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Phemx, a Novel Mouse Gene Expressed in Hematopoietic Cells Maps to the Imprinted Cluster on Distal Chromosome 7

Rhonda H. Nicholson,*[,1] Serafino Pantano,†[,1] James F. Eliason,‡ Anne Galy,‡ Sarah Weiler,§ Joseph Kaplan,¶ Mark R. Hughes,* and Minoru S. H. Ko†[,2]

*Center for Molecular Medicine and Genetics, ‡Barbara Ann Karmanos Cancer Institute, ¶Department of Pediatrics, Wayne State University School of Medicine, Detroit, Michigan 48201; and †Developmental Genomics and Aging Section, Laboratory of Genetics, and §Laboratory of Immunology, National Institute on Aging, National Institutes of Health, Baltimore, Maryland 21224

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Phemx **(Pan hematopoietic expression) is a novel murine gene expressed in developmentally regulated sites of hematopoiesis from early in embryogenesis through adulthood. *Phemx* is expressed in hematopoietic progenitors and mature cells of the three main hematopoietic lineages. Conceptual translation of the murine *Phemx* cDNA predicts a 25-kDa polypeptide with four hydrophobic regions and several potential phosphorylation sites, suggestive of a transmembrane protein involved in cell signaling. The PHEMX protein is structurally similar to tetraspanin CD81 (TAPA-1), a transmembrane protein involved in leukocyte activation, adhesion, and proliferation. *Phemx* maps to the distal region of chromosome 7, a segment of the mouse genome that contains a cluster of genes that exhibit genomic imprinting. However, imprinting analysis of *Phemx* at the whole organ level shows that it is biallelically expressed, suggesting that mechanisms leading to monoallelic expression are not imposed at this locus. The human *PHEMX* ortholog is specifically expressed in hematopoietic organs and tissues and, in contrast to murine *Phemx,* undergoes alternative splicing. The unique mode and range of *Phemx* expression suggest that it plays a role in hematopoietic cell function.** © 2000 Academic Press

INTRODUCTION

The distal end of mouse chromosome 7 and the syntenic region of human chromosome 11p15 contain a cluster of genes that undergo genomic imprinting, a mechanism of transcriptional regulation wherein only one of two alleles is expressed (reviewed in Barlow (1995); Bartolomei and Tilghman (1997); Reik and Walter (1998); Falls *et al.* (1999)). At least eight imprinted genes have been identified in this region in both human and mouse; these include *IPL, ORCTL2/IMPT1,* p57^{KIP2}, *Kvlqt1, ASCL2, Igf2, H19,* and *CD81/TAPA1* (Reid *et al.,* 1997; Paulsen *et al.,* 1998). Chromosomal breakpoints in this domain have been identified in patients with embryonal tumors and Beckwith–Wiedemann syndrome, an overgrowth disorder, suggesting that genes in this region play a role in tumor suppression and embryonic growth (Hoovers *et al.,* 1995; Feinberg, 1998).

Recently, a novel expressed sequence tag (EST), *D7Wsu37e,* was mapped to this imprinted region through a sequencing and mapping project of genes expressed in murine extraembryonic tissue (Ko *et al.,* 1998). Initial characterization of *D7Wsu37e* by Northern blot analysis detected a transcript only in the spleen, suggesting hematopoietic-specific expression.

Hematopoiesis is the complex process wherein long-term repopulating hematopoietic stem cells leave their cycle of self-renewal to differentiate and become one of eight distinct mature cell types in the lymphoid, myeloid, and erythroid lineages (Morrison *et al.,* 1995). In the mouse, hematopoiesis begins in the yolk sac at embryonic day 7 (E7) (Dzierzak *et al.,* 1998). At E10, the aorta–gonad–mesonephros region (AGM) becomes a source of definitive fetal/adult hematopoiesis. By E11 the number of hematopoietic progenitors in the AGM decreases, accompanied by an increase in progenitors in the liver. The liver is the principal site of fetal hematopoiesis from E11 through the neonatal period, when the bone marrow progressively replaces it as the primary hematopoietic tissue (Rugh, 1990).

In this report we demonstrate that *D7Wsu37e,* now designated *Phemx,* has a spatiotemporal expression pattern that follows the ontogeny of murine hematopoiesis from early in embryogenesis through adulthood. In addition, we show that the *PHEMX* human ortholog exhibits hematopoietic-specific expression as well as alternative splicing. Imprinting analyses of

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[1] These two authors contributed to this work equally.

[2] To whom correspondence should be addressed at Developmental Genomics and Aging Section, Laboratory of Genetics, NIA–NIH, 333 Cassell Drive, Suite 4000, Baltimore, MD 21224-6820. Telephone: (410) 558-8359. Fax: (410) 558-8331. E-mail: KoM@grc.nia.nih.gov.

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murine *Phemx* are also described, and its possible function is discussed in light of expression patterns and sequence motifs.

MATERIALS AND METHODS

Gene mapping. The chromosomal locations of the *H19* and *Cyp2e1* genes were determined as described (Ko *et al.,* 1998). The PCR primer pairs used for the H19 gene (*H19;* GenBank Accession No. MMH19) were 5′-ATTGCACTGGTTTGGAGTCCCG-3′ and 5′-GAGGGGCAAAGGATGAAGTAGG-3′. The PCR primer pairs used for the P450E1 gene (*Cyp2e1;* GenBank Accession No. MUSP450E1) were 5′-AATCTGAAGTCTCTGGTTGACC-3′ and 5′-TTACCCACT-GAGCCATCTCACC-3′. These primer pairs were used for genotyping 94 interspecific backcross animals from The Jackson Laboratory BSS cross (C57BL/6JEi × SPRET/Ei)F_1 × SPRET/Ei that are also typed for over 5000 other markers (Rowe *et al.,* 1994).

Northern blot analyses. Membranes for adult mouse organs, human organs, and human immune tissue were purchased from Clontech. Murine and human Northern blots were hybridized to ^{32}P-labeled *Phemx* or *PHEMX* isotype 1 cDNA probes, respectively, using the ExpressHyb hybridization solution protocol (Clontech).

Cloning and sequence analyses of cDNAs. The murine *Phemx* cDNA was cloned, and approximately 400 bp of sequence from both the 5′ and the 3′ ends were obtained as described (Ko *et al.,* 1998). The intervening *Phemx* cDNA sequence was obtained using gene-specific primers. Human *PHEMX* isotype 1 and *PHEMX* isotype 2 cDNA clones were obtained from Research Genetics (IMAGE ID 235072 and 453319) and were completely sequenced. DNA sequence analyses were performed with MacDNASIS Pro (Hitachi Software, San Francisco, CA), Lasergene (DNASTAR, Inc., Madison, WI), and the PROSITE program (Bairoch *et al.,* 1997).

Allele-specific gene expression analyses of mouse Phemx. Total RNA was isolated from various organs of F_1 hybrid offspring produced by crossing C57BL/6J females with *Mus spretus* males. Then 2.5 μg of total RNA from each sample was treated with RNase-free DNase I (Gibco BRL), and cDNA synthesis was carried out using oligo(dT) and the Superscript Preamplification System for First Strand cDNA Synthesis (Gibco BRL). To control for genomic DNA contamination, parallel cDNA synthesis reactions were performed in the absence of reverse transcriptase. For all RT-PCRs described in this paper, β-actin was used as a positive control (data not shown). Amplification of target cDNA was performed in a 50-μl reaction volume using 2 μl of the first-strand cDNA synthesis reaction. The *Phemx* oligonucleotide primers were 5′-TAATCCCAAACACGCAT-CAA-3′ and 5′-TACTGAAATGTGCAGAACCCC-3′. β-actin was used as an internal standard; the primer sequences are 5′-CCCAAC-TTGATGTATGAAGG-3′ and 5′-TTGTGTAAGGTAAGGTGTGC-3′. PCR conditions were as follows: 95°C for 60 s for 1 cycle, 94°C for 30 s, 58°C for 30 s, and 72°C for 60 s for 30 cycles, 72°C for 3 min for 1 cycle. Fourteen microliters of each *Phemx* PCR mixture was digested with 20 units of *Alu*I in a 20-μl reaction, and the digests were fractionated on a 10% polyacrylamide gel.

Expression analyses of mouse Phemx in flow-sorted bone marrow cells. Mouse bone marrow cells were flushed into nutrient medium supplemented with 5% fetal bovine serum, and they were used for fluorescence-activated cell sorting (FACS Vantage, Becton Dickinson). Aliquots containing approximately 10^5 cells were incubated with the monoclonal antibodies TER-119, B220, or GR-1 (Pharmingen, San Diego, CA) that recognize antigens on erythroid, B-lymphocyte, and granulocyte lineages, respectively. T-lymphocytes were stained with an FITC-conjugated antibody against CD3. After being washed to remove unbound antibodies, the cells were incubated with FITC-conjugated goat anti-mouse antibodies. Erythroid cells were sorted on the basis of low to medium forward and side light scatter and high FITC fluorescence. The sorted population represented 3.5% of the bone marrow cells. B- and T-lymphocytes were gated on similar scatter characteristics and represented 7.0 and 3.5% of the unfractionated population, respectively. Granulocytes were selected on the basis of low forward scatter, high side scatter plus high fluorescence and were 2.5% of the population. Lineage negative blast cells were prepared by incubating with a mixture of the antibodies TER-119, B220, MAC-1, GR-1, and anti-CD2, followed by incubation with FITC-conjugated goat anti-mouse antibodies as described above. The blast population (low intermediate forward scatter and low side scatter) was sorted on the basis of low FITC fluorescence. This represented 5% of the population. Bone marrow-derived macrophages were obtained as previously described (Li and Chen, 1995). Each sample of sorted cells was pelleted, and total RNA was extracted using 500 μl of the Trizol reagent (Gibco BRL). Total RNA from each sample was resuspended in 8 μl of RNase-free water and treated with RNase-free DNase I. First-strand cDNA synthesis was carried out as described above. Samples of cDNA were prepared from Lin^{Lo} c-$Kit^+$$Sca$-$1^+$ cells, as previously described (Weiler *et al.,* 1999).

One microliter of cDNA was used in a 10-μl PCR containing 20 mM Tris–HCl (pH 8.4), 50 mM KCl, 1.5 mM $MgCl_2$, 1 mM each dATP, dCTP, dGTP, and dTTP, 0.5 units *Taq* polymerase, and a 1 μM concentration of the primers 5′-TGCTAACTGTTCAACAAC-CCTCTAAAGCA-3′ and 5′-CCAAACACGCATCAACTAAACGCC-3′. PCR was carried out as described above, with β-actin as an internal standard.

Expression analysis of human PHEMX. All human tissues were obtained with approval from the Institutional Review Board of Wayne State University. Bone marrow mononuclear cells ($d < 1.077$ g/ml) were obtained after centrifugation through a Ficoll gradient (Pharmacia Biotech Inc., Piscataway, NJ). Monocytes and $CD34^+$ cells were obtained from the blood of cancer patients undergoing peripheral mobilization for autologous transplant. Monocytes were prepared as described (Ferlazzo *et al.,* 1999) by plastic adherence; the population contained >90% $CD14^+$ cells. $CD34^+$ cells were prepared by magnetic bead selection (Miltenyi Biotec GmBH, Sunnyvale, CA) as described (Ferlazzo *et al.,* 1999) and were routinely >85% pure. Dendritic cells were prepared by culture of $CD34^+$ hematopoietic progenitor cells in the presence of cytokines as described (Ferlazzo *et al.,* 1999). At day 14, the culture was purified with specific antibodies and flow cytometry sorting to isolate $CD1a^+$, $CD14^-$ dendritic cells on the Vantage instrument (Becton Dickinson). T-cells were obtained from blood, maintained with IL-2 for several weeks, and contained >99% $CD3^+$ T-cells. Cell lines, including Jurkat, RAMOS, U937, K562, and the nonhematopoietic cell lines SKBR3, MCF-7, 24SV48, and HUVEC, were cultured in RPMI 1640 medium (Gibco) supplemented with 10% calf serum (Hyclone, Logan, UT). PMA-stimulated K562 cells were prepared by culture for 24 h in the presence of 10 ng/ml phorbol myristate acetate (Sigma, St. Louis, MO). For monocytes, $CD34^+$ cells, dendritic cells, and cultured T-cells, cDNA was prepared from 1 μg of total RNA in a 100-μl reaction containing 80–160 units RNAsin (Promega, Madison, WI), 50 mM Tris–HCl (pH 8.3), 75 mM KCl, 3 mM $MgCl_2$, 100 mM DTT, 1 mM each dATP, dCTP, dGTP, dTTP, 2.5 μM random hexamers (Genosys), and 400 units Moloney murine leukemia virus reverse transcriptase (Promega) for 75 min at 37°C. For all other samples, cDNA was prepared from 2.5 μg total RNA using the Superscript Preamplification System for first-strand cDNA synthesis (Gibco) as described above in allele-specific gene expression by RT-PCR analyses. Amplification of target cDNA was performed in a 10-μl reaction volume using 1 μl of cDNA as described above. The *PHEMX* oligonucleotide primers were 5′-TCTTACCTACT-TCGGGGCCCACTTT-3′ and 5′-CGGACGTGGGACGTACCTTTCA-3′. PCR conditions were as follows: 95°C for 60 s for 1 cycle, 94°C for 30 s, 58°C for 30 s, and 72°C for 60 s for 30 cycles, 72°C for 3 min for 1 cycle.

In situ hybridization analyses. Digoxigenin-labeled RNA probes were prepared from the full-length cDNA (1538 bp) and were hydrolyzed with a limited alkaline hydrolysis as previously described (Wilkinson and Nieto, 1993). *In situ* hybridization on mouse embryos was carried out on 7–10 μm-thick sections according to the standard protocol (Wilkinson and Nieto, 1993). *In situ* hybridization studies on neonate organs and adult spleen were carried out on 15-μm-thick

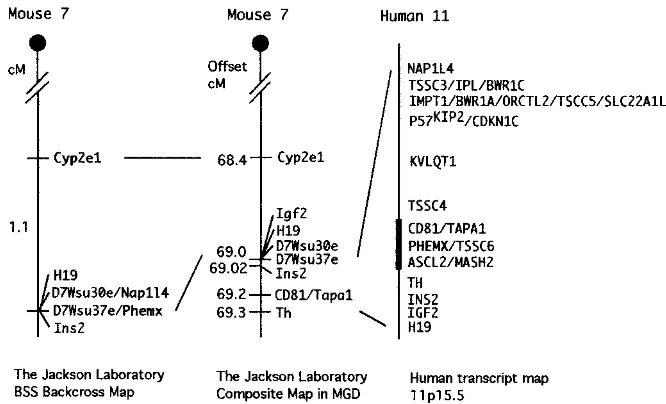

FIG. 1. Mapping of the murine *Phemx* gene. **(Left)** Genetic linkage map of chromosome 7 obtained by genotyping The Jackson Laboratory BSS Backcross Mouse Panel. **(Center)** A composite map in the Mouse Genome Database (MGD). **(Right)** A schematic physical map of human chromosome 11p15.5 (Paulsen *et al.,* 1998). A boldface line indicates the location of a completely sequenced 141-kb human PAC clone (GenBank Accession No. AC002536) that encodes *PHEMX/TSSC6, CD81/TAPA-1,* and *ASCL2/MASH2.*

cryosections according to a previously described protocol (Schaeren-Wiemers and Gerfin-Moser, 1993). No hybridization was detected in control experiments where alternate sections were hybridized to the sense probe.

RESULTS

Fine-Mapping of the Phemx Gene

In our previous work, D7Wsu37e (clone C0002D08, renamed *Phemx*) was mapped to the distal region of mouse chromosome 7 (Ko *et al.,* 1998), a region containing a cluster of genes that exhibit genomic imprinting (Reid *et al.,* 1997; Paulsen *et al.,* 1998). To localize *Phemx* relative to known genes in this region, *H19* and *Cyp2e1* were mapped on the same mapping panel. There was no recombination between *D7Wsu30e* (*Nap114*), *Phemx* (*D7Wsu37e*), *H19,* and *Ins2,* suggesting that these genes fall within about 2 Mb (Fig. 1).

During the characterization of the murine *Phemx* gene, sequences of a PAC clone from human chromosome 11p15, which carries the apparent human ortholog of murine *Phemx,* were deposited with GenBank (Accession No. AC002536). Although this gene was not annotated in the genomic DNA sequence, the sequence allowed us to localize the human *PHEMX* gene in the midst of tightly clustered imprinted genes (Fig. 1). Subsequently, we identified two human *PHEMX* cDNA clones in the public EST collection; their sequences were submitted to GenBank and they were assigned Accession Nos. AF176070 and AF176071. With the addition of Phemx, the genomic region between *TSSC3/IPL/BWR1C* and *H19* contains 12 genes, 8 of which have been clearly identified as imprinted genes. The chromosomal location of murine *Phemx* suggested that this gene could be a candidate for imprinting.

Northern Blot Analyses of Phemx/PHEMX Gene Expression

Initial characterization of murine *Phemx* mRNA by Northern blot analysis, using the 1.5-kb cDNA as a probe, showed that a transcript is detected only in the spleen on an adult mouse multiple organ Northern blot (Fig. 2A), suggestive of a hematopoietic-specific gene.

Northern blot analysis using a 1.2-kb orthologous human (*PHEMX*) cDNA (AF176070) as a probe detected four distinct transcripts ranging in size from 1.5 to 4.4 kb in peripheral blood leukocytes and spleen on an adult human multiple organ Northern blot (Fig. 2D). A similar hybridization pattern was produced in all but thymus on a human immune tissue Northern blot (Fig. 2D). The hybridization to discrete transcripts in a defined size range is indicative of alternative splicing (see below).

These results show that both murine *Phemx* and human *PHEMX* are expressed from the fetal period through adulthood and that expression in the adult is specific to hematopoietic organs and tissues.

Characterization of the Phemx/PHEMX cDNA Sequence

The mouse *Phemx* cDNA (Accession No. AF175771) (Fig. 3A) contains a translation start codon within a sequence context favorable for initiation, as defined by Kozak (1996), and encodes a polypeptide 226 amino acids in length. That the 1.5-kb *Phemx* cDNA clone contains the entire mRNA sequence is supported by the Northern analysis showing a 1.8-kb message that includes a poly(A) tail several hundred nucleotides in length (Fig. 2A).

Hydrophobicity and secondary structure profiles of the predicted murine PHEMX polypeptide reveal three hydrophobic regions suggestive of transmembrane domains. A fourth hydrophobic region is predicted to form a β-pleated sheet; this domain may cross the membrane or alternatively may be involved in intramolecular hydrophobic interactions. The predicted murine PHEMX polypeptide contains three protein kinase C consensus recognition sites at residues Thr26, Thr53, and Ser156 (Woodgett *et al.,* 1986). Additionally, there is a potential tyrosine kinase phosphorylation site at residue Tyr29 (Cooper *et al.,* 1984) and two casein kinase II consensus phophorylation sites at residues Thr53 and Thr87 (Pinna, 1990).

Human *PHEMX* cDNA (Accession No. AF176070), termed *PHEMX* isotype 1, is 1200 bp in length and encodes a polypeptide 124 amino acids in length. Human cDNA clone (Accession No. AF176071), termed *PHEMX* isotype 2, is a partial cDNA clone in that the 5′ untranslated region and initiation codon are not present. Virtual translation of the *PHEMX* isotype 2 cDNA sequence, however, predicts a polypeptide with 73% similarity to the predicted murine PHEMX protein (Fig. 3B). A nucleotide similarity search using the BLASTn program identified a third human *PHEMX*

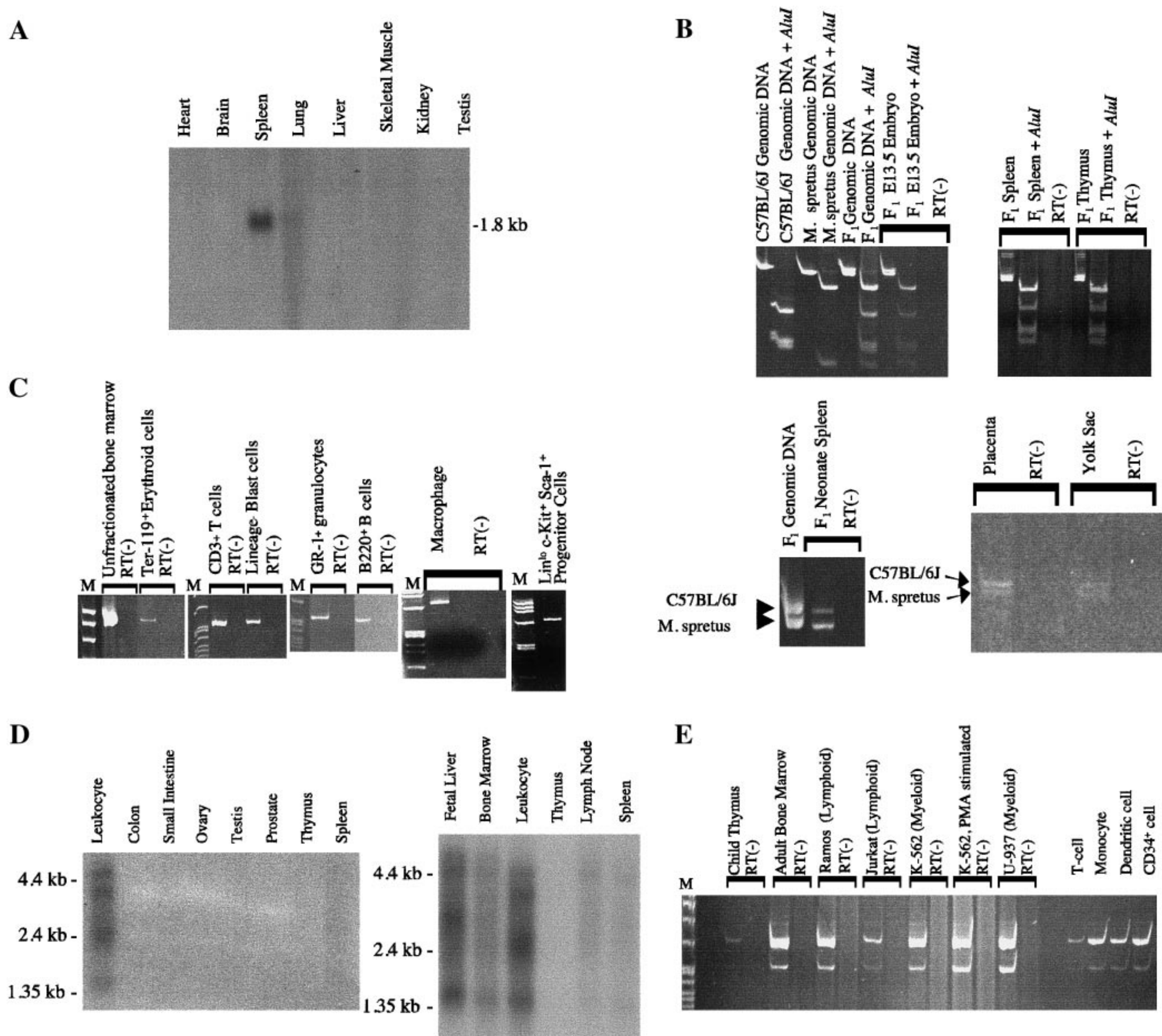

FIG. 2. Expression analysis of murine and human *PHEMX.* **(A** and **D)** Northern blot analyses where the full-length murine *Phemx* and human *PHEMX* isotype 1 cDNA sequences were hybridized, respectively, to Northern blots containing 2 μg of poly(A)$^+$ RNA per lane from **(A)** various adult mouse organs and **(D)** adult human organs and human immune tissue. **(B)** RT-PCR analysis of biallelic and organ-specific expression in F_1 (C57BL/6J female × SPRET/Ei male) E13.5 embryos and extraembryonic tissue, neonate spleen, and organs of 1-month-old animals. **(C)** Analysis of *Phemx* expression in hematopoietic progenitors and mature cells of the three main hematopoietic lineages. **(E)** RT-PCR expression analysis of human *PHEMX* in hematopoietic tissue and cell lines. Lanes containing control RT-PCRs performed without reverse transcriptase are labeled RT(−).

cDNA, named *TSSC6* (Lee *et al.,* 1999). In that report, the cDNA and predicted amino acid sequences were presented as well as the imprinting analysis of this gene in human. However, the hematopoietic expression pattern of *TSSC6* was not reported.

Comparative sequence analysis of human PAC clone AC002536 with the *PHEMX* cDNA clones and *TSSC6* revealed the intron/exon structure as well as alternative splicing in the gene, as suggested by Northern analysis (Fig. 3C). In the *PHEMX* isotype 1 cDNA clone, use of an alternative splice acceptor site in exon 4 leads to a reading frameshift that conceptually translates into a polypeptide with two predicted transmembrane domains (Figs. 3B and 3C). The *PHEMX* isotype 2 cDNA clone and *TSSC6* maintain the same open reading frame through exon 7 and are predicted to encode a polypeptide containing three putative transmembrane domains with a membrane topology similar to murine PHEMX. Alternative splicing in exon 8a of *PHEMX* isotype 2 and *TSSC6* leads to variation in their predicted carboxy-termini. Both human *PHEMX* cDNA clones and *TSSC6* share the same final exon and polyadenylation signal (Fig. 3C). Conservation of potential phosphorylation sites in both mouse and human for protein kinase C at Thr53, casein kinase II at Thr53 and Thr87, and tyrosine kinase at Tyr29 in the

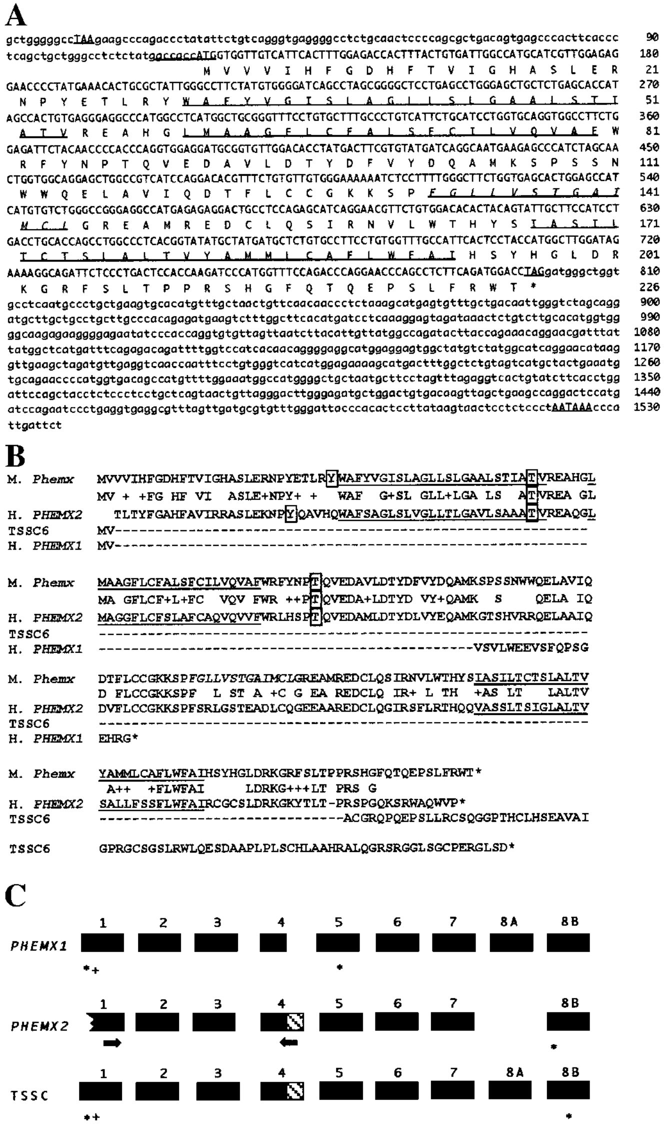

FIG. 3. Sequence analyses of murine and human PHEMX. **(A)** Nucleotide and predicted amino acid sequence of the murine *Phemx* gene. The 5′ in-frame stop codon, mammalian consensus translational start sequence, stop codon, and polyadenylation sequence are underlined. In the amino acid sequence, hydrophobic residues that are potential transmembrane domains are also underlined. The hydrophobic sequence predicted to form a β-pleated sheet is shown in italics. **(B)** Alignment of the predicted amino acid sequences of murine PHEMX, human PHEMX isotype 1 and isotype 2, and TSSC6. Identical or similar (+) amino acids shared between murine and human PHEMX are displayed between the two sequences. Identical human PHEMX and TSSC6 amino acids are denoted by a dashed line. Predicted transmembrane domains are underlined. Conserved potential phosphorylation sites for tyrosine kinase and casein kinase II are boxed. **(C)** Exon structure of human *PHEMX* splice variants and *TSSC6.* Start codons (+), stop codons (*), and positions of PCR primers (arrows) are also shown. The segment of exon 4 that maintains an open reading frame predicted to encode a polypeptide similar to murine PHEMX is shown as a hatched box.

mouse and Tyr24 in the human suggests that these residues could play a role in PHEMX function.

A BLASTp (Altschul *et al.,* 1990) search using the predicted murine PHEMX amino acid sequence as a query sequence revealed a low level of similarity (33%) to rat, mouse, and human CD81 (TAPA-1). CD81 is a member of the tetraspanin family of cell surface proteins. In addition to the predicted structural similarity, PHEMX contains two cysteine-containing sequences, CCG and EDC, that are conserved motifs in the tetraspanin family.

Allele- and Organ-Specific Expression Analysis of the Phemx Gene

To analyze the imprinting status of murine *Phemx* and to determine whether *Phemx* expression is restricted to organs involved in hematopoiesis, as suggested by Northern analysis, RT-PCR analysis was performed on organs of (C57BL/6J × SPRET/Ei)F_1 hybrid animals. The DNA sequence in the 3′-UTR of the SPRET/Ei (*M. spretus*) *Phemx* gene contains an *Alu*I site that is not present in the C57BL/6J sequence. This allowed us to distinguish the PCR product from each allele in PCRs that amplified the 3′-UTR. This region also contains a length polymorphism such that the PCR product from each allele can be resolved by 10% polyacrylamide gel electrophoresis without restriction digestion.

Analysis of *Alu*I-digested *Phemx* PCR-amplified transcripts from the E13.5 embryo and from the spleen and thymus of the adult mouse, where expression is strongest, demonstrates that *Phemx* is biallelically expressed (Fig. 2B). Biallelic expression in the F_1 neonate spleen is shown by the length polymorphism between the C57BL/6J and *M. spretus* allele-specific PCR products (Fig. 2B). The biallelic expression of *Phemx* in midgestation circulating blood cells is also indicated, as these are the probable source of the low-level allele-specific *Phemx* PCR products detected in E13.5 yolk sac and placenta (Fig. 2B). Similarly, biallelically expressed *Phemx* PCR products detected in the adult liver and other nonhematopoietic organs are likely to have originated from transcripts present in circulating blood cells. The low level of *Phemx* PCR products observed in the adult liver illustrates the downregulation of *Phemx* expression at sites no longer active in hematopoiesis (data not shown).

Expression of Phemx in Flow-Sorted Cells and Cultured Cells

To determine whether *Phemx* expression is restricted to a specific stage of hematopoietic cell differentiation or is present in only certain hematopoietic lineages or cell types, RT-PCR was carried out on RNA from C57BL/6J adult mouse bone marrow cells isolated using cell-type-specific antibodies and fluorescence-activated cell sorting. The *Phemx* PCR product is observed in unfractionated bone marrow, Terr 119^+ erythroid cells, $CD3^+$ T-cells, lineage-negative blast cells, GR-1^+ granulocytes, $B220^+$ B-cells, and cultured macrophages derived from bone marrow (Fig. 2C). The *Phemx* PCR product is also observed in Lin^-c-Kit^+Sca-1^+ cells, a population of hematopoietic progen-

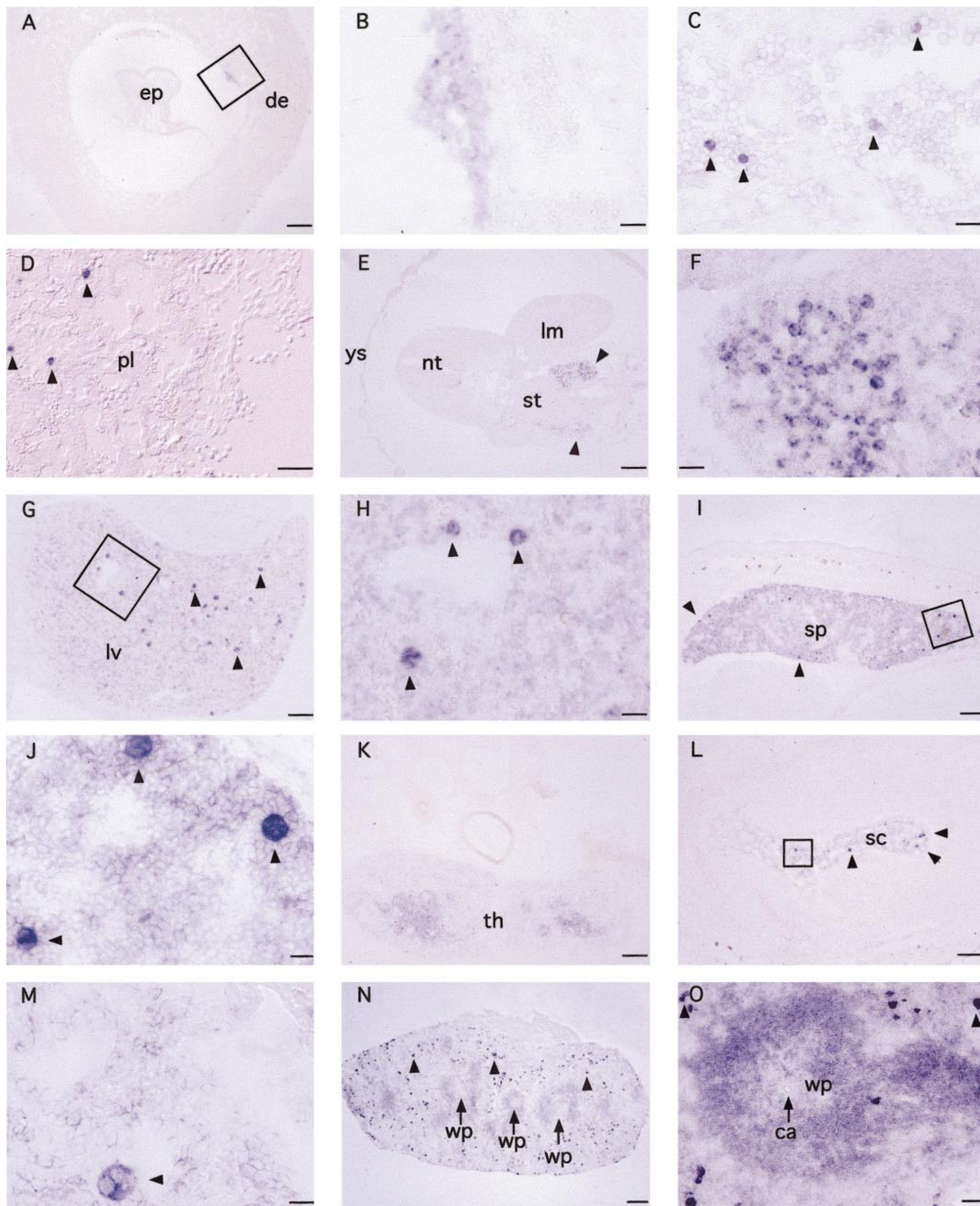

FIG. 4. *Phemx* expression analyses in the mouse by *in situ* hybridization. Cells expressing *Phemx* transcripts are indicated by arrowheads in **C, D, G, H, I, J, L, M, N,** and **O. (A)** E8.5 embryo *in utero* (frame: blood island). **(B)** Higher magnification of blood island shown framed in **A. (C)** E9.5 embryonic bloodstream. **(D)** E10.5 placenta. **(E)** E10.5 embryo; arrowheads indicate hepatic primordium wherein *Phemx*-expressing cells are localized. **(F)** Higher magnification of a segment of the hepatic primordium shown in **E. (G)** E13.5 liver. **(H)** Higher magnification of liver shown framed in **G. (I)** Neonate (day 1) spleen. **(J)** Higher magnification of spleen shown framed in **I. (K)** Neonate (day

itors that are purified from adult bone marrow and include pluripotential hematopoietic stem cells (Fig. 2C). The $Lin^-c\text{-}Kit^+Sca\text{-}1^+$ cells are defined as hematopoietic progenitors based on their ability to form day-12 spleen colony-forming units in lethally irradiated recipients and confer radioprotection through marrow repopulation (Li and Johnson, 1995). Taken together, these results suggest that *Phemx* expression in hematopoietic progenitor cells is maintained as they differentiate into mature cells of the lymphoid, myeloid, and erythroid lineages.

Expression of Human PHEMX in Hematopoietic Organs, Flow-Sorted Cells, and Cultured Cells

To examine *PHEMX* expression in human organs and cells by RT-PCR analysis, a reverse primer was designed to amplify transcripts encoding the full-length PHEMX polypeptide deduced from the sequence of the *PHEMX* isotype 2 cDNA clone and *TSSC6* (Figs. 3B and 3C). Human *PHEMX* transcripts are detected at low levels in child thymus, cultured blood T-cells, and the Jurkat T-cell line (Fig. 2E). Higher levels of transcripts appeared in adult bone marrow mononuclear cells, in the small fraction of $CD34^+$ hematopoietic progenitor cells, in blood monocytes, and in cultured purified dendritic cells (Fig. 2E). Human PHEMX transcripts were also detected in the myeloid cell lines U-937 and K-562 and in K-562 cells stimulated with phorbol myristate acetate to induce differentiation and acquisition of megakaryocytic characteristics (Tetteroo *et al.,* 1984). To confirm the human *PHEMX* hematopoietic expression suggested by Northern blots, RT-PCR analysis was carried out in nonhematopoietic cell lines. No *PHEMX* transcripts were detected in the endothelial cell line HUV-EC-C, breast epithelial cell lines MCF-7 and SK-BR-3, or SV-40-transformed thymus epithelial cell line 24SV48 (Galy *et al.,* 1993) (data not shown).

Tissue-Specific Expression Analyses of Phemx in the Developing Mouse

To examine the pattern of expression of *Phemx* RNA during mouse development, *in situ* hybridization experiments were carried out on sections of staged embryos. Although the gene was recovered from an E7.5 extraembryonic tissue cDNA library, we could not detect any signal by *in situ* hybridization at E7.5. Beginning at E8–E8.5, *Phemx* transcripts were detected, but only in rounded cells within the blood islands (Figs. 4A and 4B) of the yolk sac. No *Phemx* transcripts were detected in the flat vascular endothelial cells around the blood islands. At E9.5 and E10.5, *Phemx* transcripts were detected in single scattered cells in the bloodstream of the yolk sac (data not shown), embryo, and developing placenta (Figs. 4C and 4D). At E10.5 the hybridization signal was also clearly evident in the hepatic primordium (Figs. 4E and 4F). However, with this technique we could not detect *Phemx* expression in cells of the para-aortic splanchnopleura or the AGM, where hematopoietic stem cells are likely to arise (Wood *et al.,* 1997; Jaffredo *et al.,* 1998). Since the vitelline and embryonic circulations are linked at E8.5 (Garcia-Porrero *et al.,* 1995; Cumano *et al.,* 1996), the *Phemx*-expressing cells detected at E9.5 and E10.5 could have originated in the yolk sac. Alternatively, *Phemx* expression could define a step in hematopoietic cell differentiation that is subsequent to the pluripotent state of stem cells in the AGM. This interpretation is consistent with widespread *Phemx* expression observed in the more differentiated hematopoietic cells of the blood islands.

At E13.5 *Phemx* transcripts were detected in the liver (Figs. 4G and 4H). Here *Phemx*-expressing cells contain varying amounts of *Phemx* messenger RNA, as demonstrated by large numbers of lightly stained cells as well as single, scattered, darkly stained cells. *Phemx* transcripts were also detected in a few isolated cells in the blood vessels (data not shown). Based on these results, it is likely that *Phemx* transcripts observed in the E13.5 embryo by RT-PCR analysis originated primarily from the liver. These results also suggest that the low level of *Phemx* PCR products detected in E13.5 yolk sac and placenta came from circulating blood cells expressing the *Phemx* gene.

In the neonate spleen, *Phemx* expression is detected as a diffuse signal throughout most of the organ (Fig. 4I). Stronger signals are also detected in single, isolated cells (Fig. 4J). In the neonate thymus, *Phemx* transcripts are detected in the region of the developing medulla, located in the central portion of the thymic lobes (Fig. 4K). In contrast, there is no clear *Phemx* expression detected in the thymic cortex. In neonate bone marrow, *Phemx* expression is observed in single cells as well as cell clusters (Figs. 4L and 4M). In the adult spleen, a strong signal is detected in single cells as well as in cell groups (Figs. 4N and 4O). The diffuse signal observed in the neonate spleen is now mainly restricted to the white pulp. These data demonstrate that *Phemx* expression is restricted to certain cells of hematopoietic organs in the developing mouse.

DISCUSSION

We present here the molecular characterization of a novel mouse gene, *Phemx,* and its human ortholog.

1) thymus. **(L)** Neonate (day 1) scapula. **(M)** Higher magnification of cells shown framed in **L. (N)** Adult spleen (note the lower level of *Phemx* transcripts in white pulp). **(O)** Adult spleen: higher magnification and longer exposure of white pulp. (de) decidua, (ca) central artery, (ep) embryo proper, (lm) limb, (lv) liver, (nt) neural tube, (pl) placenta, (sc) scapula, (st) stomach, (sp) spleen, (th) thymus, (wp) white pulp, (ys) yolk sac. Scale bars are 200 μm in **A, E, I, K,** and **L;** 20 μm in **B, C, F, H, J,** and **M;** 50 μm in **D;** 80 μm in **G** and **O;** 400 μm in **N.**

Murine *Phemx* maps to the distal region of chromosome 7. Northern blot and RT-PCR analyses demonstrated that (i) *Phemx* is expressed most prominently in hematopoietic organs and (ii) *Phemx* is expressed in hematopoietic progenitor cells purified as $Lin^-c\text{-}Kit^+Sca\text{-}1^+$ cells from adult bone marrow as well as differentiated cells in the lymphoid, erythroid, and myeloid lineages. In the mouse, embryonic hematopoiesis follows a path similar to that of the adult, but developmental details remain to be investigated. For example, pluripotent hematopoietic stem cells in the very early mouse embryo may or may not correspond to the pluripotent hematopoietic stem cells in adult bone marrow. Our analyses of *Phemx* gene expression by *in situ* hybridization show that it is expressed throughout development, from the early stages of transient hematopoiesis in the blood island through the definitive stages of hematopoiesis in the adult mouse. Variable levels of transcripts detected in cells of hematopoietic organs by *in situ* hybridization suggest that *Phemx* expression is regulated. This regulation could occur in a lineage- or cell-stage-specific manner.

In the mouse, Northern analysis detects only one *Phemx* messenger RNA. To date, only a single murine *Phemx* cDNA sequence has been identified (an independent murine EST clone, AA200225, has a sequence identical to the *Phemx* cDNA clone described in this report). In contrast, the identification of human *PHEMX* splice variants suggests that mechanisms controlling human *PHEMX* expression may be more complex than in the mouse. Alternative splicing can give rise to protein isoforms with different functional properties. The human *PHEMX* splice variants described here are predicted to encode two transmembrane domains and conserved potential phosphorylation sites. Variation in the predicted carboxy-terminus of *PHEMX2* and *TSSC6* (which includes a third possible transmembrane domain) could convey different functional properties to the PHEMX protein. Whether any *PHEMX* splice variant is specific to a defined hematopoietic lineage or differentiation stage remains to be determined.

The sequence similarity of PHEMX to CD81 provides some insight into the function of this protein. CD81 is a member of the tetraspanin family of proteins that participates in multimolecular complexes on the cell membrane that influence cell function. Human CD81 forms a complex with CD21, CD19, and Leu13 in B-cells that reduces the threshold for B-cell activation (Bradbury *et al.,* 1992). Murine CD81 associates with CD4 and CD8 as part of a complex involved in T-cell maturation (Boismenu *et al.,* 1996). Human CD81 also interacts with the integrins VLA-4 and LFA-1 to affect adhesion of B-cells and thymocytes, respectively (Behr and Schriever, 1995; Todd *et al.,* 1996). CD81 lacks signaling motifs, but is proposed to influence cell behavior, such as B-cell proliferation and activation, by associating with signaling proteins (Levy *et al.,* 1998). In contrast to CD81, PHEMX contains several potential phosphorylation sites. This suggests that it may act as a signaling protein in a macromolecular complex on the hematopoietic cell surface.

Like *Phemx,* CD81 is expressed in leukocytes including B-cells, T-cells, and granulocytes. However, CD81 is also expressed in endothelial cells as well as other cell types (Nagira *et al.,* 1994; Tomlinson and Wright, 1996). In contrast, *in situ* hybridization suggests that *Phemx* is expressed neither in vascular endothelial cells nor in cells of nonhematopoietic tissues.

The location of *Phemx* suggested that it might be imprinted, like other genes in the region. However, allele-specific RT-PCR analyses showed that murine *Phemx* is biallelically expressed in the E13.5 embryo, as well as the neonate spleen, adult spleen, and adult thymus. Although the allele-specific expression analyses have been performed on a whole E13.5 embryo, *in situ* hybridization at this stage reveals *Phemx*-expressing cells primarily in the liver. Therefore, it is reasonable to conclude that the *Phemx* gene is biallelically expressed in the midgestation liver, where embryonic hematopoiesis actively takes place. Our analysis in mouse is in agreement with studies on the imprinting status of the human ortholog *TSSC6,* which also shows biallelic expression at the whole organ level (Lee *et al.,* 1999).

The presence of a nonimprinted gene in the midst of a large cluster of imprinted genes raises the possibility that imprinting changes in this region could cause pathological inactivation of one or both *PHEMX* alleles. Speculatively, a loss of biallelic expression through epigenetic silencing of one or both of the *PHEMX* alleles could be involved in leukemogenesis or lymphomagenesis. Further studies with immunohistochemical analyses, gene targeting in the mouse, and mutation analyses in the human should clarify the role of *PHEMX* in physiological and pathological hematopoiesis.

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