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

FIDA NASSAR*

Laboratoire d'Embryologie Moléculaire et Expérimentale, UMR 8080, CNRS et Université Paris Sud, Orsay, France

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 encodes 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.

***Address correspondence to:** Fida Nassar. Department of Biology, Faculty of Sciences II, Fanar, Lebanese University, Lebanon. Fax: +1-961-168-0248 . e-mail: fida.nassar@ul.edu.lb

#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*

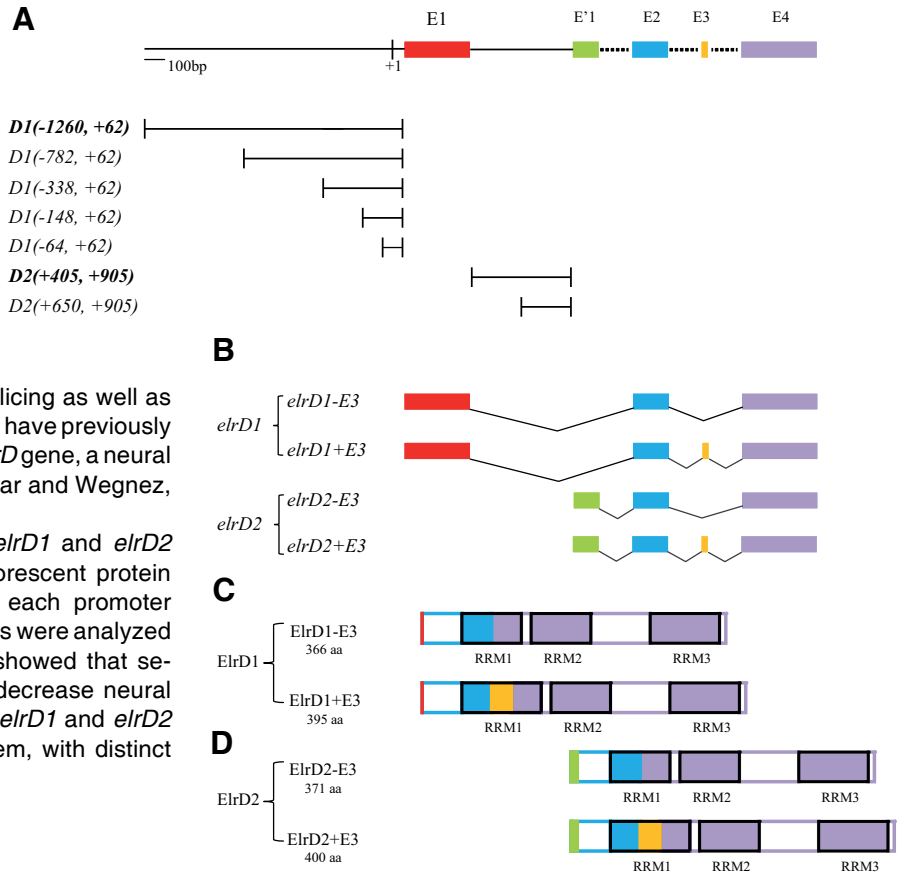

Fig. 1. Alternative promoters and splicing patterns of *elrD* gene. (A) *Genomic structure of the* elrD *gene. +1 marks the position of the first nucleotide of* elrD1*' transcript. E1:* elrD1 *exon 1, E'1:* elrD2 *exon 1, E2: exon 2, E3: exon 3, E4: exon 4. Dashes indicate the position of introns.* **(B)** 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 E'1 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 E'1 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.*

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

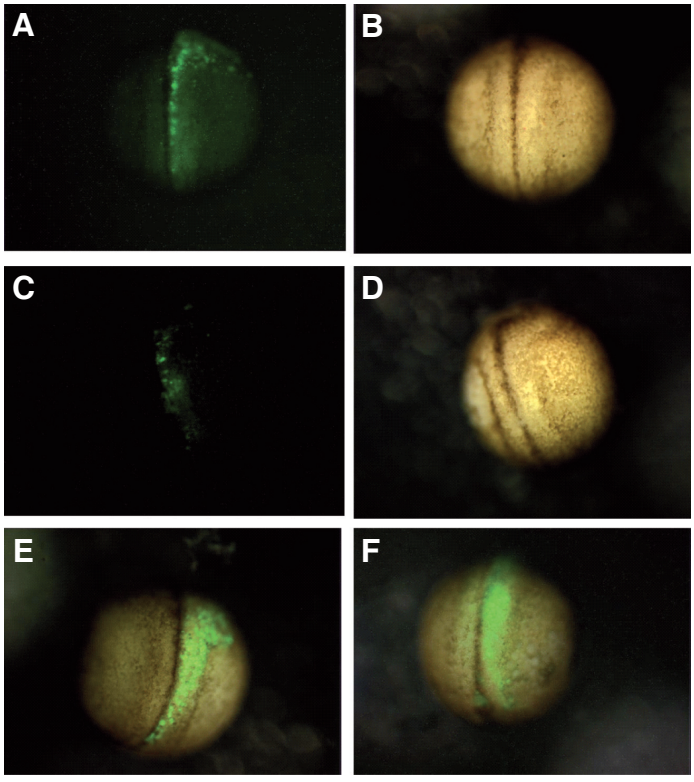

Fig. 2. Neural specificity of elr*D1* and *elrD2* promoters. *Injected embryo with* pD1-GFP **(A,B)** *and* pD2-GFP **(C,D)** *constructs into one dorsal blastomere at four-cell stage.* **(A,C)** *Embryos under blue light. GFP expression was visualized in the neural fold at stage 18. No GFP expression 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* pD1-GFP **(E)** *and* pD2-GFP **(F)** *constructs under visible and blue light. GFP expression is clearly visible in the neural fold.*

pD1 by deleting the 5' end of upstream *elrD1* region, as well as one construct containing shorter *elrD2* 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'*

TABLE 1

DELETIONS IN *ELRD1* AND *ELRD2* PROMOTERS DECREASE THE GFP EXPRESSION

Construct	Expression in the nervous system	Expression out of the nervous system	No expression	Number of scored embryos
pD1 D1(-1260,+62)	**52%**	**8%**	**40%**	**394**
D1(-782,+62)	38%	9%	53%	384
D1(-338,+62)	20%	12%	68%	392
D1(-148,+62)	35%	16%	49%	378
D1(-64,+62)	8%	15%	77%	390
pD2 D2(+450, +905)	**58%**	**6%**	**36%**	**388**
D2(+650, +905)	5%	13%	82%	398

elrD1 and *elrD2* upstream region (Table 1). These results show that *pD1-GFP* and *pD2-GFP* constructs drive neural specific expression during early *Xenopus* development. Deletions in the *elrD1* and *elrD2* 5' flanking region lead to a decrease of the level and the specificity of neural-specific GFP expression.

Expression of elrD1 *and* elrD2 *transcripts*

We compared the expression of *elrD1 and elrD2* 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 *elrD2* stable transcripts expression stronger than *elrD1*. In the adult brain, expression of the two transcripts was similar (Fig. 3A). Our results show that the relative abundance of the stable *elrD1* and *elrD2* 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 *elrD1* and *elrD2* 5'UTRs (see Materials and Methods). *elrD1* and'*elrD2* expression was coincided and restricted to the spinal cord, the brain and the cranial ganglia (Fig. 3 B,C).

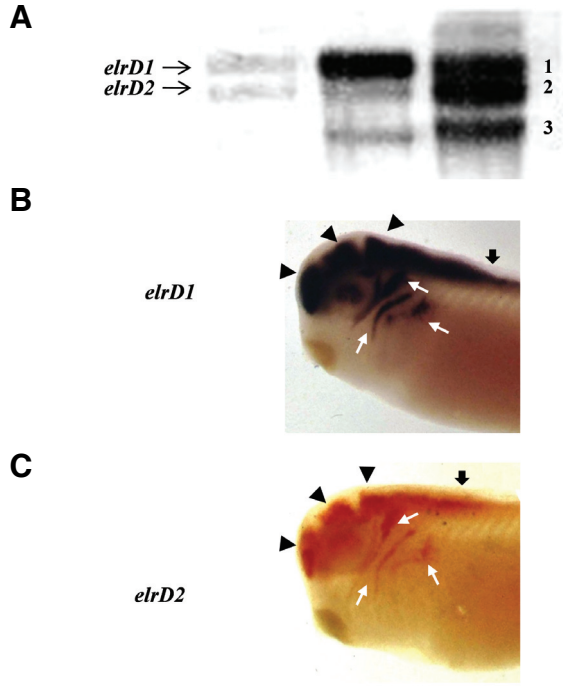

Fig. 3. Temporal and spatial expression of *elrD* transcripts. (A) *One µg of total RNA was reverse-transcribed and PCR performed by incorporating digoxigenin-dUTP. PCR products were resolved by electrophoresis in a 1% agarose gel and detected as described in Materials and Methods. Sequence analysis of the cloned products has shown that bands 1 and 2 correspond to* elrD1 *and* elrD2 *transcripts, respectively. Band 3 corresponds to an incomplete elongation of* elrD1 *and* elrD2 *transcripts. N: Neurula, T: Tailbud, B: Adult Brain.* **(B,C)** In situ *hybridization at tailbud stage with an antisense* elrD1 **(B)** *and* elrD2 **(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 product differing by 5 residues (Kim and Baker, 1993). *rbp9* produces nervous system-specific and ovary-specific transcripts, the later 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, *elrB* and *elrC* transcripts present the same pattern of expression as *elrD* (Perron *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 Brunol/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* (nrxn), 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(+650, +905)* constructs respectively. The PCR fragments were subcloned into the *pEGFP-1* basic promoterless vector (Clontech), using the *Hin*dIII and *Xho*I restriction sites present in the primers. Primers sequences are: ed1 5'-AAA A*CT CGA G*CA CCA TGA AGA GCA GCG ACT-3' (-1260, -1237); ed2 5'-GGG A*AA GCT T*GA AGC AGA GAA TGT GG-3' (+62,+43); ed4 5'-TTG T*CT CGA G*TT CTG TGT CTG CAC CTC TCG-3' (+405, +428); ed5 5'-CCA C*AA G CT T*AC ACA CAC ACA CAG TGC GCG-3' (+905, +882); ed6 5'-GCT T*CT CGA G*CA TGT GAA AGA TAG TCC C-3' (-64, -43); ed7 5'-AGA T*CT CGA G*CT TCA CTC TGC TAG AAG C-3' (-148, -127); ed8 5'-GGA C*CT CGA G*TA AAA CAG TTT GCA GGC T-3' (-338, -317); ed9 5'-ATA G*CT CGA G*GT TAA TAA TCG CAG CCC C-3' (-782,-760); ed10 5'-GGT T*CT CGA G*CT GTC ATT GTG TTT CTG C-3' (+650,+671). The sequence of the 5'-region of *elrD* gene is accessible in the Genbank database under the accession no. AF329448.

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 coolsnap camera.

Detection of elrD *transcripts by labeling 5'RACE PCR products with digoxygenin*

The 5' rapid amplification of cDNA ends (5'RACE) was conducted according to the procedure described in the SMART™ 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/chloroforme 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 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, o,3% Tween 20 and the alkaline phosphatase activity was revealed using BCIP/NBTaccording to Harland (1991). 5'RACE products were also gel-purified using the Nucleo 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 (E'1: from +917 to +1045 nucleotides from ts1). *elrD1* and *elrD2* specific probes were generated by PCR using primers D1s/D1as and D2 s/D2 as, respectively. D1s 5'-GCG GGG *AAG CTT* ATA TCT ACA CCC TAC TTG-3', D1as 5'-AAT T*CT CGA G*GA GTC TTG CTG GAC TTC G-3', D2s 5'-GGG G*AA GCT T*TA GCA TCG CTT GCC AGC A-3', D2as 5'-CAT C*CT CGA G*TT CAA GCC ATT CCA CTC C-3'. The PCR fragments were subcloned into pbluescript vector using the *Hin*dIII and *Xho*I 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-digoxygenin antibody alkaline phosphatase conjugate. The alkaline phosphatase was revealed using BCIP/NBT according to Harland (1991).

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