Regulation and expression of elrD1 and elrD2 transcripts during early Xenopus laevis development#

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ABSTRACT The Xenopus laevis elrD (elav-like ribonucleoprotein D) gene is a member of the elav/Hu family which encodes RNA-binding proteins. Most of the elav/Hu genes are expressed in the nervous system, where they are implicated in the development and maintenance of neurons. The regulation of elrD gene expression involves two promoters, pD1 and pD2. In this study, we analyzed the neural specificity directed by both promoters. They were fused to the gene encoding green fluorescent protein, and their ability to drive neural expression in injected Xenopus embryos was examined. We show that both promoters direct neural expression and that whole promoter sequences are needed to induce neural specific expression. Finally, we analyzed the spatial and temporal localization of the two elrD transcripts, elrD1 and elrD2. We found that the two transcripts present the same tissue-specific pattern of expression, with distinct developmental regulation. Our results show a complex regulation of the elrD gene and suggest that different transcripts resulting from alternative splicing of the elrD gene probably define different neurons.

KEY WORDS: regulation, promoter, alternative splicing, elav, Xenopus

The elav/Hu genes constitute a multigenic family implicated in the post-transcriptional control of gene expression in neuronal cells. In Drosophila, three members of the elav/Hu have been isolated: elav (Yao et al., 1993), rbp9 (Kim and Baker, 1993) and fne (Samson and Chalvet, 2003). Members of this family have also been identified in human (Szabo et al., 1991), in the mouse (Okano and Darnell, 1997), in Xenopus (Good, 1995), in chickens (Wakamatsu and Weston, 1997), in zebrafish (Park et al., 2000) and in Caenorhabditis elegans (Fujita et al., 1999). Expression of elav/Hu genes is generally restricted to all or parts of the nervous system, except for elrB/HuB whose transcripts are also expressed in the ovary and testis and elrA/HuA whose transcripts are ubiquitously expressed (Good, 1995; Okano and Darnell, 1997; Wakamatsu and Weston, 1997).

All elav/Hu genes encode RNA-binding proteins (RBPs) containing three RNA recognition motifs (RRMs). RRM-containing proteins are known to be involved in many post-transcriptional and translational events (Pascale et al., 2008).

In Drosophila, genetic analysis of elav suggests a role in the differentiation and maintenance of neurons (Yao et al., 1993). Consistently, overexpression of a wild type form of HuB or HuC in the mouse embryonic neural tube induces ectopic expression of neuronal markers, whereas a dominant negative form suppresses the differentiation of motor neurons (Akamatsu et al., 1999). In vitro studies also suggest the implication of ELAV/Hu proteins in neuronal differentiation. Misexpression of cHuD in cultured chicken neural crest cells also induces neuronal differentiation (Wakamatsu and Weston, 1997). In addition, overexpression of elrB in Xenopus embryos induces severe defects in the neural tube (Perron et al., 1999). These genes thus appear to be implicated in neuronal differentiation and or maintenance of neuronal markers.

Because all these proteins contain RRMs it is though that they may promote neuronal differentiation by regulating gene expression at a post transcriptional level (Perrone-Bizzozero and Bolognani, 2002). Consistently, Drosophila ELAV function has been shown to be important for the formation of the neuron-specific transcripts of neuroglian, erect wing and armadillo by regulating their 3’UTR (Lisbin et al., 2001; Soller and White, 2005). The Elav/Hu proteins are also implicated in regulating mRNA stability and mRNA export (Pascale et al., 2005). Indeed, HuD and HuR can decrease protein expression by inhibiting p27 translation (Kullmann et al., 2002). HuD also appears to act as a...

Abbreviations used in this paper: elrD1, elav-like ribonucleoprotein D 1; elrD2, elav-like ribonucleoprotein D 2; pD1, elrD1 promoter; pD2, elrD2 promoter; GFP, green fluorescent protein.

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#Note: In memory of my late supervisor, Dr. Maurice Wegnez.

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major determinant of GAP-43 and Musachi 1 expression (Pascale et al., 2004; Ratti et al., 2006). Nova-1 mRNA stability and translation are strongly controlled by ELAV proteins (Ratti et al., 2008). Moreover, in Drosophila, elav autoregulates through a mechanism requiring the 3’UTR of its own mRNA (Samson, 1998). It has also been shown that fne autoregulates and interact with elav by binding the same sequences (Borgeson and Samson, 2005) and that Hu proteins can also interact with themselves (Kasashima et al., 2002).

The regulation of elav/Hu family gene expression is complex and involves alternative splicing as well as alternative promoters (Kim and Baker, 1993). We have previously characterized two alternative promoters of the elrD gene, a neural specific elav/Hu genes of Xenopus laevis (Nassar and Wegnez, 2001).

In this work, we studied the specificity of elrD1 and elrD2 promoters (pD1 and pD2) using the green fluorescent protein (GFP) as a reporter. Expression directed by each promoter construct as well as deletions promoter constructs were analyzed by microinjection into Xenopus embryos. We showed that sequential deletions in pD1 and pD2 constructs decrease neural expression and that both elrD transcript types, elrD1 and elrD2 are expressed specifically in the nervous system, with distinct developmental regulation.

Results

elrD1 and elrD2 promoter induces neural expression

The genomic structure of elrD gene revealed a gene with two alternative promoters, pD1 and pD2 (Nassar and Wegnez, 2001). The elrD gene is composed of at least 5 exons and 4 introns (Fig. 1A) with various forms of transcripts resulting from alternative splicing (Fig. 1C). The different elrD transcripts potentially encode alternative forms of proteins with different N-termini and an alternative splicing of exon 3 (Fig. 1D).

In order to test the neural expression of pD1, D1(-1260,+62) and pD2, D2(+405,+905) (Fig. 1B), we fused them to the GFP reporter gene, leading to pD1-GFP and pD2-GFP constructs. Injections of pD1-GFP and pD2-GFP constructs were performed into one dorsal or one ventral blastomere of four-cell stage embryos, knowing that the nervous system derives essentially from dorsal blastomeres. No expression of either construct was detected before stage 18 (mid neurula). GFP fluorescence was detected in the neural tube specifically in the embryos where dorsal injections of pD1-GFP and pD2-GFP were performed (Fig. 2). GFP expression appears as a mosaic due to unequal distribution of the DNA templates between cells during the course of development, but it is clearly restricted to the neural tube (Fig. 2). Embryos injected with pEGFP-1 (promoterless GFP), as well as those injected ventrally did not express GFP.

We also studied expression directed by pD1-GFP and pD2-GFP constructs by injecting them into one blastomere at the animal pole of two-cell stage. pD1-GFP and pD2-GFP drive GFP expression in the neural tube in 52% and 58% of injected embryos respectively (Table 1). Coinjections of pD1-GFP or pD2-GFP with SV40-lacZ constructs (lacZ gene under the control of the SV40 promoter) were also performed. About 60% of injected embryos expressed GFP exclusively in the neural tube at the neurula stage while beta-galactosidase expression in these embryos was observed within and outside the neural tube (data not shown). These experiments confirmed that expression of GFP is restricted to the nervous system and that both pD1 and pD2 promoter regions are specifically regulated during early neurogenesis.

Deletions in elrD1 and elrD2 promoters decrease GFP expression

In order to determine the promoter region essential to drive neural expression, four deletion constructs D1(-782,+62), D1(-338,+62), D1(-148,+62) and D1(-64,+62) were produced from

Fig. 1. Alternative promoters and splicing patterns of elrD gene. (A) Genomic structure of the elrD gene. +1 marks the position of the first nucleotide of elrD1 transcript. E1: elrD1 exon 1, E1’: elrD2 exon 1, E2: exon 2, E3: exon 3, E4: exon 4. Dashes indicate the position of introns. (B) pD1, D1(-1260,+62), and pD2, D2(+405,+905) deletion constructs. All deletions were fused to the GFP reporter gene. (C) Variants elrD transcripts. elrD1 and elrD2 have different exon 1, E1 and E1’ respectively. They share the same E2 and E4. E3 is alternatively spliced. The structure of the elrD transcripts were deduced from the structure of the elrD cDNAs. (D) Predicted ElrD protein isoforms. E1 encodes the first 3 amino acids of the ElrD1 protein while E1’ encodes the first 8 amino acids of the ElrD2 protein. E3 encodes 29 amino acids in RRM1, RNA Recognition Motif 1. The presence of intron in E4 is not determined. RRM2: RNA Recognition Motif 2. RRM3: RNA Recognition Motif 3. aa: amino acids.
Regulation and expression of elr\(D\) transcripts

p\(D1\) by deleting the 5' end of upstream elr\(D1\) region, as well as one construct containing shorter elr\(D2\) 5' upstream region D2(+650, +905) (Fig. 1B). These constructs were fused to the GFP reporter gene and injected into one blastomere of two-cell stage embryos. No GFP fluorescence was detected for all injected constructs before neurula stage. The GFP expression begins at neurula stage in the neural tube (Table 1). The proportion of fluorescent embryos, as well as the neural tube expression, decreased when we injected constructs containing deleted 5' elr\(D1\) and elr\(D2\) upstream region (Table 1). These results show that p\(D1\)-GFP and p\(D2\)-GFP constructs drive neural specific expression during early Xenopus development. Deletions in the elr\(D1\) and elr\(D2\) 5' flanking region lead to a decrease of the level and the specificity of neural-specific GFP expression.

**Expression of elr\(D1\) and elr\(D2\) transcripts**

We compared the expression of elr\(D1\) and elr\(D2\) transcripts at different developmental stages and in the adult brain. Since the two transcripts differ in size at their 5'ends and share the same 3'ends, we used the 5'RACE PCR to discriminate between them. No expression was found at gastrula stage (data not shown). The two transcripts were first detected at neurula stage with identical expression (Fig. 3A). Expression was also detected at tailbud stage with elr\(D2\) stable transcripts expression stronger than elr\(D1\). In the adult brain, expression of the two transcripts was similar (Fig. 3A). Our results show that the relative abundance of the stable elr\(D1\) and elr\(D2\) transcripts changes during development, revealing one specific level of regulation.

In order to determine the spatial expression of the two transcripts at the tailbud stage, we performed in situ hybridization with RNA probes specific to elr\(D1\) and elr\(D2\) 5'UTRs (see Materials and Methods). elr\(D1\) and elr\(D2\) expression was coincided and restricted to the spinal cord, the brain and the cranial ganglia (Fig. 3 B,C).

### Table 1

<table>
<thead>
<tr>
<th>Construct</th>
<th>Expression in the nervous system</th>
<th>Expression out of the nervous system</th>
<th>No expression</th>
<th>Number of scored embryos</th>
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<td>p(D1)</td>
<td>52%</td>
<td>8%</td>
<td>40%</td>
<td>394</td>
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<tr>
<td>D(1(-1260,+62))</td>
<td>38%</td>
<td>9%</td>
<td>53%</td>
<td>368</td>
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<tr>
<td>D(1(-782,+62))</td>
<td>20%</td>
<td>12%</td>
<td>68%</td>
<td>392</td>
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<tr>
<td>D(1(-148,+62))</td>
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<td>16%</td>
<td>49%</td>
<td>378</td>
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<tr>
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<td>8%</td>
<td>15%</td>
<td>77%</td>
<td>390</td>
</tr>
<tr>
<td>p(D2)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>D(2(+450,+905))</td>
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<td>6%</td>
<td>36%</td>
<td>388</td>
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<tr>
<td>D(2(+650,+905))</td>
<td>5%</td>
<td>13%</td>
<td>82%</td>
<td>398</td>
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</tbody>
</table>

### Fig. 2. Neural specificity of elr\(D1\) and elr\(D2\) promoters

Injected embryo with p\(D1\)-GFP (A,B) and p\(D2\)-GFP (C,D) constructs into one dorsal blastomere at four-cell stage. (A,C) Embryos under blue light. GFP expression was visualized in the neural fold at stage 18. No GFP expression was detected in the non-injected side that was used as a control (left part of the embryos). (B,D) Embryos under visible light. (E,F) Embryo expressing p\(D1\)-GFP (E) and p\(D2\)-GFP (F) constructs under visible and blue light. GFP expression is clearly visible in the neural fold.

### Fig. 3. Temporal and spatial expression of elr\(D\) transcripts

(A) One μg of total RNA was reverse-transcribed and PCR performed by incorporating digoxigenin-dUTP. PCR products were resolved by electrophoresis in a 1% agarose gel and detected as described in Materials and Methods. Sequence analysis of the cloned products has shown that bands 1 and 2 correspond to elr\(D1\) and elr\(D2\) transcripts, respectively. Band 3 corresponds to an incomplete elongation of elr\(D1\) and elr\(D2\) transcripts. N: Neurula, T: Tailbud, B: Adult Brain. (B,C) In situ hybridization at tailbud stage with an antisense elr\(D1\) (B) and elr\(D2\) (C) probes. expression is seen in the brain vesicles (arrowheads), the cranial ganglia (arrows) and the spinal cord (block arrow).
Discussion

Regulation of elrD gene expression by alternative promoters

The elrD gene through the use of alternative promoters generates at least four alternative transcripts, predictive of four alternative protein isoforms. Deletion series in the promoter regions reveals that long sequences upstream of the start of transcription are required to direct efficient and specific neural expression of GFP reporters microinjected in embryos. In zebrafish, two GC-rich boxes were found to play a role in controlling the neuronal specific expression of zHuC (Zhao et al., 2006). In Xenopus, the sequences of such boxes regulating elrD gene were not identified. Injection of neurogenin, a bHLH transcription factor, induces expression of zHuC (Kim et al., 1997). Little is known about the regulation of elav/Hu genes. Our results provide elements that are needed to identify specific regulators that bind to the 5’ flanking region of elrD genes.

It is known that the elav gene family has evolved new functions remarkably rapidly through standard gene duplication and retrotransposition (Samson, 2008). Of particular relevance here is one of the three fly elav/Hu paralogs, rbp9, which includes 3 promoters, and produces 2 alternative forms of protein differing by 5 residues (Kim and Baker, 1993). rbp9 produces nervous system-specific and ovary-specific transcripts, the latter being essential to female fertility (Kim-ha et al., 1999). Promoter duplication/specification might provide a mechanism for functional diversification of the elav family by changing the cell specificity of expression rather than the structure of the protein product, whether in specific cell types (elrC) or tissues (rbp9).

Alternative forms of elrD transcripts

The elrD gene, similar to most but not all of its orthologs, is specifically expressed in the nervous system. In situ hybridization with elrD1 and elrD2 transcript-specific probes at the tailbud stage shows coincided expression in the brain, the spinal cord and the cranial ganglia.

Similar expression was described when using a RNA probe corresponding to the entire elrD cDNA. At this stage, elrBand elrC transcripts present the same pattern of expression as elrD (Per- ron et al., 1999). Nevertheless, different combinations of elav/Hu transcripts were found in subset of neurons in the mouse, zebrafish and Xenopus, suggesting that groups of neurons are defined by the expression patterns of elav/Hu (Okano and Darnell, 1996; Wakamatsu and Weston, 1997; Perron et al., 1999).

The ELAV/Hu related family Bruno/CeLF genes are also differentially expressed during neurogenesis and are essential for proper neural development (Wu et al., 2010). During neurogenesis, several genes such as the Sox family of transcription factors, neurexin (nrnx), and CRMP gene also present differential expression pattern suggesting differential regulation (Cunningham et al., 2008; Souopgui et al., 2007; Zeng et al., 2006).

The different elrD transcripts potentially encode alternative forms of proteins with different N-termini and an alternative splicing of exon 3 that does not correlate with alternative promoter use. The alternative forms of elrD transcripts as well as the alternative alternative ElrD protein isoforms might be specific for different neurons.

Our results suggest a complex regulation of elrD gene; not only different elav/Hu members but also alternative transcripts from a given member can possibly define different neurons. The two elrD promoters provide an interesting experimental system to identify factors that regulate the elav/Hu genes and present a tool that can be used as a marker of neural expression in Xenopus laevis embryos.

Materials and Methods

Construction of GFP plasmids

The pD1 and pD2 constructs were generated by PCR amplification using ed1/ed2 and ed4/ed5 primers to give D1(-1260, +62) and D2(+405, +905) constructs. Smaller fragments of pD1 and pD2 constructs were generated using ed9/ed2, ed8/ed2, ed7/ed2, ed6/ed2 and ed10/ed5 primers, to give D1(-782, +62), D1(-338, +62), D1(-148, +62), D1(-64, +62) and D2(-850, +905) constructs respectively. The PCR fragments were subcloned into the pEGFP-1 basic promoterless vector (Clontech), using the HindIII and Xhol restriction sites present in the primers. Primers sequences are: elrD(-1260, -1237); elrD(-338, -148, +62); elrD(-64, -43); elrD(-50, -43).

Microinjections

Fertilized Xenopus eggs were dejellied in 2% cysteine, pH 7.6 and transferred into 0.1x modified Barth Saline (MBS), 5% Ficoll solution. Plasmid constructs (300pg) were injected with a pressure-driven injector into the animal pole of two-cell stage embryos or into one dorsal or ventral blastomere of four-cell stage embryos. About 120 embryos were injected for each tested construct. Experiments were repeated four times. Embryos were cultured at 18-24°C in 0.1x MBS. They were staged according to Nieuwkoop and Faber (1967).

Microscopy

Embryos were observed using a Leica MZFL III stereomicroscope equipped with a GFP plus filter fluorescence device and photographed using a cool snap camera.

Detection of elrD transcripts by labeling 5’RACE PCR products with digoxigenin

The 5’ rapid amplification of cDNA ends (5’RACE) was conducted according to the procedure described in the SMARTTM RACE cDNA amplification kit (Clontech). RNA was isolated from Xenopus laevis embryos and adult brains. Ten stage 10-11 or stage 18 embryos, five stage 30 embryos (late tailbud) and three adult brains were homogenized in 1ml of 4M guanidine isothiocyanate, 0.5% sarcosyl, 25mm sodium citrate, pH7.0. RNA was extracted with phenol/chloroform and recovered by ethanol precipitation. Genomic DNA and polysaccharides were removed by LiCl precipitation. Prior to reverse transcription, the samples were treated with two units of RNase-free DNase I. One μg of total RNA was used as the template of MMLV reverse transcriptase Superscript II to synthesize the first strand cDNA with an oligo(dT) primer. The cDNA was tailed with dCTP and amplified by PCR using elrD-specific antisense oligonucleotide primer 5’-GGG GGC CAG GAT AAC GTC TGT TGG GGG ATT GGT AGA GAA G-3’ corresponding to the linker region between RRM2 and RRM3. This PCR was performed using a dNTP mix with 1/19
digoxigenin-11-dUTP to dTTP ratio. 5’ RACE products were separated on a 1% agarose gel and transferred to a nylon membrane. Nucleic acids were fixed by baking for 30 min at 80°C. The membrane was rinsed in MAB (100 mM maleic acid, 150 mM NaCl, pH 7.5), incubated 2h in MAB, 1% BMBR and then 2h in MAB, 1% BMBR, 1/5000 antidigoxigenin antibody-alkaline phosphatase conjugate. The membrane was then washed twice in MAB, 0.3% Tween 20 and the alkaline phosphatase activity was revealed using BCIP/NBT according to Harland (1991). 5’ RACE products were also gel-purified using the Nuclear Trap Gel Extraction kit, cloned into T/A-type PCR cloning vector pGEM-T, and sequenced.

Whole mount in situ hybridization

Whole mount in situ hybridization was performed with digoxigenin-labeled probes according to Harland (1991). The elrD1 probe corresponds to the elrD1 exon sequence (E1 from +13 to +329 nucleotides from the transcription start site ts1) and elrD2 probe to elrD2 exon 1 sequence (E1: from +917 to +1045 nucleotides from ts1). elrD1 and elrD2 specific probes were generated by PCR using primers D1s/D1as and D2s/D2as, respectively. D1s 5’-GCG GAG AAT TTA TCT ACA CCC TAC TTG-3’, D1as 5’-AAT TAC TTG-3’, D2s 5’-AAT TCT CGA GAG TTC GTC GAG CAC TGG-3’, D2as 5’-GTA TGC TTG CTG GAC TTC G-3’. The PCR fragments were subcloned into plasmid vector using the HindIII and XhoI restriction sites present in the primers and then used as a template to generate anti-sense and sense RNA probes with T7 and T3 RNA polymerase, respectively. The embryos were sequentially incubated with the probes (1μg/ml) and anti-digoxigenin antibody alkaline phosphatase conjugate. The alkaline phosphatase was revealed using BCIP/NBT according to Harland (1991).

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References


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