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Regulation of photoreceptor gene transcription via a highly conserved transcriptional regulatory element by *vsx* gene products

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Purpose: The photoreceptor conserved element-1 (PCE-1) sequence is found in the transcriptional regulatory regions of many genes expressed in photoreceptors. The *retinal homeobox (Rx or Rax)* gene product functions by binding to PCE-1 sites. However, other transcriptional regulators have also been reported to bind to PCE-1. One of these, *vsx2*, is expressed in retinal progenitor and bipolar cells. The purpose of this study is to identify *Xenopus laevis vsx* gene products and characterize *vsx* gene product expression and function with respect to the PCE-1 site.
Methods: *X. laevis vsx* gene products were amplified with PCR. Expression patterns were determined with in situ hybridization using whole or sectioned *X. laevis* embryos and digoxigenin- or fluorescein-labeled antisense riboprobes. DNA binding characteristics of the *vsx* gene products were analyzed with electrophoretic mobility shift assays (EMSAs) using in vitro translated proteins and radiolabeled oligonucleotide probes. Gene transactivation assays were performed using luciferase-based reporters and in vitro transcribed effector gene products, injected into *X. laevis* embryos.
Results: We identified one *vsx1* and two *vsx2* gene products. The two *vsx2* gene products are generated by alternate mRNA splicing. We verified that these gene products are expressed in the developing retina and that expression resolves into distinct cell types in the mature retina. Finally, we found that *vsx* gene products can bind the PCE-1 site in vitro and that the two *vsx2* isoforms have different gene transactivation activities.
Conclusions: *vsx* gene products are expressed in the developing and mature neural retina. *vsx* gene products can bind the PCE-1 site in vitro and influence the expression of a rhodopsin promoter-luciferase reporter gene. The two isoforms of *vsx* have different gene transactivation activities in this reporter gene system.

Photoreceptor gene transcription is regulated by a set of transcription factors that operate through a set of conserved cis-acting elements [1-5]. One of these, photoreceptor conserved element-1 (PCE-1) or Ret1, has been found to interact with the *retinal homeobox (Rx or Rax)* gene product [6]. This interaction is essential for normal retinal development and photoreceptor maintenance [7]. Other transcription factors can also bind to the PCE-1 site, including Erx [8] and Chx10/Vsx2 [9]. Visual system homeobox 2 (Vsx2) and Vsx1 are the prototypical members of a subgroup of homeodomain-containing gene products [10]. Vsx gene products contain a conserved domain, the CVC domain, that functions to strengthen DNA binding by the homeodomain [11]. *vsx2* is expressed in retinal progenitor cells in the developing vertebrate retina, and *vsx1* and *vsx2* are expressed in bipolar cells in the mature retina [12-18]. *rax* is expressed in all retinal progenitor cells in the developing vertebrate retina and the ciliary marginal zone (CMZ) of cold-blooded animals and in differentiated photoreceptors [19]. Thus, *vsx2* and *rax* are coexpressed in retinal progenitor cells but not in differentiated retinal cells. We thought it curious that a PCE-1-binding protein, such as Vsx2, was expressed in cells where PCE-1-containing genes are not expressed. We hypothesized that Vsx2 may repress expression of PCE-1-containing genes.

To learn more about the potential role of Vsx1 and 2 in controlling transcription of PCE-1-regulated genes, we sought to characterize the expression and function of *vsx1* and *2* in *Xenopus laevis* retinal development. Here we report the expression patterns of *X. laevis vsx1* and *vsx2* in the retina, that both gene products can bind the PCE-1 site in vitro, and that Vsx2 can repress expression of a *Xenopus* rhodopsin promoter-based reporter.

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METHODS

Plasmids: We originally identified *vsx1* as an expressed sequence tag (EST) with homology to *rax* within the homeodomain. This EST apparently represented unspliced

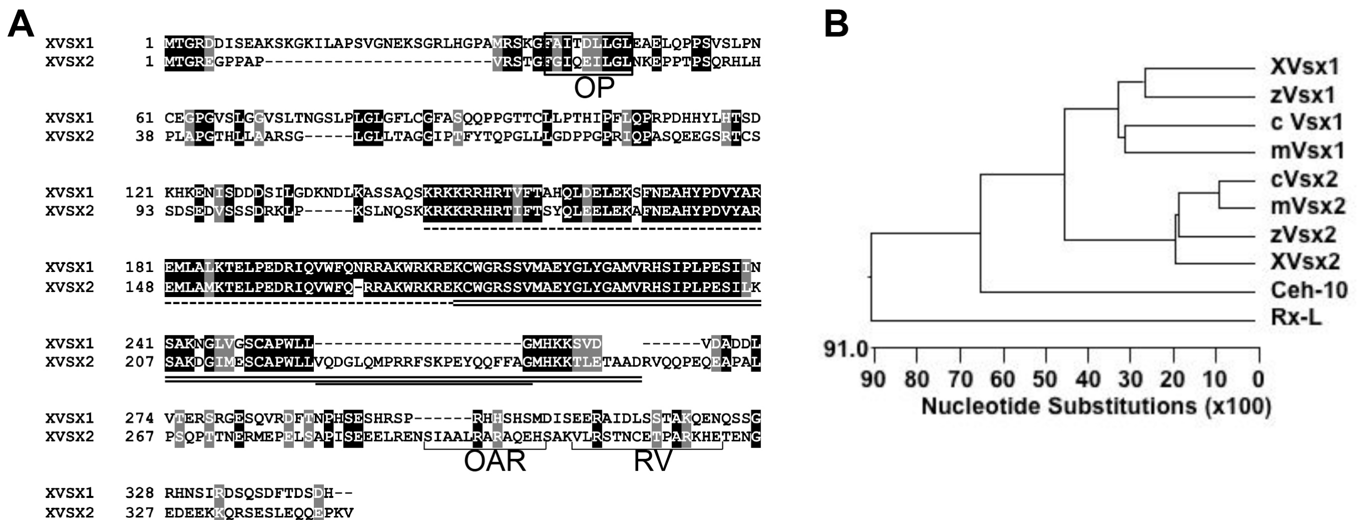


Figure 1. Identification of *Xenopus laevis* Vsx1 and Vsx2. **A**: Predicted protein sequences of *X. laevis* Vsx1 and Vsx2. Sequences were aligned using ClustalW. The black and gray backgrounds indicate amino acid identity and similarity, respectively. The highly conserved octapeptide motif (OP; box), homeodomain (dashed line), and CVC domain (double underline) are indicated. The orthopedia-aristaless-Rx (OAR; Vsx2) and Rinx-Vsx1 (RV; Vsx1) domains are also indicated. The triple underline indicates the region of Vsx2 lacking from the short form. **B**: Phylogenetic representation of ClustalW alignment of the Vsx1 and Vsx2 amino acid sequences from *X. laevis*, zebrafish, chicken, and mouse, *Caenorhabditis elegans* Ceh-10, and *X. laevis* Rx-L.

genomic DNA (BC044049). We amplified the *vsx1* coding region from cDNA prepared from st 37/38 embryo head RNA using the SMART cDNA Synthesis and Library construction Kit (Clontech, Mountain View, CA) using primers designed from the EST sequence. The primers contained exogenous recognition sequences (underlined; *vsx1* CDS F: 5′-GAT CGA ATT CAT GAC CGG GCG AGA TGA C-3′; *vsx1* CDS R: 5′-GAT CCT CGA GTC AGT GGT CAC TGT CAG T-3′). We identified an EST encoding *vsx2* with a BLAST search using the mouse CVC domain as the query (AAH58806). The coding sequence included in the EST was used to design primers for 3′-RACE (Rapid Amplification of cDNA Ends). Two rounds of 3′-RACE were performed using st 37/38 embryo head cDNA using the SMART RACE cDNA Amplification Kit (Clontech): first round primer: 5′-GTT TGG TTC CAG AAC AAA CGA GCG AAA TG-3′; second round primer: 5′-CTG AAT ATG GTC TGT ATG GGG CAA TGG TTC-3′. The sequence of the 3′-RACE product was used to design primers for 5′-RACE: 5′-CAC AGG ACT CCA TTA TGC CAT CCT TCG-3′. Finally, primers were designed to amplify the entire coding region from st 37/38 embryo head cDNA (restriction sites used for subcloning are underlined): forward: 5′-GAT CGG ATC CGG GAG TTG CAC TGG GAC CAG AAA GG-3′; reverse: 5′-GAT CCT CGA GGA CTC CCC TGG CAT TCT CCT TAC CC-3′.

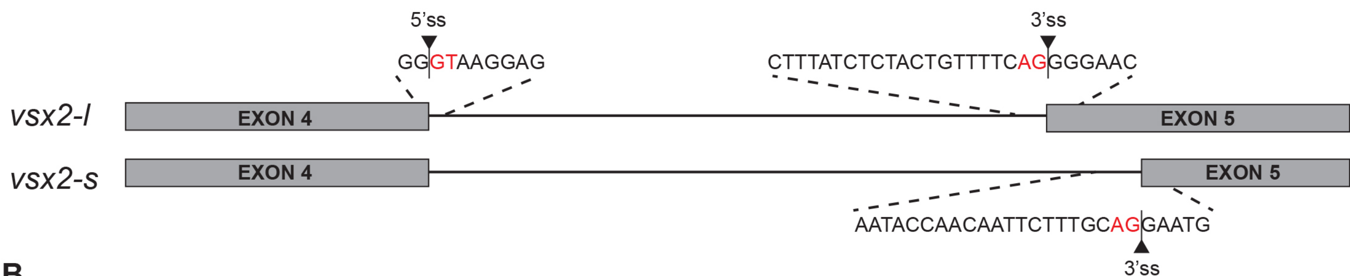
Splice site analysis: vsx2 genomic DNA encompassing exons 4 through 5 was identified with a BLAST search of *X. laevis* genomic DNA using the *vsx2* cDNA sequence (Genomes) and imported via the FASTA sequence into Splice Site Prediction by Neural Network and Alternative Splice Site Predictor and analyzed with default cutoff values for the 5′ and 3′ splice sites.

In situ hybridization: Antisense riboprobes against *vsx1* and *vsx2* were prepared by in vitro transcription as described previously from the following plasmids, linearized, and transcribed using the indicated enzymes: pCS2/Vsx1 (EcoRI/T7), pCS2/Vsx2 (BamHI/T7). In situ hybridization using whole or sectioned embryos was performed as described previously [20,21].

Double section in situ was performed following the section in situ protocol as described previously [21] with some modifications (buffers and solutions are from this reference). Sections were hybridized simultaneously with a mixture of digoxigenin (DIG) and fluorescein-labeled riboprobe probes. After the DIG-labeled probe was detected using the BM purple alkaline phosphatase (AP) substrate (Roche, Indianapolis, IN), AP was inactivated by incubation at 70 °C for 10 min. The slides were then reblocked and incubated with anti-fluorescein-AP antibody (Roche 11711400), washed, and the second color was developed with a different AP substrate solution, Fast Red (Sigma, St Louis, MO, F4523).

Luciferase assays: Embryos were injected at the two-cell stage with 25 pg XOP-Luc DNA [22], pRLtkLuc (encoding

A.



B.

	5'ss	*vsx2-l* 3'ss	*vsx2-s* 3'ss
NN Score	0.99	0.92	0.43
ASSP Score	11.62	8.18	5.67

Figure 2. *vsx2* undergoes alternative splicing of exon 5. **A**: A graphic depiction of the long (*vsx2-l*) and short (*vsx2-s*) forms of *vsx2* showing alternative 3′ splice site use in intron 4. The locations and sequences of the splice sites (arrow; red text) are indicated by their position relative to the exons (gray boxes) and intron (black lines). **B**: The splice site matrix scores of the splicing signals found in intron 4 according to the Neural Network (NN) and Alternative Splice Site Predictor (ASSP) bioinformatics programs. Both programs recognize *vsx2-l* as a strong 3′ splice site (high homology to consensus splicing signals) of intron 4 and *vsx2-s* as a weaker 3′ acceptor splice site (intermediate homology to splicing signals) for intron 4. ss, splice site.

Renilla luciferase as an internal control), and RNAs encoding effectors as indicated in the figure legends. Embryo lysates were prepared at st 11 as described previously [22,23] and assayed for luciferase activity using the Stop n Glo Dual Luciferase Assay Kit (Promega, Madison, WI). Firefly luciferase activity values were normalized against *Renilla* luciferase activity values and presented as fold activation compared to the baseline activity of the reporter (without coinjected effectors). Statistical significance was tested using Student t-test (Microsoft Excel). P-values lower than 0.05 were considered to be significant.

Electrophoretic mobility shift assays: Electrophoretic mobility shift assays were performed as described previously [22,24] using radiolabeled oligonucleotides representing wild-type and mutated mouse PCE-1 sequences [6]. Oligonucleotides representing mouse BAT and Ret4 sequences were designed based on the XOP sequence [3,22]. Vsx proteins were prepared with in vitro translation using a linked transcription and translation kit (TNT; Promega) and pCS2/Vsx1 and pCS2/Vsx2 plasmids.

RESULTS

To characterize the *vsx* gene products in *Xenopus laevis,* we isolated orthologs of *vsx1* and *vsx2* (Figure 1A). *X. laevis vsx1* has recently been independently isolated [13]. As found in *vsx* genes from other species, *X. laevis vsx1* and *vsx2* encode highly conserved homeo- and CVC domains. *vsx2* encodes an OAR domain (named after the first three gene products where this conserved domain was identified: orthopedia-aristaless-Rx), while *vsx1* does not; *vsx1* encodes an RV domain (named after the first gene products where this conserved domain was identified, Rinx-Vsx1) domain while *vsx2* does not. The *X. laevis vsx1* and *vsx2* genes we isolated are most closely related to *vsx1* and *vsx2/chx10* genes from other vertebrate species than they are to each other (Figure 1B). Both are more highly related to other vertebrate *vsx* genes than to *Caenorhabditis elegans Ceh10* or another paired-type homeobox gene, *X. laevis Rx-L*.

Interestingly, we were able to identify two forms of *vsx2*; a longer form, *vsx2-l*, encoded a 21 amino acid fragment interrupting the CVC domain. This fragment was not found in a shorter form of *vsx2* (*vsx2-s*) or in *vsx1*. cDNAs representing the longer and shorter forms of *vsx2* have been reported in zebrafish [25] and chicks [16] and can be found in ESTs from other species (for example, in the mouse, accession numbers NP_031727 and AAH58806). Alignment of the *X. laevis vsx2* cDNA sequences with the *X. laevis* genomic DNA sequence revealed that *vsx2-l* and *vsx2-s* corresponded to the same stretch of genomic DNA. *vsx2-l* is the major isoform generated from this locus (Figure 2A). *vsx2-s* lacks 63 nucleotides from the 5′ end of exon 5 (Figure 2A). To determine whether the alternatively-spliced transcripts of *vsx2* result from the use of alternative splice site selection, we analyzed the genomic locus surrounding

intron 4 using splice site prediction software. We used two bioinformatics prediction programs: Alternative Splice Site Prediction (ASSP) [26] and Neural Network (NN) from the Berkeley Drosophila Genome Project [27]. These programs use neural networks, a machine learning technique, to scan a given sequence for putative splice sites. When the score of the respective matrix indicates a hit, the putative splice sites are classified through a backpropagation network and generate a score for a potential splice site. For ASSP, the cutoff values for correctly identifying a donor and acceptor splice site are 4.5 and 2.2, respectively. For NN, the scores range from 0 to 1 with a cutoff of 0.4 for the donor and acceptor splice sites. Therefore, an output value exceeding these thresholds indicates the presence of a putative splice site with a low probability of false positivity.

Our analyses revealed that the donor 5′ splice site in intron 4 of *vsx2* has high matrix scores, 0.99 (NN) and 11.62 (ASSP), indicating that it is a constitutive splice site (Figure 2B). Similarly, the prediction software identified the site for *vsx2-l* as the constitutive splice 3′ acceptor splice site in intron 4 with scores of 0.92 (NN) and 8.18 (ASSP; Figure 2B). Importantly, both prediction algorithms detected an alternative 3′ acceptor splice site corresponding to *vsx2-s* with matrix scores of 0.43 (NN) and 5.67 (ASSP; Figure 2B). Taken together, these data indicate the *vsx2-s* acceptor 3′ splice site is a bona fide alternative splice site capable of being recognized by core splicing machinery.

In addition to splice site prediction, we analyzed *vsx2* for predicted binding sites for known splicing regulatory proteins using Human Splice Finder [28]. Interestingly, we detected predicted binding sites for RNA-binding splicing regulators SRSF5 (SRp40) and SRSF6 (SRp55) situated on the 3′ acceptor splice site for *vsx2-s*. Thus, the SRSF4 and SRSF5 predicted binding sites are appropriately located for regulation of this alternative splicing event, and it is interesting

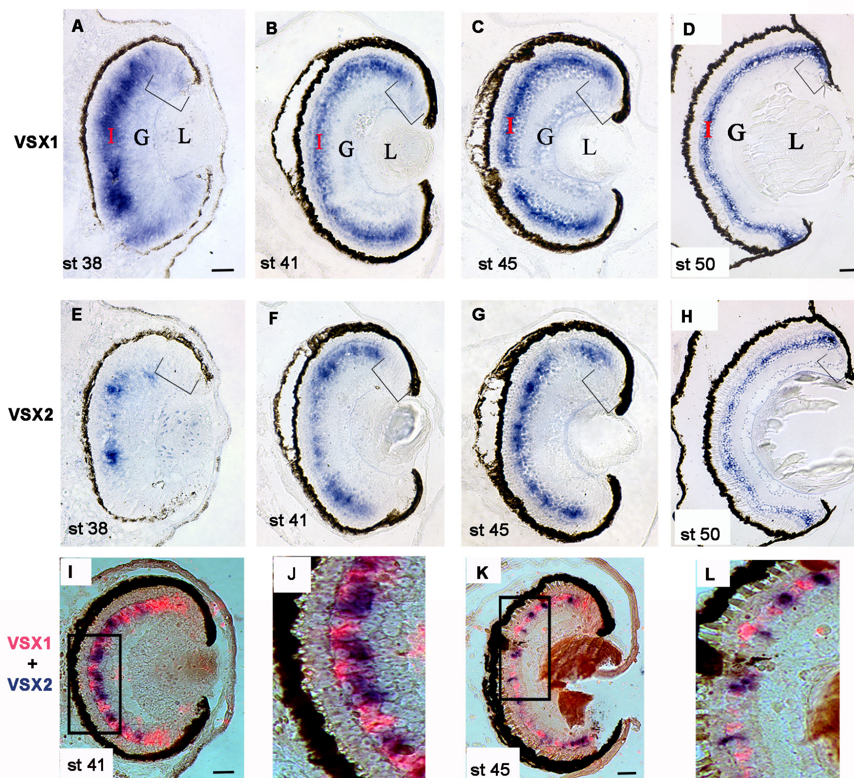


Figure 3. *vsx1* and *vsx2* are expressed in the INL of the maturing *Xenopus laevis* retina. **A**–**H**: Expression of *vsx1* (**A**–**D**) and *vsx2* (**E**–**H**) at st 38, 41, 45, and 50 (as indicated). Brackets indicate the ciliary marginal zone (CMZ). **I**–**L**: Overlapping expression of *vsx1* and *vsx2* decreases as the retina matures. Double in situ hybridization was performed on sections prepared from paraffin-embedded tadpoles using differently labeled probes for *vsx1* (red) and *vsx2* (blue). I, inner nuclear layer; G, ganglion cell layer; L, lens. Scale bars = 1 µM. Scale bar in **A** applies to **B**, **C**, **E**, **F**, and **G**; scale bar in **D** applies to **H**.

to speculate whether the binding of Srsf5 and/or Srsf6 can mediate splicing of *vsx2-s*.

The *X. laevis vsx* genes are expressed in distinct cells in the outer inner nuclear layer (OINL; Figure 3), consistent with the expression of *vsx* genes in bipolar cells in other species [12-18]. At st 38–50, *vsx1* is expressed in the retinal progenitor cells of the ciliary marginal zone (CMZ) and throughout the OINL (Figure 3A–D). We did not observe expression of *vsx2* in the CMZ (Figure 3E–H). This differs from *vsx* gene expression in warm-blooded vertebrates, where *vsx2* is expressed in retinal progenitor cells but is consistent with the previously published *X. laevis vsx1* expression pattern [13]. When we directly compared expression of the two *vsx* genes with double in situ hybridization, we found that *vsx2* expression is largely localized to a subset of the *vsx1*-expressing cells at st 41 (Figure 3I,J). This expression pattern is essentially the opposite of that of the mammalian retina, where *vsx1* is expressed in cone bipolar cells and is necessary for their development [14,29] while *vsx2* is necessary for the development of all bipolar cells [30]. Interestingly, as development progresses and the retina matures toward a fully differentiated state, the overlap between *vsx1*- and *vsx2*-expressing cells decreases, and cells are observed that express *vsx1* or *vsx2* but not both (Figure 3 K,L).

We found that *X. laevis* Vsx1 and Vsx2 were capable of binding to the PCE-1 sequence in vitro (Figure 4A). Incubation of each protein with a labeled PCE-1 oligonucleotide probe resulted in a mobility shift that could be effectively competed with an excess of unlabeled wild-type, but not mutated, PCE-1 oligonucleotide (Figure 4A, compare lane 1 to lanes 2 and 3 for Vsx1 and lane 6 to lanes 7 and 8 for Vsx2). The binding of Vsx2 with the PCE-1 site is consistent with previously published reports involving the mouse ortholog [9]. Vsx1 and Vsx2 preferentially bound the PCE-1 site in an EMSA and did not appear to appreciably bind BAT and RET4 oligonucleotide competitors. Interestingly, the short and long

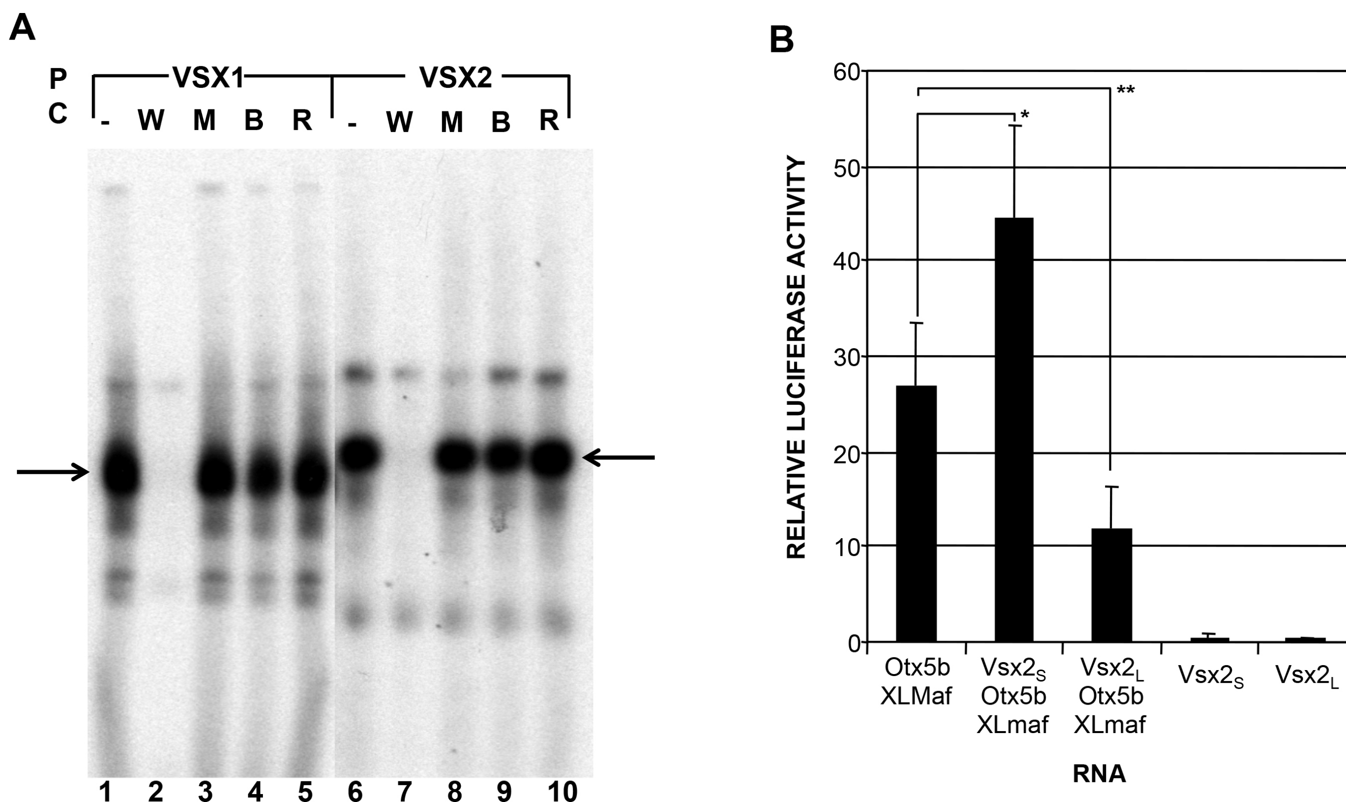


Figure 4. DNA binding activity of Vsx1 and 2 and transcriptional transactivation activity of Vsx2 isoforms. **A**: Vsx1 and Vsx2 can bind the photoreceptor conserved element-1 (PCE-1) site in vitro. Electrophoretic mobility shift assay (EMSA) performed using a radiolabeled PCE-1 probe, in vitro translated Vsx1 or Vsx2 proteins, and unlabeled wild-type (W) or mutant (M) PCE-1, BAT (B), or Ret4 (R) oligonucleotide competitors. Arrows indicate specific DNA–protein complexes. **B**: $Vsx2_L$, but not $Vsx2_S$, inhibits activation of XOP-Luc by otx5b and XLmaf. The luciferase assay was performed using lysates from embryos coinjected with the XOP-Luc reporter plasmid and otx5b + XLmaf and/or $Vsx2_L$ or $Vsx2_S$ RNAs as shown. Values are presented as fold mean normalized luciferase activity (relative to the reporter only, no effector control); error bars denote standard deviation from the mean. P, protein; C, competitor. * p<0.03; ** p<0.01.

forms of Vsx2 exhibited different transcriptional regulatory activities (Figure 4B). The long form, Vsx2$_L$, inhibited activation of XOP-Luc by otx5b and XLmaf, similar to the report by Dorval and colleagues [9]. Unexpectedly, the short form, Vsx2$_S$, cooperated with otx5b and XLmaf to activate XOP-Luc expression. Neither Vsx2$_L$ nor Vsx2$_S$ activated XOP-Luc alone. These results suggest that a form of Vsx2, Vsx2$_L$, can inhibit the activation of XOP and, perhaps, other photoreceptor-specific genes.

DISCUSSION

In this paper, we presented data suggesting that *vsx* gene products may play a role in regulating PCE-1-containing promoters. *rax* is expressed in photoreceptors of the maturing retina while *vsx* genes are not. This is consistent with the involvement of *rax* in activation of PCE-1-containing promoters and *vsx* gene products potentially being involved in repression of those promoters. We found that Vsx2$_L$ was capable of inhibiting activation of a reporter gene containing a rhodopsin promoter by Otx5b and XLmaf. *vsx2* is thought to play a role in blocking photoreceptor differentiation, since loss-of-function resulted in an increase in photoreceptors at the expense of bipolar cells [31]. Further, *vsx2* is associated with PCE-1-containing promoters, such as those of the *rod arrestin* and *interphotoreceptor retinoid binding protein* genes, in vivo [9]. It is interesting to speculate that while Rax and Rx-L promote activation of PCE-1-containing promoters in photoreceptors, Vsx gene products may function not only to promote the bipolar cell fate but also to suppress the photoreceptor cell fate and directly repress photoreceptor-specific promoters in other parts of the retina.

There is evidence that suggests that PCE-1-binding proteins do not strictly bind to only the canonical PCE-1 sequence. It has also been documented that Rax can bind to the BAT site, although with lower affinity compared to the binding of Rax to the PCE-1 site [6]. Additionally, Vsx gene products also have been reported to bind to non-PCE-1 sequences [32]. It will be interesting to determine whether the variation in Rx and Vsx binding sites depends on context, interactions with transcription factors that bind neighboring sites, or lends specificity to regulation of the expression of genes expressed early or late in retinal development.

Vsx2 can inhibit the activation of PCE-1-containing promoters by Crx [9]. The same report used a chromatin immunoprecipitation (ChIP) assay to demonstrate that Vsx2 is associated with a subset of PCE-1-containing promoters in vivo, not including the rhodopsin promoter. Together, these results support the principle that non-photoreceptor PCE-1-binding transcription factors, such as Vsx family members, may negatively regulate the activity of PCE-1-containing promoters in non-photoreceptor cells.

We found that only the long form of *vsx2* inhibited the activation of XOP-Luc by Otx5b and XLmaf. The short form actually enhanced this activity while *vsx1* had no consistent statistically significant effect (data not shown). The difference between Vsx2$_L$ and Vsx2$_S$ is a 21 amino acid fragment that is found in the CVC domain of Vsx2$_L$. The CVC domain has been shown to be involved in ubiquitin-mediated control of Vsx2 protein stability [9]. The CVC domain is necessary for full ubiquitination of Vsx2; however, it is not known what effect interruption of the CVC domain by the 21 amino acid fragment would have on ubiquitination or protein stability. Additionally, the CVC domain is important for the strength of homeodomain-dependent DNA binding [11], but the effect of disruption of the CVC domain, as found in Vsx2$_L$, on DNA binding is unknown. It is difficult to understand the significance of the differential activities of Vsx2$_L$ and Vsx2$_S$ since it is not known if the two forms are differentially expressed in the developing retina. It is interesting that Vsx2$_S$ did not inhibit the activation of XOP-Luc by otx5b and XLmaf in early *Xenopus* embryos.

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