; and Cita Nicolas, Maria Ambrus, and Lawryn Kasper for expert technical assistance.

These studies were supported by grants to M.L.C. from the National Institutes of Health (CA42971, CA70704, and CA90735) and to L.S. from the March of Dimes and Birth Defects Foundation (6-FY03-071) and the National Institutes of Health (HD043997-01 A1).

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