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Cloning and characterization of the *Xenopus laevis p8* gene

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The *p8* gene encodes a transcription factor with a basic helix-loop-helix motif and has been cloned in rat, mouse and human. It is upregulated in acute pancreatitis. In the present study, the *Xenopus laevis* homolog of *p8* (*Xp8*) was isolated by PCR. The full-length *Xp8* cDNA consists of 677 bp and encodes 82 amino acids. The basic helix-loop-helix region is well conserved between *X. laevis* and mammals. Overexpression of an Xp8-EGFP fusion protein indicated that Xp8 was localized to nucleus, as in mammals. Analysis by RT–PCR showed that expression of *Xp8* was increased after gastrulation and maintained into later developmental stages. Expression was detected in the prospective neural region at the neurula stage by whole-mount *in situ* hybridization. At the larval stage, *Xp8* was expressed in central nervous regions, such as ventrolateral brain, trigeminal nerve, vestibulocochlear nerve and dorsal neural tube. Expression was also detected in the basal cement gland, olfactory placode, ear vesicle, notochord and anus. This specific pattern of expression suggests that Xp8 may play a role in the development of the central nervous system in *X. laevis*.

Key words: basic helix-loop-helix, development, nerve, *p8*, *Xenopus*.

Introduction

p8 was first cloned from a rat pancreatic cDNA library as a gene that was upregulated in pancreatic acinar cells during the acute phase of pancreatitis (Mallo *et al*. 1997). Rat *p8* encodes an 80-amino acid polypeptide with a basic helix-loop-helix (bHLH) motif and shows homology to several homeotic genes (Mallo *et al*. 1997). *p8* homologs have subsequently been cloned and sequenced in human and mouse (Vasseur *et al*. 1999a,b). Human p8 (hp8) has DNA-binding activity, which is increased when the protein is phosphorylated (Encinar *et al*. 2001). hp8 also has sequence similarity to the transcription factor HMG-I/Y (Bustin & Reeves 1996; Encinar *et al*. 2001). Analysis of the mouse *p8* promoter has identified putative transfactor binding sites (Vasseur *et al*. 1999b). In adult, p8 is expressed in various normal organs, such as the pancreas, lung, liver and gut (Mallo *et al*. 1997; Vasseur *et al*. 1999a), and is upregulated after injury and during regeneration, and in pancreatic cancer (Su *et al*. 2001). p8 is also structurally similar to candidate of metastasis-1, a novel factor in human breast cancer (Ree *et al*. 1999).

In a search for novel genes expressed in the *X. laevis* embryo using degenerate oligonucleotide primers 'GRRMFP' and 'VTAYQN', complementary to a DNA-binding sequence (Hayata *et al*. 1999), we isolated a clone with a bHLH motif, which was sequenced and found to be the *X. laevis* homolog of *p8*. We obtained a full-length clone and characterized its expression pattern in early *X. laevis* development.

Materials and Methods

Embryos

Xenopus laevis eggs were obtained from females injected with 400 units of human chorionic gonadotropin (Gestron; Denka Seiyaku Co., Kawasaki, Japan) and were fertilized *in vitro* with minced testis, then cultured in 1 × Steinberg's solution (58 mM NaCl, 0.67 mM KCl, 0.34 mM $Ca(NO_3)_2$, 0.83 mM $MgSO_4$, 100 mg/L kanamycin sulfate and 5 mM Tris-HCl (pH 7.4)). The *X. laevis* embryos were staged according to Nieuwkoop and Faber (1967). The jelly coats were removed with 3% cysteine hydrochloride in 1 × Steinberg's solution (pH 7.8) and the vitelline membranes were removed manually with fine forceps. All operations were carried out under sterile conditions.

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Cloning of full-length X. laevis p8 *cDNA*

A phagemid library containing approximately 2×10^6 p.f.u. was prepared from *X. laevis* larvae cultured for 3 days. To isolate a full-length *X. laevis p8* cDNA, we used a modified polymerase chain reaction (PCR) method. Phage were eluted from NZY medium to SM buffer (100 mM NaCl, 10 mM $MgSO_4$-H_2O, 0.01% gelatin, 50 mM Tris-HCl, pH 7.5) and then positive pools were identified by PCR with primers 5′-AACG-TCGTACATTGAGGCC-3′ (forward) and 5′-CTTCTT-CTTGCACTCGCTGC-3′ (reverse) designed against the original *Xp8* partial clone. In the first round, phage were plated on 15 NZY plates at 5×10^4 p.f.u./plate, cultured and screened by PCR. Second-round screening involved the transfer of soft agar from the positive plate to 16 tubes of SM buffer (500 µL) with sterile wooden toothpicks, to give less than 5×10^3 p.f.u./tube. Tubes were screened by PCR, then a 1 µL aliquot was taken from a positive tube and diluted 1×10^{-3}, 1×10^{-4} and 1×10^{-5} and 100 µL of each dilution was plated onto NZY plates. A plate with approximately 5×10^3 p.f.u. was screened in a third round, and three further rounds of screening were needed to obtain a single positive plaque.

Overexpression of enhanced green fluorescent protein

The *Xp8* coding region linked to enhanced green fluorescent protein (*EGFP*) or only *EGFP* was inserted into the pCS2 plasmid vector (Fig. 3a). *Xp8-EGFP* and *EGFP* transcripts were generated *in vitro* and 0.1–1 ng of mRNA was injected into the animal pole of each blastomere at the 2-cell stage and cultured. The putative ectoderm region, called the animal cap, was cut away at stage 9 and immediately observed under a fluorescent microscope.

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GCACGAGCAG ACAGACTCTG TGACACTGAG CTTGTATACA ACTCCCAGCA GCCAGACAAC   60
TCCCAGCAGC CAGACAACTC CCAGCAGCCA GAGAAGCCGC ACTCACTGAG CAACATGAAA  120
                                                          M  K

ACGTCGTACA TTGAGGCCAA CAAAGTGCAG CCCACAGACT TCGAGGTGCA ATACTTTGAT  180
T  S  Y  I   E  A  N   K  V  Q   P  T  D  F   E  V  Q   Y  F  D

GAGTACGAAT ATTACAACCT GACCGACAGA TACTCACTGC CCACGGCTGC CAGGAAGGGC  240
E  Y  E  Y   Y  N  L   T  D  R   Y  S  L  P   T  A  A   R  K  G
                                                    Basic region
CGCACCAAGA AGGAGGCTGA AGCCAACACC AACAGAGAGA GCCCATGTGG CCACGAGAGG  300
R  T  K  K   E  A  E   A  N  T   N  R  E  S   P  C  G   H  E  R
          Helix I                      Loop                Helix II
AAGATCAGTA GCAAACTGCA GCGCAGCGAG TGTAAGAAGA AGCTGAAAGT GGCCAAGGTG  360
K  I  S  S   K  L  Q   R  S  E   C  K  K  K   L  K  V   A  K  V

TGAGGACAGG ACAAACCTCC GGATGGCACA AGACACACAC AGACCAATGG CAACGAGACG  420
*

GACCAAGAGA ACTGGTACAG ACTGATGTGG TGAGACACAA GCTCCGCCCC TCACAGACAC  480
CAGAGCAAGG AGGACAGCAG GACAGATGAG TGCAGAGTAT GACTCCTCTA GTCCGGGAGC  540
AGCAGAGCCT CCTTTAACCT CTTCATGGCT GTAGCTAGAA AGACTGGGAA GAGTGGGGCA  600
CGCTGTTAAC TCCTTCAATG CTGGGGAGAG CTGCATGTGA GAATCAGTTG TGTATGAAAT  660
AAACTGAATG TTCTGCT                                                 677
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Fig. 1. Sequence of *Xenopus laevis p8* cDNA and the deduced peptide sequence. The polyadenylation site AATAAA is underlined. A full-length *Xp8* clone was obtained by a modified PCR method. The *Xp8* cDNA is 677 bp in length and encodes 82 amino acids, including a basic helix-loop-helix motif. It has 246 bp in the ORF, 114 bp in the 3′ UTR and 317 bp in the 5′ UTR.

(a)

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X-P8  -MKTSYIEAN KV-QPTDFEV Q--YFDEYEY YNLTDRYSLP TAARKGRTKK EAEANTNRES PCGHERKISS KLQRSECKKK LKVAKV  82
H-P8  -MATFPPATS APQQPPGPED EDSSLDESDL YSLAHSYLG- GGGRKGRTKR EAAANTNRPS PGGHERKLVT KLQNSERKKR G--ARR  82
M-P8  -MATLPPTAN PSQQPLNLED EDGILDEYDQ YSLAHPCVV- GGGRKGRTKR EAAANTNRPS PGGHERKLVT KFQNSERKK- ---AWR  80
R-P8  MMATLPPTAH TSQQPVNIED EDGILDEYDQ YSLAQSYVV- GGGRKGRTKR EAAANTNRPS PGGHERKLLT KFQNSERKK- ---AWR  81
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(b) Homology (total)

	Xenopus	Mouse	Rat
Human	38%	73%	73%
Rat	38%	91%	
Mouse	39%		

(c) Homology (bHLH)

	Xenopus	Mouse	Rat
Human	75%	96%	96%
Rat	75%	100%	
Mouse	75%		

Fig. 2. Comparisons of p8 peptide sequences among *Xenopus laevis*, human, mouse and rat. (a) Alignments of Xp8 and the three mammalian p8 peptides. Conserved amino acids are highlighted. A putative basic helix-loop-helix (bHLH) region is illustrated under the sequence. The region defined by arrows indicates a nuclear targeting sequence (Mallo *et al.* 1997). (b) Full-length amino acid sequence identity between the four species. (c) Sequence identity within the p8 bHLH regions of the four species. Comparison of the full-length and bHLH regions indicates that only the bHLH region is well conserved. Arrowheads show conserved serine, threonine and tyrosine residues, which may be targets for phosphorylation.

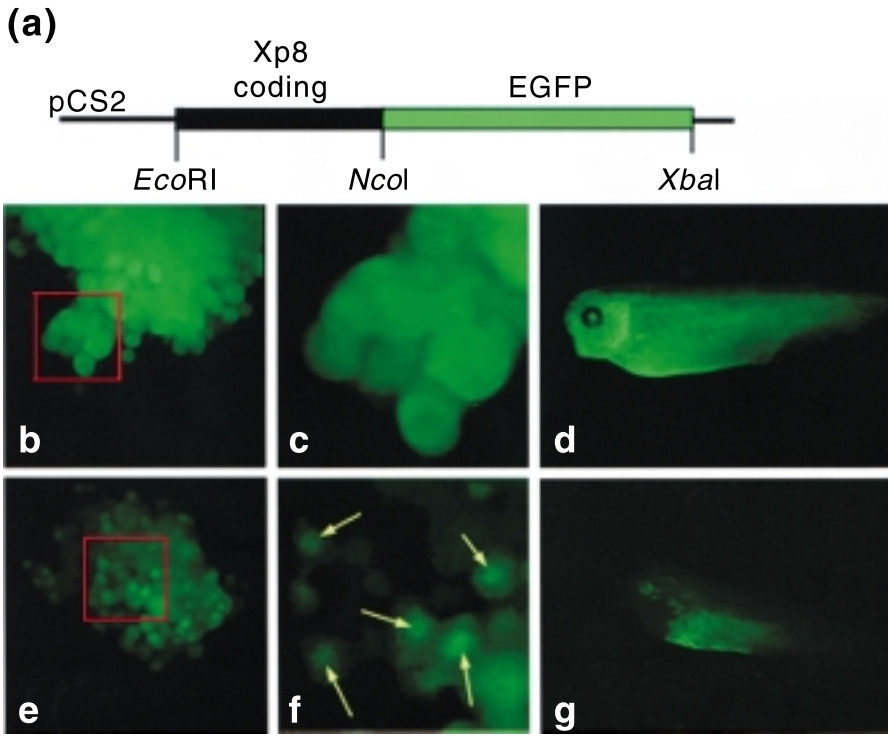

Fig. 3. Intracellular localization of the Xp8. (a) *Xp8-EGFP* construct. An *Xp8-EGFP* or *EGFP*-only sequence was ligated into the pCS2 plasmid vector. Enhanced green fluorescent protein (EGFP) or Xp8-EGFP transcripts were injected into the animal pole at the 2-cell stage. The enclosed regions in (b) and (e) are enlarged in (c) and (f), respectively. Animal cap cells injected with 2 ng *EGFP* had fluorescence throughout the cytoplasm (b,c), whereas Xp8-EGFP was specifically localized to the nucleus (e,f). Arrows show the nucleus. Injected embryos were cultured to stage 38 and observed. Larvae injected with 0.2 ng *EGFP* mRNA or 2 ng *Xp8-EGFP* mRNA are shown in (d) and (g), respectively. (d) The fluorescence remained strong in whole embryo. (g) Only low level fluorescence was observed on the abdominal surface.

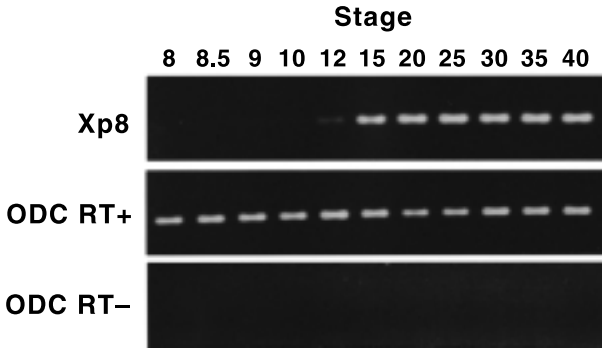

Fig. 4. Reverse transcription–polymerase chain reaction analysis of *Xp8* expression. Expression of *Xp8* mRNA was increased from stages 12 to 15 and was maintained until stage 40. Maternal *Xp8* mRNA was detected at very low levels.

Reverse transcription–polymerase chain reaction

Reverse transcription–polymerase chain reaction (RT–PCR) was used to detect *Xp8* mRNA. The following primers were used: *Xp8* 5′-AGACGGACCAAGAGAACTGG-3′ (forward) and 5′-CAACTGATTCTCACATGCAGC-3′ (reverse); *ODC* 5′-GTCAATGATGGAGTGTATGGATC-3′ (forward) and 5′-TCCATTCCCTCTCCTGAGCAC-3′ (reverse). Both genes were amplified using an annealing temperature of 56°C for 30 cycles.

Whole-mount in situ *hybridization*

Whole-mount *in situ* hybridization was performed according to standard methods (Harland 1991) on *X. laevis* albino embryos using a full-length *Xp8* cDNA as a template to synthesize RNA probe. The RNA probe (200 ng) was added to 1 mL hybridization buffer. Hybridized embryos were stained with BM purple at room temperature for 3 h and at 4°C overnight. Embryos were refixed with Bouin's fluid, dehydrated in ethanol series, replaced in xylene, embedded in paraffin and sectioned at 12 µm.

Results

Cloning of the X. laevis p8 *gene*

A full-length *Xp8* clone comprising 677 nucleotides was isolated from a 3-day-old *X. laevis* embryo cDNA library using a modified PCR method. *Xp8* is predicted to encode an 82 amino acid polypeptide with a bHLH motif (Fig. 1). A putative polyadenylation signal (AATAAA) is present at 20 bp upstream of the poly(A) extension. There were 114 nucleotides and 317 nucleotides in the 5′ and 3′ untranslated regions, respectively. Comparison of the *X. laevis* p8 amino acid sequence with that of rat, mouse, and human (Fig. 2) showed a 38–39% similarity to the mammalian sequences (Fig. 2b). The bHLH motif in Xp8 (Fig. 2a,

Mallo *et al.* 1997) had 75% similarity with the mammalian p8 sequences (Fig. 2c). In addition, the candidate phosphorylated amino acids, serine, threonine and tyrosine, were well conserved in all species (Fig. 2a).

Intracellular localization of Xp8

Animal cap cells were injected with *Xp8-EGFP* mRNA and observed under fluorescent microscope. Xp8-EGFP fluorescence was stronger in the nucleus than in the cytoplasm (Fig. 3f), suggesting that Xp8 is localized in the nucleus. Some injected embryos were cultured to stage 38. Although strong EGFP fluorescence was maintained to this stage (Fig. 3d), Xp8-EGFP fluorescence was weak (Fig. 3g).

Pattern of Xp8 expression in early development

Analysis by RT–PCR was used to detect *Xp8* mRNA expression in the developing embryo (Fig. 4). The expression of *Xp8* increased from stage 12 to stage 15 and then remained at a steady level until stage 40.

Whole-mount *in situ* hybridization was performed to determine the spatial pattern of *Xp8* expression in

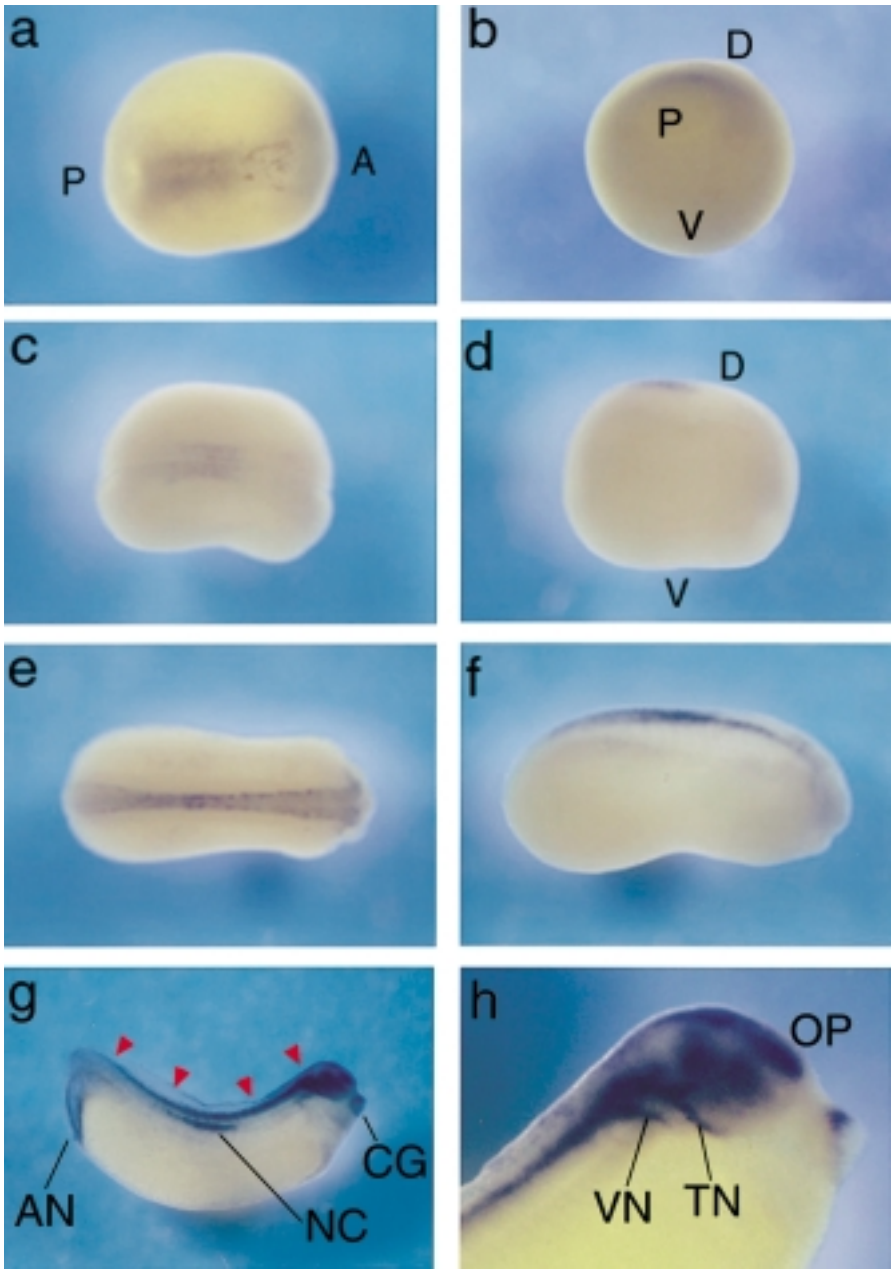

Fig. 5. Whole-mount *in situ* hybridization. Expression pattern of *Xp8* at stages (a, b) 14, (c, d) 18, (e, f) 22 and (g, h) 30. (a,c,e) Dorsal (D) view, (d,f–h) lateral view, (b) ventroposterior view. The anterior (A) is situated on the right side in all parts except (b). Arrowheads show the central nervous system. V, ventral; P, posterior; AN, anus; NC, notochord; CG, cement gland; VN, vestibulocochlear nerve; TN, trigeminal nerve; OP, olfactory placode.

developing embryos (Figs 5,6). *Xp8* expression was detected in the middle body of the prospective neural region at stage 14 (Fig. 5a,b), where it was maintained to stage 18 (Fig. 5c,d). Expression in the prospective neural region extended to the anteroposterior area and branched in the brain area at stage 22 (Fig. 5e,f). The neural tube showed patchy signals, which were thought to be along the somites. At stage 30, signals were present in the central nervous regions, including the ventrolateral brain (Fig. 6B,C), vestibulocochlear nerve (Fig. 5h), trigeminal nerve (Fig. 5h) and dorsal neural tube (Fig. 5g). Expression was also detected in the basal cement gland, olfactory placode, notochord and anus (Fig. 5g). *Xp8* expression was detected in the notochord at stage 30, but thereafter moved anteriorly and then disappeared (data not shown). Embryos were sectioned (Fig. 6) and expression was detected throughout the brain and eye, but was particularly strong in the dorsal and ventrolateral brain and dorsal and ventral retina (Fig. 6B). The dorsal ear vesicle adjacent to the hindbrain also showed a strong signal (Fig. 6C). In the bulbar region, Xp8 was expressed in the dorsal and ventrolateral neural tube (Fig. 6D).

Discussion

This is the first report of a non-mammalian *p8* gene. *Xp8* is a small gene with a full-length sequence of only 677 bp, encoding 82 amino acids. The *Xp8* product, consistent with other *p8* proteins, has no signal peptide or transmembrane regions, but contains a bHLH motif. The sequence of this bHLH region showed significant similarity between *X. laevis* and mammals (Fig. 2c) and, given that Xp8 is localized to the nucleus, it is likely to be a transcription factor. The DNA-binding activity of human p8 is increased with phosphorylation (Encinar *et al*. 2001) and, while we could not identify any putative phosphorylation sites on Xp8, there were a couple of well-conserved amino acids outside the bHLH region (Fig. 2a) that may be targets for phosphorylation.

The *Xp8* clone reported here was isolated by a modified PCR screening method. This method has several advantages over standard filter hybridization methods. Screening time is significantly reduced: it took only 48 h to isolate one positive plaque by this method. The method is also technically simple, with no requirement for radiolabeled or fluorescent probes or hybridization filters. In addition, the rate of signal to noise is very high, decreasing the rate of false positives.

Analysis by RT–PCR showed that *Xp8* expression was increased after gastrulation and persisted to stage 40 (Fig. 4). Reports of *p8* expression in rat (Mallo *et al*. 1997) and human adult organs (Vasseur *et al*. 1999a) have suggested that *Xp8* may be expressed in endoderm during development and in adult *X. laevis*. However, whole-mount *in situ* hybridization (Figs 5,6) showed that *Xp8* is expressed in the prospective neural region in *X. laevis* embryos, and then in limited regions of nerves. These results suggest that Xp8 may have different functions in development and in the adult, as has been seen for the *Pax* genes, which also have a HLH motif (Dohrmann *et al*. 2000).

Injection of *Xp8-EGFP* mRNA into 2-cell stage embryos led to very minimal fluorescence in the larva. Microinjection of *Xp8* mRNA into *X. laevis*

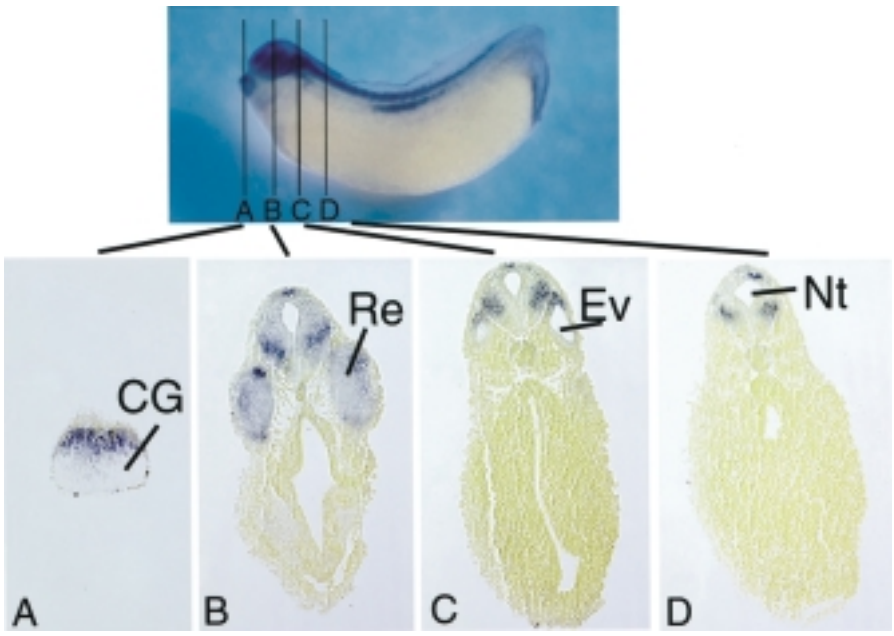

Fig. 6. After whole-mount *in situ* hybridization, embryos were embedded in paraffin and sectioned at 12 µm. The larva shown (stage 30) was sectioned at lines A–D and sections are shown in (A–D). CG, cement gland; Re, retina; Ev, ear vesicle; Nt, neural tube.

embryos led to no identifiable mutant phenotype. The pattern of *Xp8* expression in the embryo suggested a possible role in neural development, but other experimental strategies, such as loss-of-function or dominant-negative approaches may be needed to further clarify the role of this gene in *X. laevis* development.

Acknowledgments

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