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Isolation and Characterization of *Vsx1,* a Novel Mouse CVC *paired-like* Homeobox Gene Expressed during Embryogenesis and in the Retina

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Gastrula stage mouse embryo RNA was screened by degenerate RT-PCR to yield a novel *paired-like* homeobox gene. The open reading frame encoded by the cDNA was most similar to human *VSX1.* Mouse *Vsx1* encodes a protein of 363 amino acid residues that contains a CVC domain that was originally identified as a conserved motif among mouse CHX10, goldfish VSX-1 and *C. elegans* CEH-10. Linkage analysis showed that mouse *Vsx1* mapped to the distal region of chromosome 2. RT-PCR analysis detected mouse *Vsx1* transcripts from gastrulation and post-gastrulation stage mouse embryos, suggesting a role for *Vsx1* during mouse embryogenesis. Analysis of the eyes of mouse chimeras generated with embryonic stem cells in which a *lacZ* reporter was targeted to the *Vsx1* locus suggested that *Vsx1* is expressed in the inner nuclear layer of the retina.

Key Words: **paired-like class homeodomain; CVC domain; gastrula; retina.**

Homeoproteins are nuclear transcription factors containing the homeodomain, a DNA binding motif consisting of 60 amino acid residues. Homeobox genes are classified into two superclasses according to their distribution on chromosomes; i.e., clustered or dispersed. Dispersed superclass homeobox genes are further subdivided into at least 16 subclasses according to the primary structure of the homeodomain and its flanking sequences (1). Homeobox genes have been identified from various multicellular organisms, indicating that they are evolutionarily conserved and play important roles for organized cell proliferation and differentiation.

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In vertebrates, gastrulation is an important step for subsequent tissue and pattern formation (2). The gastrulation process results in the generation of the three primary germ layers, the ectoderm, mesoderm and endoderm. A subset of homeobox genes have been shown in the mouse to be required during gastrulation stages, including *Otx2* and *Lim1* (3–6). It is likely that other homeobox genes exist that also regulate this important embryological process.

One subclass of homeoproteins contains an additional motif known as the CVC domain. The CVC domain was originally identified as a conserved motif among mouse CHX10, goldfish VSX-1 and *C. elegans* CEH-10 (7). CVC domain-containing proteins have been identified from various species including zebrafish, Japanese medaka fish, chicken, mouse, and human (8–12). The CVC domain consists of approximately 50–60 amino acid residues except Japanese medaka fish VSX2, that has a CVC domain that is 75 amino acids in length. The larger CVC domain of medaka VSX2 is derived from an alternative splice form that encodes an additional 21 amino acid residues in the CVC domain that is also found in zebrafish ALX and chicken CHX10 (8, 9). The function of the CVC domain is still unknown although a possible involvement in DNA binding and/or protein–protein interaction has been proposed (7, 10). Because the CVC domain is hydrophobic, it might be important for protein folding or protein–protein interactions. Recently, it was suggested that the CVC domain of zebrafish VSX-1 is important for efficient ubiquitination that probably causes the degradation of VSX-1 by the 26 S proteasome (13).

Mouse *Chx10* encodes a paired-like class CVC homeoprotein that is involved in ocular-tissue formation (14, 15). *Chx10*-null mutant mice manifest an ocular retardation phenotype due to a defect in retinal progenitor proliferation and bipolar cell differ-

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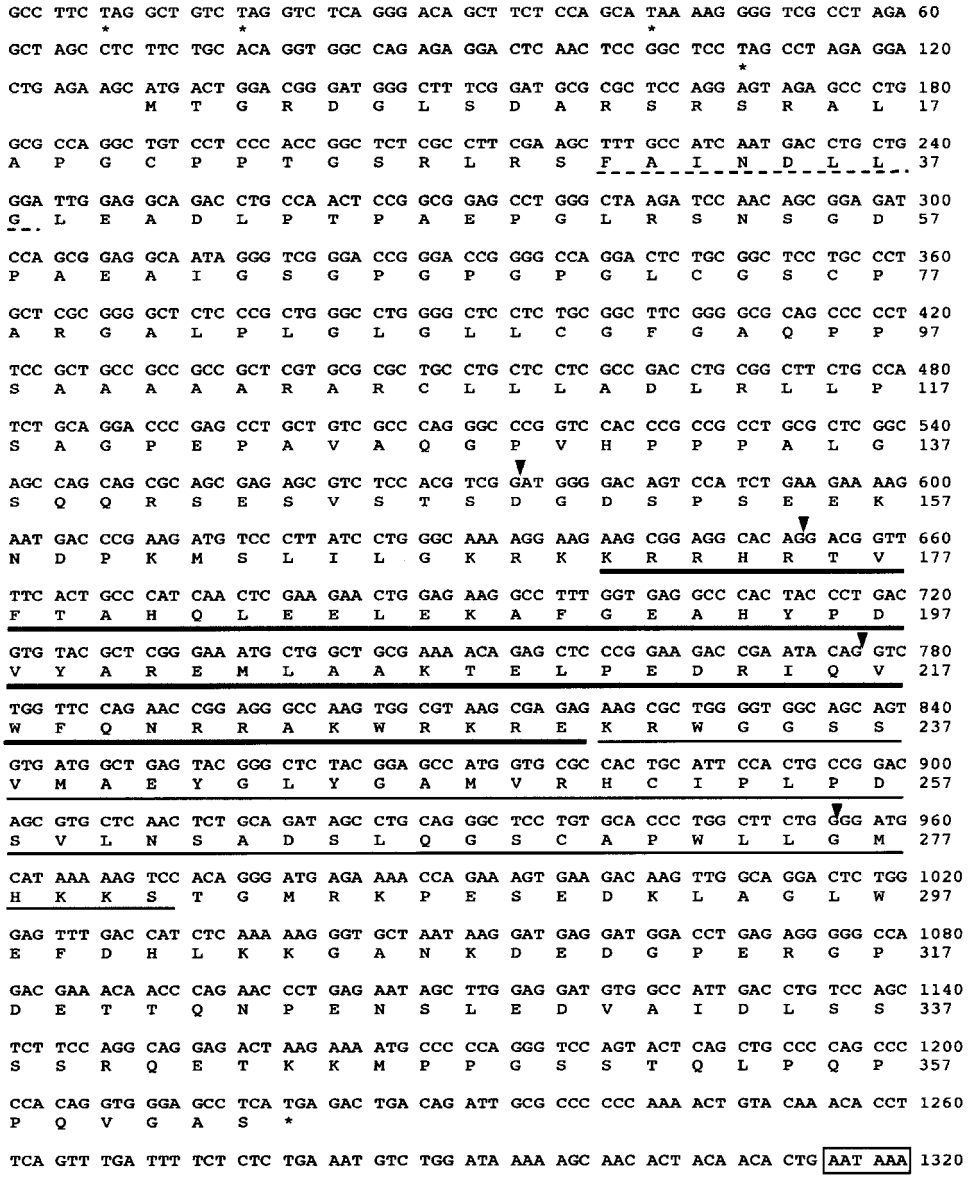
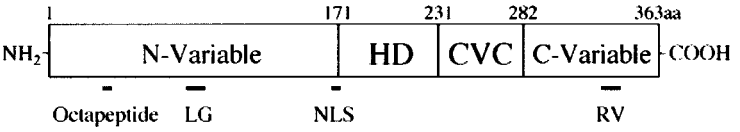


FIG. 1. cDNA and deduced amino acid sequence of mouse *Vsx1*. Dashed, thick, and thin lines underlining the amino acid residues indicate the octapeptide, homeodomain, and CVC domain sequences, respectively. A putative polyadenylation signal is boxed. In-frame stop codons in the 5′ untranslated region are marked *. ▼ shows the position of introns.

FIG. 2. Schematic structure, hydropathy plot, and the sequence comparisons of mouse VSX1. (A) Schematic structure of mouse VSX1. Mouse VSX1 consists of 363 amino acid residues and can be divided into four regions, the homeodomain (HD), CVC domain, amino-terminal, and carboxy-terminal variable regions. The numbers above the schematic protein structure refer to the first amino acid position of the four regions. The positions of the octapeptide sequence, LG sequence, a putative nuclear localization signal (NLS), and RV domain are underlined. (B) The hydrophobicity of mouse VSX1 is scored by the Kyte–Doolittle method (33). (C–G) Sequence comparisons among CVC domain-containing proteins. (C) Homeodomain, (D) CVC domain, (E) octapeptide sequence, (F) LG sequence, (G) RV domain. *M. musculus* (Mm) VSX1 (GenBank Accession No. AF395732) and CHX10 (L34808); *H. sapiens* (Hs) VSX1 (AF176797) and CHX10 (16); *B. taurus* (Bt) RINX (AF251032); *G. gallus* (Gg) CHX10 (AF178671) and CHX10-1 (AF178670); *D. rerio* (Dr) VSX-1 (AF025348) and ALX (U62898); *C. auratus* (Ca) VSX-1 (U07056) and VSX-2 (AF004318); *O. latipes* (O1) VSX1 (AJ250403) and VSX2 (AJ250404); *C. elegans* (Ce) CEH-10 (U19995); *D. melanogaster* (Dm) CG15782 (AE003434) and CG4136 (AE003434). (H) Sequence similarity between mouse VSX1 and other CVC domain-containing proteins. (I) Phylogenetic tree of CVC domain-containing proteins. Sequence alignments, comparison, and phylogenetic analysis were performed by the Jotun Hein method (34).

A



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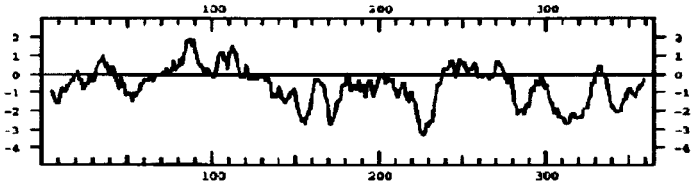

C Homeodomain

```
MmVsx1      KRRH-RTVFTA HQLEELEKAF GEAHYPDVYA REMLAAKTEL PEDRIQVWFQ NRRAKWRKRE
MmChx10     ····-··I··S Y········· N·········· ·····M···· ·········· ··········
HsVSX1      ····-····· ·········· S·········· ·····V···· ·········· ··········
HsChx10     ····-··I··S Y········· N·········· ·····M···· ·········· ··········
BtRINX      ····-····· ·········· S·········· ·····M···· ·········· ··········
GgChx10     ····-··I··S Y········· N·········· ·····M···· ·········· ··········
GgChx10-1   ····-····· ·········· N·········· ·····V···· ·········· ··········
DrVsx-1     ····-·····S ·········· N·········· ·····M···· ·········· ··········
DrAlx       ····-··I··S YE·····V·· N·········· ·····M···· ·········· ··········
CaVsx-1     ····-·····S ·········· H·········· ·····M···· ·········· ··········
CaVsx-2     ····-··I··S Y········· N·········· ·····M···· ·········· ··········
CeCeh-10    ····-··I··Q Y·ID····V· QDS····I·· ··V··G···· Q·········· ·····V··T·
OlVsx2      ····-··I··S Y········· N·········· ·····M···· ·········· ··········
DmCG15782   ····S··I··S Y···K··E·· K·········· ····SL···· ·········· ·····V··T·
DmCG4136    ····G··I··S S········· K·····V·S· ··L·SM··G· A·····V·Y· ·····V··T·
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D CVC domain

```
MmVsx1      KRWGGSSVMA EYGLYGAMVR HCIPLPDSVL NSA-DSL-QG SCAPWLLG-- ---------- ---------M HKKS--
MmChx10     ·C··R····· ·········· ·S····E·I· K··K·GI-MD ·········· ----------· ····LE
HsVSX1      ·C··R····· ·········· ·S····E·I· K··K·GI-MD ·········· ----------· ····LE
HsChx10     ·C··R····· ·········· ·S····E·I· K··K·GI-MD ·········· ----------· ····LE
BtRINX      ·C··R····· ·········· ·S····E·I· K··K·GI-MD ·········· ----------· ····LE
GgChx10     ·C··R····· ·········· ·S····E·I· K··K·GI-ME ·········· ----------· ····LE
GgChx10-1   ·C··R····· ·········· ·S····E·II ···KSG·-V· ·········· ----------· ····ME
DrVsx-1     ·C··R····· ·········· ·S····E·II ···KSGM-M· ·····V···· ----------· ····ME
DrAlx       ·C··R····· ·········· ·S····E·I· K··K·GI-MD ·········· ----------· ····LE
CaVsx-1     ·C··R····· ·········· ·S····E·II ···KNGM-M· ·····V···· ----------· ····LE
CaVsx-2     ·C··R····· ·········· ·S····E·I· K··K·GI-MD ·········· ----------· ····LE
OlVsx1      ·C··R····· ·········· ·S····E·I· ···KNGM-M· ·····V···· ----------· ····LE
OlVsx2      ·C··R·T··· ·········· ·S····E·I· K··K·GI-ME ·····V···· ----------· ····ME
CeCeh-10    ·T··K·TI·· ·········· ·SL···ETIT K··EAADP·Q ·A·····V·· ----------· ····ME
DmCG15782   ·V····TI·· ·········· ·SL····TI· K··K·---ND AV·····V·· ----------· ····LE
DmCG4136    ·C··H·TK·· ·········· ·SL···ETII K··KE---DE ·V·····V·· ----------· ····LE
```

E Octapeptide

```
MmVsx1      FAINDLLG
MmChx10     ·G·QEI··
HsVSX1      ···T····
HsChx10     ·G·QEI··
BtRINX      ···T····
GgChx10     ·G·QEI··
GgChx10-1   ···T····
DrVsx-1     ···T····
DrAlx       ·G·QEI··
CaVsx-1     ···T····
OlVsx2      ·G·QEI··
CeCeh-10    ···HEI··
DmCG4136    ···QEI··
```

H

	N-Variable	Homeodomain	CVC domain	C-Variable	Total
MmChx10	19%	91%	78%	12%	42%
HsVSX1	69%	96%	90%	57%	74%
HsChx10	19%	91%	78%	13%	43%
BtRINX	67%	96%	86%	53%	72%
GgChx10	22%	91%	78%	7%	40%
GgChx10-1	51%	96%	80%	26%	56%
DrVsx-1	37%	95%	78%	22%	52%
DrAlx	20%	90%	78%	18%	43%
CaVsx-1	38%	95%	78%	22%	51%
OlVsx2	21%	91%	76%	12%	40%
CeCeh-10	21%	78%	67%	11%	38%

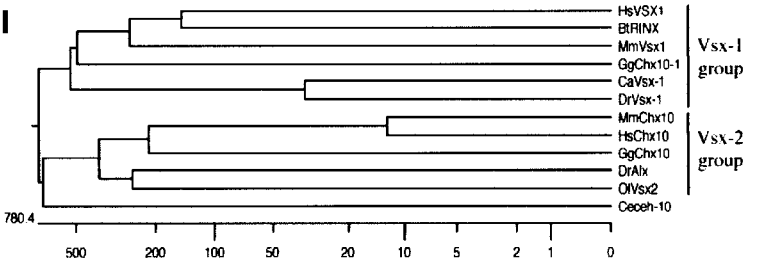
F LG sequence

```
MmVsx1      GALPLGLGLLCGFG
HsVSX1      ··············
BtRINX      ··············
GgChx10-1   ···G·····LA
DrVsx-1     ·S·····F··SLA
CaVsx-1     ·S··I···F··SLA
```

G RV domain

```
MmVsx1      EDVAIDLSSSSRQET
HsVSX1      ··········A····
BtRINX      ···V·····T····
GgChx10-1   ··T·····RTAK··K
DrVsx-1     ··M·····T·K··N
CaVsx-1     ··M·····T·K··S
Olvsx1      ·E·····K·QQ
```

I



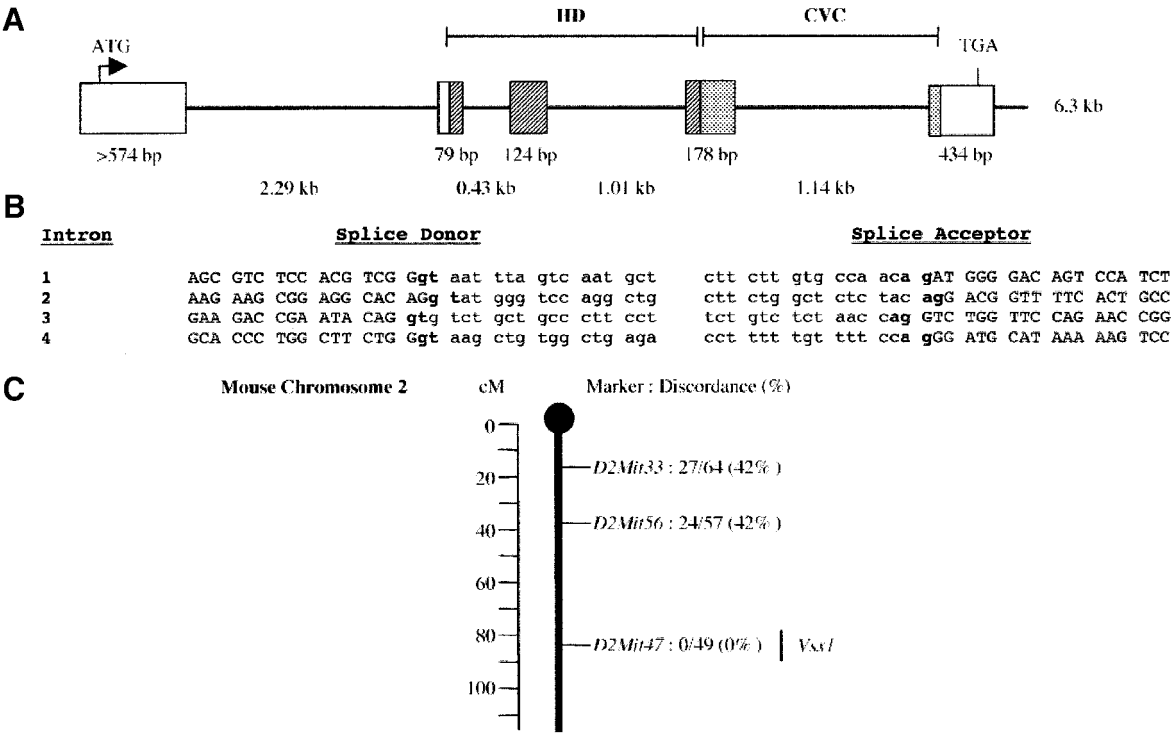


FIG. 3. (A) Genomic structure of mouse *Vsx1*. Coding exons and introns are indicated by boxed regions and lines, respectively. Homeodomain (HD) and CVC domain (CVC) are hatched and dotted, respectively. The first coding exon may larger than 574 bp because it is probably incomplete. (B) The sequences of the exon–intron junctions of mouse *Vsx1*. Conserved residues in splice donor and acceptor sites are indicated in bold. (C) Chromosomal localization of mouse *Vsx1* and other DNA markers.

entiation (14). In human, two CVC homeodomain-encoding genes, *CHX10* and *VSX1*, have recently been isolated (11, 16). Human *CHX10* is expressed in the developing and mature retina (16). A loss of function allele of *CHX10* was found to be associated with microphthalmia, demonstrating a conserved function in mammals (8, 14, 16). *VSX1* was isolated from a human embryonic craniofacial cDNA library and is expressed in the adult retina and cornea, implicating *VSX1* in the development and maintenance of ocular tissues (11). Thus, it appears that a set of CVC homeobox genes have conserved roles in eye development.

To isolate novel *paired-like* class homeobox genes expressed in mouse gastrula stage embryos, we performed a degenerate PCR using a homeobox sequence and cDNA prepared from embryonic day 7.5 (E7.5) embryos as a template. We have isolated a second mouse gene encoding a CVC domain-containing paired-like class homeodomain, *Vsx1*. Here, we describe the cDNA and deduced amino acid sequence, genomic structure, chromosomal mapping, and expression analysis of mouse *Vsx1*. These results are consistent with the idea that *Vsx1* has a role during mouse embryogenesis and later in the retina.

MATERIALS AND METHODS

RNA preparation and cDNA cloning. Total RNA was extracted from E7.5 mouse embryos using the acid guanidinium thiocyanate-phenol-chloroform extraction method (17). cDNA was prepared by reverse transcription using oligo dT primers. To obtain a DNA fragment encoding a novel paired-like class homeoprotein, PCR was performed using AmpilTaq (Applied Biosystems) and degenerate primers, 5′-cag gt(g/a/t/c) tgg tt(c/t) ca(a/g) aac-3′ and 5′-tcc tcc ca(c/t) gag tcc a(a/g)c-3′. The PCR conditions were denature at 95°C for 2 min 30 s followed by 30 cycles of denature (95°C for 30 s), anneal (48°C for 1 min) and extension (72°C for 1 min). The last extension was for 10 min. The sequences of fragments obtained after two rounds of the PCR were examined. To obtain the 3′ end of the cDNA, 3′ RACE was performed using the 3′ RACE System for Rapid Amplification of cDNA Ends kit (Life Technologies, Inc) and the primer, 5′-ata aaa agt cca cag gga tg-3′. The cDNA encoding the entire ORF was obtained by a PCR reaction using primers, 5′-gga att ctg ctc cct gct gat tgg c-3′ and 5′-gga att caa act gaa ggt gtt tgt ac-3′ and cDNA prepared from E7.5 embryos as a template. The cDNA sequence has been deposited in GenBank (accession number AF395732).

Screening of genomic DNA. Mouse strain 129/SvEv genomic (18) and BAC (Roswell Park Cancer Institute) libraries were screened to isolate genomic clones using a 3′ cDNA probe.

Linkage analysis. A mouse interspecific backcross DNA panel between *M. musculus* (strain SB/Le) and *M. spretus* was used to map the chromosomal location of *Vsx1* (19). Backcross panel samples were PCR-amplified using *Vsx1* primers, 5′-ata aaa agt cca cag gga tg-3′ and 5′-cgg gat cca gac att tca gag ag-3′. The resulting PCR products were digested with *Pvu*II and separated by agarose gel electrophore-

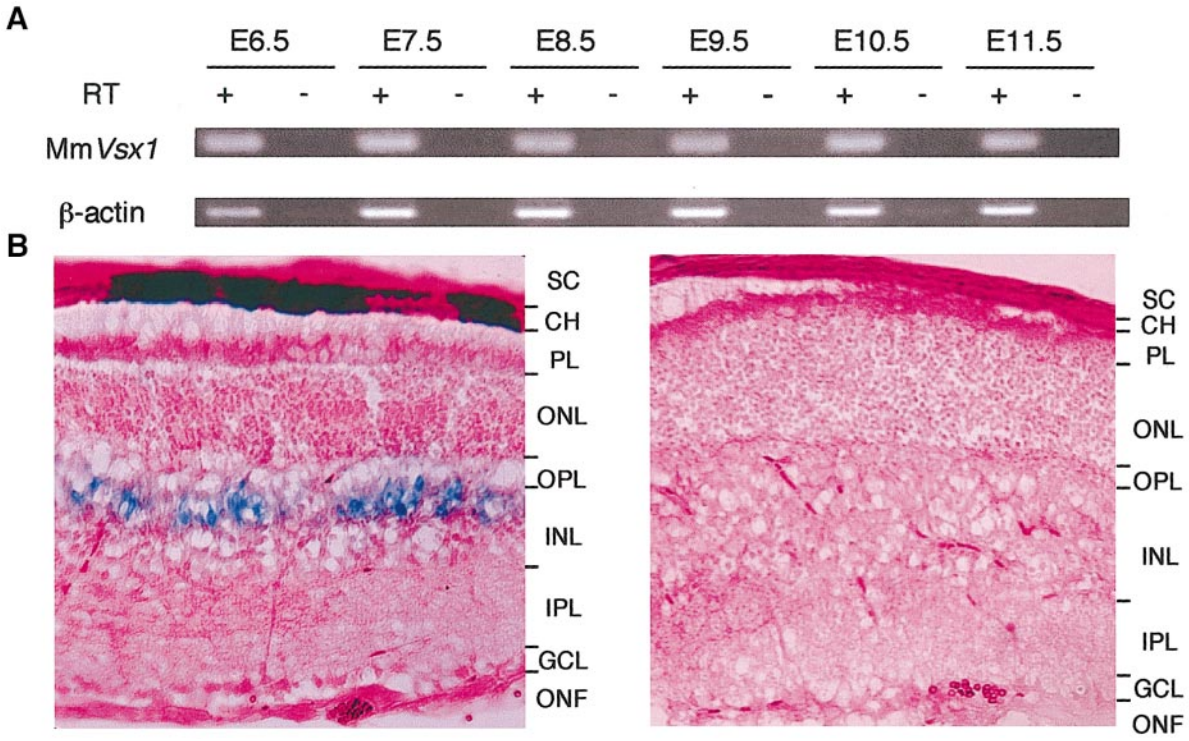


FIG. 4. (A) RT-PCR analysis of mouse *Vsx1*. Mouse *Vsx1* transcripts from various embryonic stages (E6.5–E11.5) were detected. Reactions were run with (+) and without (−) reverse transcriptase (RT). RT-PCR using mouse β-actin primers is presented as a control. (B) *Vsx1*-directed expression in the retina of postnatal chimeric mice. (Left) The eye from a chimeric mouse generated with ES cells carrying the *lacZ* gene targeted to the *Vsx1* locus was subjected to X-gal staining and 20-μm frozen sections were prepared. The presence of patches of pigmentation in the choroid layer is indicative of the extent of chimerism because the ES cell-derived cells are from a pigmented mouse and the host blastocyst is from an albino mouse. SC, sclera; CH, choroid; PL, photoreceptor layer; ONL, outer nuclear layer; OPL, outer plexiform layer; INL, inner nuclear layer; IPL, inner plexiform layer; GCL, ganglion cell layer; ONF, optic nerve fiber.

sis. *M. spretus* DNA yielded a 335 bp fragment, whereas *M. musculus* DNA products were digested into two fragments of 108 and 227 bp. Linkage and recombination distances were analyzed using the program MapManager Version 2.6 (Kenneth F. Manley, Roswell Park Cancer Institute, Buffalo, NY). Gene order is determined by minimizing the number of recombination events that are required to explain the allele distribution patterns.

RT-PCR. Total RNA and cDNA from mouse embryos were prepared as described above. The first PCR was performed using primers, 5′-gga aga ccg aat aca ggt c-3′ and 5′-ggt ttt ctc atc cct gtg-3′. The PCR conditions were 40 cycles of 94°C for 30 s, 55°C for 1 min and 72°C for 1 min. One twenty-fifth of the first PCR products were subjected to the second reaction (25 cycles) of PCR with a nested primer 5′-gtg cac agg agc cct gca g-3′, and 5′-ggt ttt ctc atc cct gtg-3′. The PCR products were separated by agarose gel electrophoresis. β-actin was used as a control for RNA integrity.

LacZ staining and histological analysis. Mouse embryonic stem (ES) cells that have a *lacZ* gene targeted to the *Vsx1* locus were established (A. Ohtoshi and R. Behringer, unpublished) and injected into blastocysts derived from Swiss albino mice to generate chimeras. Eyes were isolated from the adult chimeric mice and stained for β-galactosidase activity (20). Stained samples were rinsed in PBS, equilibrated into a solution of 30% sucrose, and embedded in OCT compound (Sakura Finetek, Inc.). Twenty-micrometer frozen sections were prepared and counterstained with eosin Y.

RESULTS

To identify novel paired-like class homeobox genes involved in mouse gastrulation, we performed degenerate PCR using cDNA prepared from E7.5 embryos and isolated a DNA fragment that encoded a portion of a novel protein. A cDNA that encodes an entire open reading frame (ORF) was subsequently isolated by PCR and 3′ RACE. The complete cDNA and deduced amino acid sequence are shown in Fig. 1. The nucleotide sequence was confirmed by comparisons with the sequence from genomic clones. Because we used PCR to isolated the 5′ region of the cDNA, the 5′ untranslated region is probably still incomplete. However, we believe that we have isolated the entire ORF sequence because the putative first ATG sequence has a purine residue at the −3 position, which corresponds to the first-AUG rule (21) and in-frame stop codons exists in the 5′ upstream region. The 3′ end of the cDNA was isolated by 3′ RACE and a putative poly-adenylation signal was identified.

Mouse VSX1 consists of 363 amino acid residues and contains a homeodomain and a CVC domain (Fig. 2A). The homeodomain sequence of mouse VSX1 is most likely a paired-like class homeodomain because it lacks the serine residue at position 50, the ninth position of Helix III, that is conserved among paired-class homeoproteins (1). Sequence comparisons revealed that the homeodomain of mouse VSX1 is most similar to that of CVC domain-containing paired-like class homeoproteins (Fig. 2C). In addition to the homeodomain and CVC domains (Figs. 2C and 2D), mouse VSX1 also has conserved sequence stretches among CVC domain-containing protein family members such as an octapeptide sequence, LG sequence, a putative nuclear localization signal and RV domain (Fig. 2A). The octapeptide sequence is conserved among all CVC domain-containing homeoproteins (Fig. 2E), however, the LG sequence and RV domain are only found in mouse VSX1, human VSX1, bovine RINX, chicken CHX10-1, zebrafish VSX-1 and goldfish VSX-1 (Figs. 2F and 2G).

The genomic DNA that contains mouse *Vsx1* was isolated to determine the *Vsx1* gene structure (Fig. 3A). Mouse *Vsx1* consists of 5 coding exons, that spans an approximately 6.3 kb region of genomic DNA. The exon-intron structure is completely conserved between mouse *Vsx1* and human *VSX1* (11), indicating that they share a common ancestor. Figure 3B shows the exon-intron junction sequences of mouse *Vsx1* that correspond to the GT-AG splicing donor-acceptor rule.

The chromosomal localization of mouse *Vsx1* was determined by linkage analysis using an interspecific backcross panel. Mouse *Vsx1* was mapped to the distal region of chromosome 2 (Fig. 3C). Analysis of 49 N2 DNA samples showed 100% concordance between mouse *Vsx1* and *D2Mit47*. Thus, mouse *Vsx1* is very tightly linked to *D2Mit47* within 0.05–7.11 cM with 95% confidence and 0.35–3.60 cM with 68% confidence. Mouse *Vsx1* showed about 42% discordance with other markers on chromosome 2, *D2Mit33* and *D2Mit56* which are located at the 17 cM and 38 cM positions, respectively (Fig. 3C).

The expression of mouse *Vsx1* was examined by RT-PCR analysis (Fig. 4A). Because mouse *Vsx1* was isolated from E7.5 embryos, we performed RT-PCR using gastrulation and post-gastrulation stage embryos. Mouse *Vsx1* transcript were detected in all stages examined in a reverse transcriptase-dependent manner, suggesting that mouse *Vsx1* is transcribed at least from E6.5 to E11.5. However, the abundance of *Vsx1* transcripts at these stages of embryogenesis appears to be very low because detection required nested PCR amplification.

Because human *VSX1,* bovine *RINX,* chicken *Chx10-1,* zebrafish *Vsx-1* and goldfish *Vsx-1* are detected in the developing and adult retina (9–11, 22–24), we examined the expression of mouse *Vsx1* in the eyes of postnatal chimeric mice that have a *lacZ* gene targeted to the *Vsx1* locus. *lacZ*-positive cells were detected in the outer half of inner nuclear layer (Fig. 4B). This expression pattern is consistent with bovine *RINX,* chicken *Chx10-1,* zebrafish *Vsx-1* and goldfish *Vsx-1* (9, 10, 22–24). Recently, one EST clone from mouse retina, which has a similar sequence to mouse *Vsx1,* was deposited in GenBank (Accession No. BG298137). We have also detected mouse *Vsx1* transcripts in the adult mouse eye by RT-PCR (data not shown). These results suggest that mouse *Vsx1* is most likely expressed in the retina.

DISCUSSION

Here we describe the identification and characterization of the mouse *Vsx1* gene. Mouse VSX1 is a novel member of the CVC domain-containing paired-like class of homeoproteins. CVC domain containing proteins have been isolated from various species including human, mouse, chicken, fish and *C. elegans.* Recently, the *Drosophila* genome sequence was determined revealing the existence of two genes encoding CVC domain-containing proteins (25). Furthermore, the recently identified bovine *RINX* gene also encodes a CVC domain (22). These observations suggest an evolutionary conserved function of the CVC domain.

Because the homeodomain and CVC domain are located adjacent to each other, the CVC domain is predicted to influence transcriptional regulation through DNA-binding, protein–protein interaction and/or protein degradation processes (7, 10, 13). It is possible that the CVC domain is necessary for protein folding or protein-protein interaction because this domain is hydrophobic. Although the function of the CVC domain is still not clear, the domain seems to be required for protein function because in *C. elegans ceh-10,* point mutations in the CVC domain are known to abolish protein function (26).

Apparently, all vertebrates, including mouse, human, chicken, zebrafish, goldfish and Japanese medakafish, have two CVC homeobox genes. These vertebrate CVC homeobox genes can be classified into two groups, the Vsx-1 group and the Vsx-2 group, according to sequence similarity. The characteristic features of the Vsx-1 group are the existence of a RV domain and LG sequence and the lack of a Paired-Tail/OAR domain. The RV domain was identified as a conserved sequence between human RINX (VSX1) and goldfish VSX-1 (22). Another characteristic of the VSX-1 group is the existence of an LG sequence. This region is a leucine and glycine-rich sequence, and has high-hydrophobicity. The LG sequence may contribute to the specific function of the VSX-1 group proteins. Instead of an RV domain, the Vsx-2 group which includes mouse CHX10, chicken CHX10, zebrafish ALX,

goldfish VSX-2 and Japanese medakafish VSX2 have a conserved 14 amino acid motif called the Paired-Tail or OAR domain (11, 24, 27, 28). These specific domain differences may correlate with the functional specificity of each group. Although the functional difference between these two groups has not yet been identified, similarities and differences in the temporal and spatial expression patterns of Vsx-1/Chx10-1 and Vsx-2/Chx10 have been described in zebrafish, goldfish and chicken, suggesting that each protein has overlapping and unique functions (9, 10, 23, 24).

Mouse *Vsx1* is the second gene encoding a CVC domain-containing protein identified in mice and is a member of the Vsx-1 subgroup. It is unknown if mouse VSX1 functions as a transcriptional activator, however, proline and acidic residue-rich regions exist that suggest a transcriptional activation domain (29, 30). The first mouse CVC homeobox gene isolated was *Chx10* from a newborn mouse eye cDNA library (15). Mouse *Chx10* is expressed in the optic vesicle, optic cup and mature retina as well as developing thalamus, hindbrain and spinal cord (15). The generation of *Chx10* knockout mice has shown that *Chx10* plays an essential role in eye development because these mice develop an ocular retardation phenotype (14). Recently, it was reported that human *CHX10* is also involved in the eye development and a loss of function allele is associated with microphthalmia (16). Sequence comparisons revealed that mouse *Vsx1* is most similar to human *VSX1*. Furthermore, chromosomal mapping suggests that mouse *Vsx1* is located on the distal part of chromosome 2. The homologous gene cluster region of the distal part of mouse chromosome 2 is located on human chromosome 20 where human *VSX1* is mapped (11). The sequence similarity, gene structure, and chromosomal position suggest that mouse *Vsx1* is a mouse orthologue of human *VSX1*. Human *VSX1* was isolated from a human embryonic craniofacial cDNA library and exclusively expressed in adult retina and cornea, indicating that human *VSX1* plays some role(s) in the ocular development (11). Interestingly, the mouse *blind-sterile* mutation maps to the same position as mouse *Vsx1* (31). This indicates that mouse is a candidate genes of the *blind-sterile* mutation. However, we have sequenced the coding region of *Vsx1* from *blind-sterile* mutant mice and do not find any sequence differences (unpublished observations).

Our RT-PCR analysis suggests that mouse *Vsx1* is expressed at low levels during gastrulation and post-gastrulation stages of mouse embryogenesis, suggesting that *Vsx1* may have roles in the gastrulation process and later in embryogenesis. Unfortunately, numerous attempts using whole mount in situ hybridization with multiple *Vsx1* DNA fragments as probes on mouse embryos at different developmental stages did not result in any specific hybridization signals. Therefore, we employed an indirect method to study *Vsx1* expression. Gene knock-ins generated by targeting heterologous genes to endogenous gene loci in mice can be used to express foreign gene products in the pattern of the targeted gene (32). We have introduced the *lacZ* gene into the *Vsx1* locus in mouse ES cells to report *Vsx1*-directed expression by X-gal staining (A. Ohtoshi and R. Behringer, unpublished results). Analysis of chimeras generated using these cells revealed that *lacZ*-positive cells were detected in the outer half of inner nuclear layer where bipolar cells are predominantly located, suggesting that mouse *Vsx1* is possibly transcribed in retinal bipolar cells. Interestingly, mouse *Chx10* is also expressed in the outer edge of inner nuclear layer and the loss of *Chx10* leads to an ocular retardation phenotype that lacks differentiated bipolar cells (14, 15). The generation of *Vsx1* mutant mice will reveal whether this CVC homeobox gene has a required role in mouse embryogenesis and retinal development.

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