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Original Article

Misexpression of the RNA-binding protein ELRB in *Xenopus* presumptive neurectoderm induces proliferation arrest and programmed cell death

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ABSTRACT Proteins of the ELAV/Hu family share the presence of three RNA binding domains. In *Xenopus,* three nervous system-specific *elav*/Hu related genes, *elrB, elrC* and *elrD,* have been identified so far. The temporally regulated expression patterns of *elrB, elrC* and *elrD* suggest their involvement at different steps of neural differentiation. In the present study we misexpressed *elrB* by RNA injection in early *Xenopus* embryos and analyzed morphologically and molecularly its effects on neural development. We showed that heterochronous expression of *elrB* in presumptive neurectoderm down-regulates the expression of neural markers, such as *N-tubulin,* as well as that of other *Xenopus elav*-like genes, *elrC* and *elrD,* whereas ectopic expression of *elrB* in presumptive mesoderm has no effect on *MyoD*. Misexpression of *elrB* also induces severe defects in neural tube development, associated with massive cell loss resulting from early cell cycle arrest and programmed cell death. Our results are discussed in the context of early neural differentiation.

KEY WORDS: *Xel-1, elav, Hu, RNA-binding protein, nervous system, apoptosis*

Introduction

The spatial and temporal control of gene expression during development occurs at the transcriptional, post-transcriptional and post-translational levels. Post-transcriptional control in development has been shown to take place in the nucleus, i.e. alternative splicing, polyadenylation or export of mRNAs, and in the cytoplasm, i.e. mRNA stability or translation, mRNA transport to different cellular compartments (reviewed in Bashirullah *et al.*, 1998). Post-transcriptional regulation involves a large number of proteins which share the ability to bind RNAs. One large subclass of RNA-binding proteins has been identified by virtue of the presence in these proteins of one or more putative RNA recognition motifs (RRM) of 80-90 amino acids, containing an octapeptide consensus sequence RNP1 and an hexapeptide consensus sequence RNP2 (Adam *et al.*, 1986; Kenan *et al.*, 1991; Nagai *et al.*, 1995). Expression of some of the RRM-containing proteins is restricted to a given developmental stage or tissue. For instance, neural-specific RNA-binding proteins have been identified both in *Drosophila* and vertebrates, which contain either one (*Couch potato*; Bellen *et al.*, 1992), two (*musachi/nrp1;* Richter *et al.*, 1990; Nakamura *et al.*, 1994; Sakakibara *et al.*, 1996) or three RRMs (ELAV/Hu family, review in Antic and Keene, 1997; Good, 1997).

The first *elav*/Hu gene was identified in *Drosophila*. Null mutations of *Drosophila elav* lead to disorganization of neurites as evidenced by interrupted longitudinal connectives and missing commissures in the nerve cord (Campos *et al.*, 1985). Inactivation of *elav* in flies carrying a temperature-sensitive allele induces neuronal degeneration, thereby indicating that *elav* function is required for the maintenance of the nervous system throughout the lifetime of the fly (Campos *et al.*, 1985; Homyk *et al.*, 1985). In humans, the involvement of ELAV/Hu proteins in the maintenance of the nervous system is illustrated by the correlation between the presence of anti-Hu antibodies and a paraneoplastic encephalomyelitis syndrome (reviewed in Posner, 1994). Inactivation of HuD by antisense RNA experiments in rat pheochromocytoma PC12 cells leads to inhibition of neurite outgrowth (Dobashi *et al.*, 1998), which demonstrates that vertebrate ELAV/Hu proteins are also involved in neuronal differentiation.

elav/Hu genes constitute a small multigenic family of varying size depending on the species. Two *elav*/Hu genes have been

Abbreviations used in this paper: ARE, AU-rich element; BrdU, Bromodeoxyuridine; ELG, *elav*-like gene; RNP, ribonucleoprotein particle; RRM, RNA Recognition Motif; TUNEL, terminal deoxynucleotidyl transferase-mediated dUTP nick end-labeling; UTR, untranslated region.

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identified so far in *Drosophila* (*elav*: Campos *et al.*, 1985, 1987; Robinow *et al.*, 1988; *rbp9*: Kim and Baker, 1993). Four genes homologous to *elav* (*elav*-like genes: ELGs) have been identified in humans (Szabo *et al.*, 1991; King *et al.*, 1994; Sakai *et al.*, 1994; Ma *et al.*, 1996) as well as in *Xenopus* (Good, 1995; Perron *et al.*, 1995), and mouse (Abe *et al.*, 1994, 1996a,b; Okano and Darnell, 1997) and three in chicken (Wakamatsu and Weston, 1997). The expression of the majority of ELGs is typically restricted to all or parts of the nervous system except for HuR/*elrA* homologs whose transcripts are ubiquitously expressed (Good, 1995; Okano and Darnell, 1997; Wakamatsu and Weston, 1997). In addition to being spatially restricted, the expression of neural specific ELGs is developmentally regulated in *Drosophila* (Robinow and White, 1988, 1991; Kim and Baker, 1993; review in Yao *et al.*, 1993). Developmental studies in chicken, mouse and *Xenopus* demonstrated the differential spatio-temporal expression of ELGs in the developing nervous system as well as specific expression of some ELGs in proliferating migrating cells or in post-mitotic cells (Marusich *et al.*, 1994; Perron *et al.*, 1995, 1999; Okano and Darnell, 1997; Wakamatsu and Weston, 1997).

The subcellular distribution of different ELAV/Hu proteins is variable between the nucleus and the cytoplasm, suggesting that these proteins may be involved in different aspects of RNA processing (Robinow and White, 1991; Kim and Baker, 1993; Marusich *et al.*, 1994; Gao and Keene, 1996; Perron *et al.*, 1997). Studies in *Drosophila* demonstrate the functional specificities of different ELGs in the control of RNA metabolism. Since ELAV co-localizes with snRNPs in nuclear structures called coiled bodies (Yannoni and White, 1997) and *elav* function is required for neuron-specific splicing of neuroglian mRNA (Koushika *et al.*, 1996), a role for ELAV in splicing regulation seems likely. In contrast, the finding that RBP9 binds to poly(U) of the 3'-untranslated region (3'UTR) of *extra macrochaetae (emc)* mRNA and that *rbp9* function is required for the down regulation of *emc* mRNA (Park *et al.*, 1998b) together suggest that RBP9 controls mRNA stability rather than splicing.

A search for ELAV/Hu partners and RNA targets in mammals also indicates a role for these proteins in at least two steps of RNA metabolism. Random RNA selection and *in vitro* binding assays showed that Hu proteins bind preferentially to the 3'UTR of mRNAs containing AU-rich elements (ARE). These elements are known to regulate mRNA stability (reviewed in Chen and Shyu, 1995). Some ELAV/Hu proteins were also identified in a search for regulatory factors for mRNA turn-over (Chagnovich and Cohn, 1996; Chagnovich *et al.*, 1996; Myer *et al.*, 1997). Subsequently several ELAV/Hu proteins were shown to increase the *in vivo* stability of ARE-containing mRNAs (Chagnovich *et al.*, 1996; Fan *et al.*, 1997; Myer *et al.*, 1997; Fan and Steitz, 1998; Peng *et al.*, 1998). Another cytoplasmic function of ELAV/Hu proteins in translational regulation is suggested by the demonstration that Hel-N1 protein associates with polysomes and microtubules (Gao and Keene, 1996; Antic and Keene, 1998). A role in translational regulation is also suggested by the finding that ectopic expression of Hel-N1 in adipocytes increases the stability and the translation efficiency of GLUT1 mRNA (Jain *et al.*, 1997). Taken together, all these data indicate a differential use of ELAV/Hu RNA-binding proteins at various levels of RNA metabolism including splicing, RNA stability and translational regulation during normal development.

Functional studies as well as *in vitro* binding studies suggest that ELAV/Hu homologs may have homologous target RNAs. For instance, inactivation of neurite outgrowth in PC12 cells by HuD antisense RNA is reminiscent of the alteration of connectives and commissures in *elav* mutant embryos in *Drosophila* (Jimenez and Campos-Ortega, 1987). At the molecular level, RBP9 binds to *emc* mRNA in *Drosophila* and Hu proteins bind to *Id* mRNA (an *emc* helix-loop-helix transcriptional repressor homolog) in vertebrates (King *et al.*, 1994).

We chose *Xenopus* as an experimental system to address, *in vivo*, the role of ELAV/Hu proteins during vertebrate development. We had previously shown that *elrB* expression is detected from the early tailbud onwards, and that it is restricted to the nervous system (Perron *et al.,* 1995). Strikingly, we found that heterochronic expression of *elrB* down regulates the expression of neural specific genes including two ELGs, *elrC* and *elrD* but not *MyoD*, a mesodermal marker. We show that severe morphological defects in neural tube development resulting from misexpression of *elrB* are associated with early cell cycle arrest and programmed cell death.

Results

Expression of elrB in the presumptive nervous system leads to morphological and cellular defects in tailbud embryos

To address the function of *Xel-1/elrB* (that we will call simply *elrB*) in neural development, we tested for possible effects of an altered expression of *elrB* in neural presumptive cells. Synthetic mRNA was microinjected into one of the two animal-dorsal blastomeres of *Xenopus* embryos at the 8-cell stage, and subsequent development was monitored. This experimental procedure is equivalent to a gain of function mutation of ELRB, leading to both heterochronic expression and overexpression, and is therefore referred to below as misexpression. The morphology of injected embryos did not appear to be perturbed at early stages, since gastrulation and neurulation proceeded without any detectable defects. At the tailbud stage however, embryos injected with *elrB* RNA showed abnormal eye development (Fig. 1A). As a control, *LacZ* RNA was injected at comparable doses. None of the *LacZ* RNA-injected embryos showed any developmental perturbation (data not shown), thereby indicating that abnormal development of *elrB* injected embryos is not an artefact inherent to our experimental procedure. We also injected an epitope-tagged form of *elrB*, ($elrB_{NT}$, previously called $Xel\text{-}1_{NT}$ in Perron *et al.*, 1997), which gave rise to similar phenotypes (Fig. 1B) as those observed for *elrB*. Therefore, the MYC epitope provides a way to trace directly cells that received the injected RNA. In all embryos displaying eye alteration after injection of $elrB_{NT}$ RNA, the corresponding area stained positively for the MYC epitope (Fig. 1C). These results show that the presence of an eye abnormality correlates with *elrB* misexpression. Also, since the same phenotype is obtained with both *elrB* and $elrB_{NT}$, it suggests that the MYC epitope does not interfere with *elrB* misexpression.

The frequency of abnormal embryos was scored according to the dose of $elrB_{NT}$ RNA injected. At a dose of 10 pg of RNA per embryo, 33% of the injected embryos displayed eye abnormalities. Between 50 pg and 200 pg per embryo, the number of affected embryos remained approximately constant, 60 to 66% of injected embryos displaying one abnormal eye. Abnormal embryos can be separated into two classes according to the severity of their

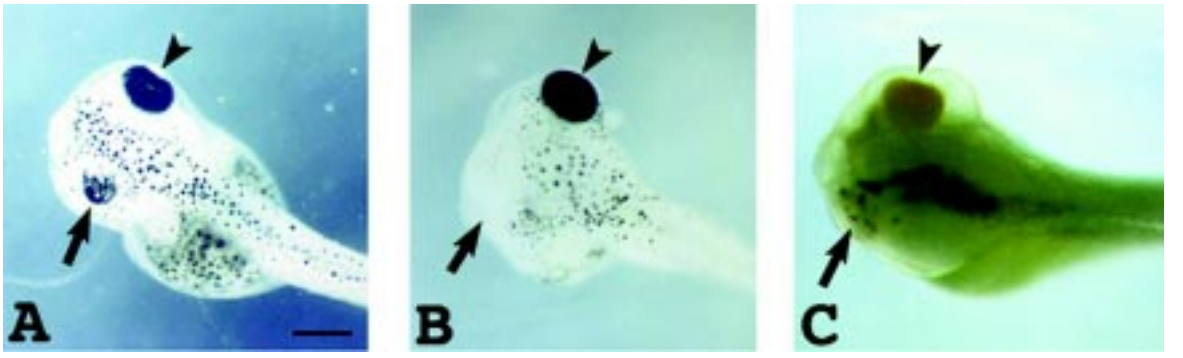

Fig. 1. Developmental effect of *elrB* misexpression. *Embryos were injected with elrB* **(A)** *or elrB$_{NT}$* **(B,C)** *RNA at the 8-cell stage. The injected embryos were fixed and either photographed without further processing* **(A,B)** *or whole-mount immunostained with anti-MYC antibody* **(C)**, *which revealed the injected side (arrow), whereas the uninjected side served as a control (arrowhead). All panels represent dorsal views of injected embryos at stage 45. Panel A shows altered development of an eye that remains visible (arrow) whereas Panel B shows a complete deficiency of the eye (arrow) in a different embryo. Arrowheads indicate a normal eye on the other side.* **(C)** *Immunodetection of exogenous ELRB$_{NT}$ showing the correlation between phenotype and ELRB$_{NT}$ misexpression (arrow). A normal eye is found on the uninjected side (arrowhead). Bar, 1 mm.*

phenotype. A first class comprises embryos which show only eye defects. The embryos of the second class, in addition to the eye defect, are curved along the antero-posterior axis towards the side of the injected blastomere. Thus, whereas the frequency of affected embryos remains stable above 50 pg/embryo, the severity of the phenotype depends upon the RNA concentration (40, 60 and 75% of embryos with a severe phenotype when 50, 100 and 200 pg of RNA/embryo were injected, respectively).

To further analyze the phenotypes, a histological study was performed on paraffin cross-sections of immunostained embryos misexpressing *elrB$_{NT}$* (50 pg/embryo). At stage 33 on the control side, the neural tube is organized into two main layers easily visualized after Hoechst staining. The inner or ventricular layer, where the density of nuclei is high, corresponds to the proliferative zone. The thicker outer layer, where the density of nuclei is low, corresponds to differentiating postmitotic cells. The eye is already well structured with a lens and a layered retina. On the injected side, both the neural tube and the eye area appear disorganized (Fig. 2A-B). Later in development, the abnormal organization of the neural tube due to *elrB* misexpression is accentuated (Fig. 2C-F). The density of nuclei in the ventricular layer of the injected side is not distinguishable from that of the outer layer of the neural tube. Nuclei in the myc-stained area and in the corresponding area on the control side were counted. At stage 33, a loss of 22% to 32% of nuclei was found on the injected side (4 sections of different embryos). At stage 36, this loss reaches 50% to 68% (2 sections of different embryos). Such a drastic reduction in cell density is never observed when *elrB* is ectopically expressed in the epidermis (Fig. 2).

Loss of cells correlates with early arrest of proliferation and apoptosis in elrB misexpressing embryos

The perturbation of the proliferative zone on the injected side of tailbud embryos and the drastic reduction in the number of nuclei suggest cell loss that could be due to an early arrest of proliferation and/or the induction of cell death. We assayed the potential of cell proliferation on the injected side versus the uninjected side using BrdU incorporation into replicating DNA. BrdU$^+$ cells were scored both in the *elrB* misexpressing area and in the symmetrical area of the uninjected side in stage 24 and 30 embryos. When the number of BrdU positive cells on the control side is compared to that on the injected side, a 50% reduction of BrdU$^+$ cells is found on the injected side (2 stage 24 and 4 stage 30 embryos, Fig. 3A-B). This result demonstrates an early arrest of proliferation due to *elrB* misexpression, which may account by itself for the lower number of nuclei on the injected side. However, at late tailbud stages, *elrB* misexpressing cells also display aberrant nuclear fragmentation (Fig. 3C-D), a phenotype reminiscent of programmed cell death. We tested further for the presence of apoptotic cells in *elrB* misexpressing embryos by using terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL; Gavrieli *et al.*, 1992), a method that allows detection, *in situ*, of the chromosomal fragmentation characteristic of programmed cell death. At stage 24, no apoptotic cells were detected in the neural tube, neither on the control nor on the injected side (data not shown). At stage 30, no apoptotic cells were detected on the control side of the neural tube. In contrast, apoptotic cells were present on the side misexpressing ELRB$_{NT}$ (4 to 6 labeled cells per section on the injected side of 2 stage 30 embryos, Fig. 3E-G). Later in development (stage 35), whereas no apoptotic cells were detected on the control side, the number of apoptotic cells on the injected side increased (10 to 15 labeled cells per section on the injected side of 4 embryos, Fig. 3H-J).

Taken together, these results show that the severe morphological perturbations of the neural tube development observed when *elrB* is misexpressed are associated at the cellular level with cell loss that correlates with premature arrest of proliferation and apoptosis.

elrB misexpression abolishes the expression of neural markers

Since injection of *elrB* RNA in the animal-dorsal blastomere produces heterochronic expression in proliferative neural precursors of a neuronal marker normally expressed in post-mitotic neurons, we addressed the question whether the early arrest of proliferation was linked to early neuronal differentiation. Therefore, the expression pattern of nervous system-specific markers was assayed at the neurula stage in *elrB* injected embryos. As described above, *LacZ* RNA and *elrB*+*LacZ* RNAs were injected in one of the two animal-dorsal blastomeres at the 8-cell stage, and development was allowed to proceed. At subsequent stages, embryos were fixed and processed for *in situ* hybridization with the appropriate probe.

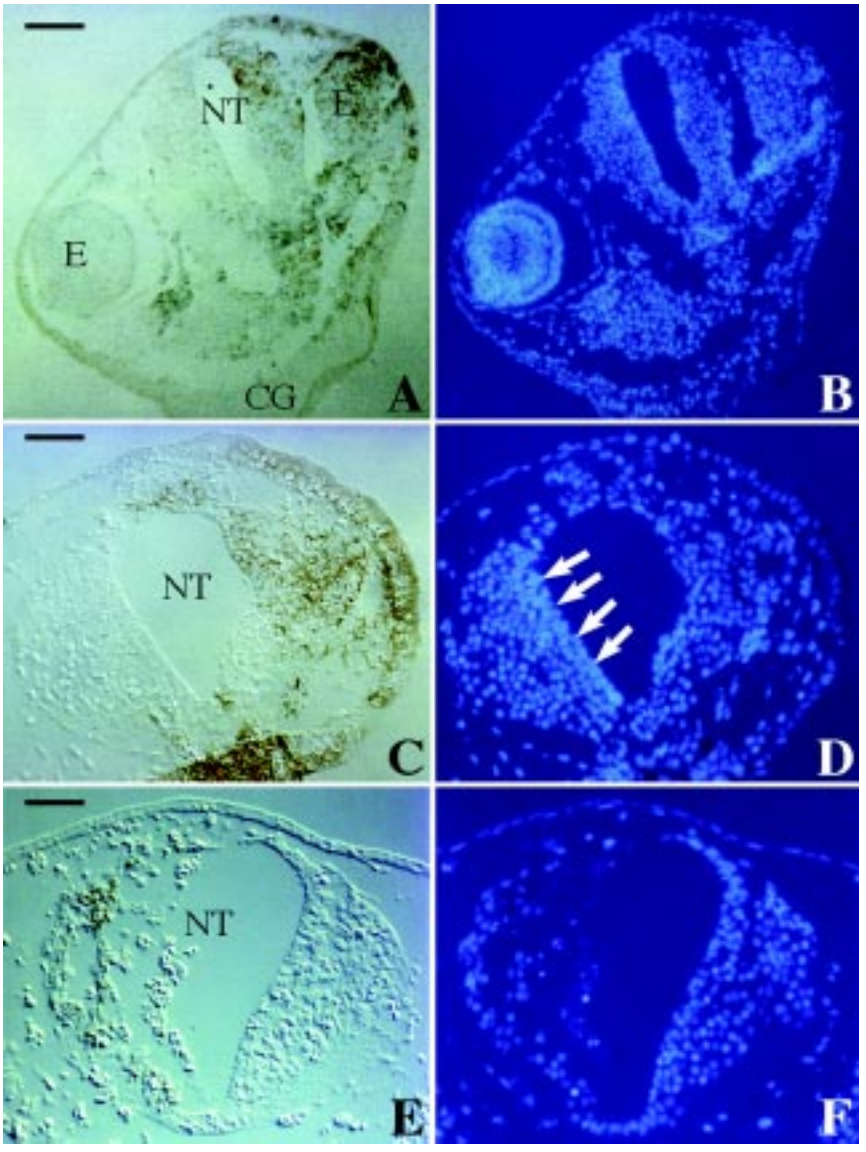

Fig. 2. Histological analysis of embryos misexpressing *elrB*. *Transverse sections of tailbud embryos injected with $elrB_{NT}$ RNA into a single animal-dorsal blastomere at the 8-cell stage. $ELRB_{NT}$ was immunodetected and visualized in brown* **(A,C,E)**, *and nuclei were visualized with Hoechst dye* **(B,D,F)**. *All sections shown are at the level of the eye vesicles (labeled E in Panel A), and $ELRB_{NT}$ protein is detected on the right side* **(A-D)** *or left side* **(E,F)**. **(A,B)** *Stage 33. Cell layers in the neural tube and eye vesicle expressing $ELRB_{NT}$ are disorganized when compared to the corresponding regions on the other side.* **(C,D)** *Stage 36. Arrows indicate the proliferative ventricular epithelium on the normal side of the neural tube, a region that cannot be recognized on the half of the neural tube expressing $ELRB_{NT}$ protein. Note the difference between the thick dorsal epidermis on the right side expressing exogenous ELRB and the thin epidermal layer on the control side. It is noticeable that no reduction in cell density is observed when elrB is ectopically expressed in the epidermis. However, the shape of cells in the epidermis is abnormal when compared to that on the control side.* **(E,F)** *Stage 38. The cellular organization is severely affected at stage 38 on the side of the neural tube expressing $ELRB_{NT}$.* **(F)** *The number of nuclei is lower on the left side when compared to that on the right side. CG, cement gland; E, eye vesicle; NT, neural tube. Bar in A, 100 μm, in C, 50 μm, in E, 60 μm.*

N-tubulin is an early marker of neural commitment (Richter *et al.*, 1988). At stage 14, *N-tubulin* is expressed in normal embryos in the posterior neural plate in three longitudinal stripes (medial, intermediate and lateral), on either side of the dorsal midline. Cells that differentiate in these domains correspond to motor neurons, inter-neurons and sensory neurons, respectively (Chitnis *et al.*, 1995). Anteriorly, *N-tubulin* is expressed in the trigeminal placodes. This pattern of *N-tubulin* was not affected in embryos injected with *LacZ* RNA (12/12 embryos examined, data not shown). In contrast, after injection of *elrB* + *LacZ* RNAs (Fig. 4 A), a strong decrease in the expression of *N-tubulin* was found in the medial and intermediate stripes. Staining in both the lateral stripe and the trigeminal placode also disappeared in the area where exogenous *elrB* expression overlapped these domains (22/34 embryos examined).

In order to know whether these effects are general or neural specific, we looked at the effects of *elrB* misexpression on a mesodermal marker, *MyoD*. Therefore we injected *LacZ*+*elrB* RNAs in one of the two animal-dorsal blastomeres at the 4-cell stage in the marginal zone. Zygotic expression of *MyoD* begins in early gastrula and is restricted to the gastrula mesoderm and to the somites of neurula and tailbud embryos (Hopwood *et al.*, 1989). We found that *MyoD* expression was not altered in the injected side (5/5 embryos, Fig. 4B), suggesting that misexpression of *elrB* specifically affects neural tissue.

Two nervous system-specific *elav*/Hu genes, *elrC* and *elrD*, are expressed before *elrB* (at stage 12 and stage 17, respectively), and thereby provide stage specific markers of neural development (Good, 1995; Perron *et al.*, 1999). We analyzed the expression of *elrC* in *elrB* injected embryos at stage 14, 19 and 32 (Fig. 5). In the case of *elrD*, we analyzed the effect of *elrB* misexpression at stage 19 and 32 (Fig. 5) since *elrD* expression is detected normally only from stage 17 onwards. When *LacZ* RNA was injected as a control, no effect was detected on either *elrC* or *elrD* expression (12/12 and 9/9 embryos examined, data not shown). In contrast, at all stages tested, a decrease in *elrC* expression was found on the injected side (10/14 stage 14 embryos; 5/7 stage 19 embryos; 8/11 stage 32 embryos). Similarly, a decrease in *elrD* expression was observed on the injected side (6/8 stage 19 embryos; 3/4 stage 32 embryos).

We performed similar misexpression experiments with the two other ELGs, *elrC* and *elrD*. In both cases, we observed down regulation of *N-tubulin* transcripts, a disorganization of the nervous system accompanied with cell loss and nuclear fragmentation at the tailbud stage (data not shown), as observed for *elrB* misexpression. Misexpression of *elrB*, *elrC*, or *elrD* thus does not induce ectopic or heterochronic expression of neural markers in presumptive neural cells. On the contrary, it down regulates the expression of *N-tubulin*. Therefore, it affects the identity of early neural precursors.

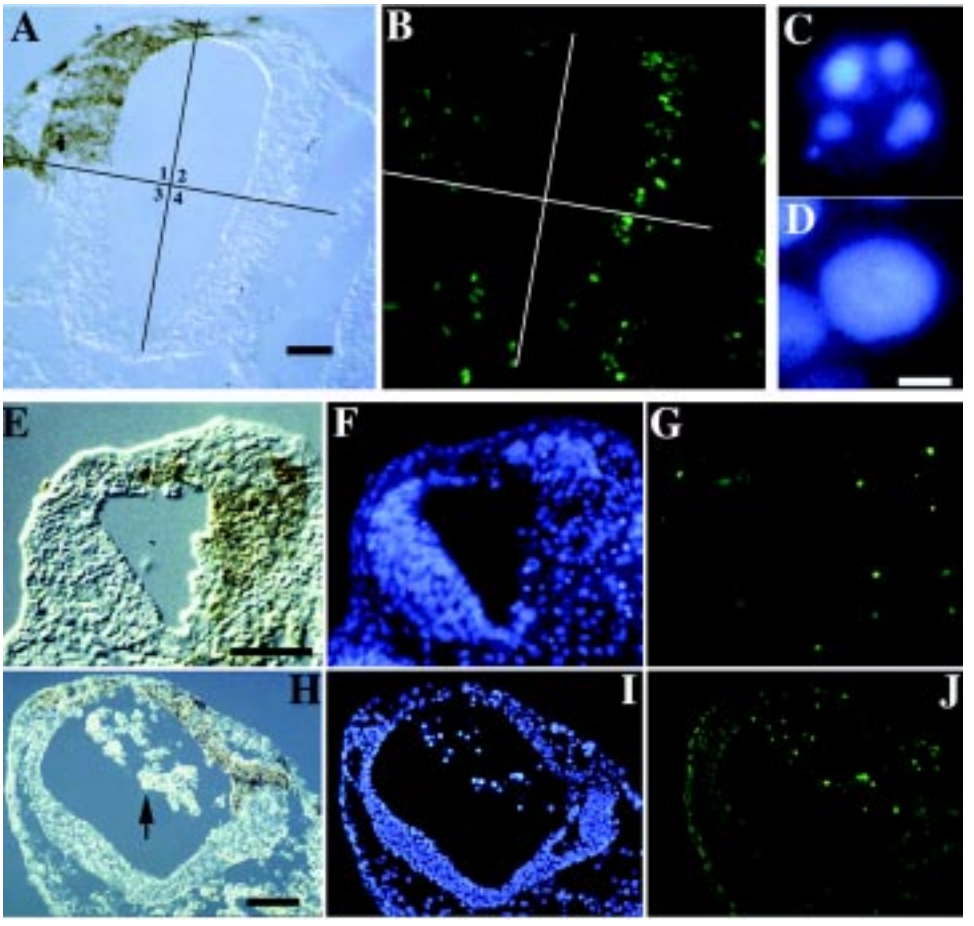

Fig. 3. Detection of cell proliferation and apoptosis in embryos misexpressing *elrB*. **(A,B)** *Transverse section of a representative stage 30 embryo injected at the 8-cell stage with $elrB_{NT}$ RNA, then injected at stage 30 with BrdU solution.* **(A)** *$ELRB_{NT}$ was immunodetected and visualized in brown, indicating the injected side on the left.* **(B)** *BrdU uptake is visualized under fluorescence in green. In the neural tube, fewer cells are $BrdU^+$ on the injected side when compared to the same area on the uninjected side (area 1: 15 $BrdU^+$ cells; area 2: 30 $BrdU^+$ cells; area 3: 11 $BrdU^+$ cells; area 4: 10 $BrdU^+$ cells). Bar, 125 μm.* **(C,D)** *Nuclei of a stage 36 embryo injected with $elrB_{NT}$ RNA. After immunodetection of $ELRB_{NT}$, nuclei were visualized on sections with Hoechst dye.* **(C)** *A typical nucleus on the injected side displaying a vesicular aspect indicating nuclear fragmentation.* **(D)** *A nucleus on the control side. Bar, 10 μm.* **(E,J)** *Transverse sections of stage 30* **(E-G)** *and stage 35* **(H-J)** *embryos injected with $elrB_{NT}$ RNA at the 8-cell stage. $ELRB_{NT}$ was immunodetected and visualized in brown indicating the injected side on the right* **(E,H)**. *Nuclei were visualized with Hoechst dye in blue* **(F,I)**. *Apoptotic cells were detected using the TUNEL method and visualized under fluorescence in green* **(G,J)**. *At both stage 30 and 35, few cells undergo apoptosis on the injected side of the neural tube, whereas no apoptotic cells are seen on the non-injected side. At stage 35, many cells floating in the luminal portion of the neural tube are labeled by TUNEL (arrow in H). Bar, 100 μm.*

Discussion

Three neural specific *elav*/Hu genes (ELGs) are known in *Xenopus*, *elrB/Xel-1*, *elrC* and *elrD* (Good, 1995; Perron *et al.*, 1995). Their high sequence similarity suggests that they share common properties. Transcript analysis using RT-PCR and Northern analysis (Good, 1995), as well as *in situ* hybridization (Perron *et al.*, 1999) showed that they have temporally and spatially regulated expression patterns. Despite numerous studies on neural-specific *elav*/Hu homologs, little is known about their function in normal development. In an attempt to address *elrB* function in development of *Xenopus*, we injected mRNAs encoding ELRB in a single animal-dorsal blastomere of 8-cell stage embryos. This approach leads to the heterochronic expression of a neural specific post-mitotic gene in presumptive neural tissues.

Expression of elrB in presumptive neural cells interferes with the normal developmental program of neurogenesis

Embryos injected with *elrB* RNA into a single animal-dorsal blastomere show no morphological alterations at early stages and proceed through gastrulation and neurulation. However, minor non external visible effects cannot be excluded. Molecularly, the first event detected in these embryos is the down regulation of *N-tubulin* and *elrC* in primary neurons at neurula stage 14, when endogenous *elrB* is not expressed. Subsequently, *elrD* expression is also down regulated in stage 19 embryos.

Since *elrA*, *elrC*, and *elrD* are normally expressed before *elrB* (Good, 1995, Perron *et al.*, 1999), it is thus possible that the heterochronic expression of ELRB protein competes for the targets of the three other ELGs. Indeed, several ELAV/Hu proteins were shown to share identical RNA binding specificities *in vitro*. For instance, Hel-N1 and HuR both bind the ARE of c-*myc* (Levine *et al.*, 1993; Ma *et al.*, 1996) and Hel-N1, HuR and HuD all three bind the ARE of c-*fos* (Levine *et al.*, 1993; Liu *et al.*, 1995; Chung *et al.*, 1996; Ma *et al.*, 1996). No such information is available on the RNA binding specificity of the three neural specific ELGs in *Xenopus*. However, Wu *et al.* (1997) have shown that the ubiquitous *Xenopus* ELRA protein binds specifically to the CPE (Cytoplasmic Polyadenylation Element), a property that it does not share with ELRB. According to these results, the effect of *elrB* misexpression on *Xenopus* development would not be due to interactions with ELRA target RNAs, but rather with those of ELRC and/or ELRD.

Among ELG targets might be their own mRNA, as demonstrated for Mel-N1 in mouse (Abe *et al.*, 1996b). ELGs may thus down regulate their own product, as it is the case for *elav* in *Drosophila* (Samson, 1998). At the neurula stage, endogenous *elrB* is not expressed. However, exogenous ELRB would be available to compete with a possible autoregulatory binding of other ELG products (i.e., ELRC and ELRD) on their own mRNAs. Our finding that *elrB* heterochronic expression down regulates the expression of both *elrC* and *elrD* is compatible with this hypothesis. Down regulation of *elrC* and *elrD* transcripts by ELRB may also reflect

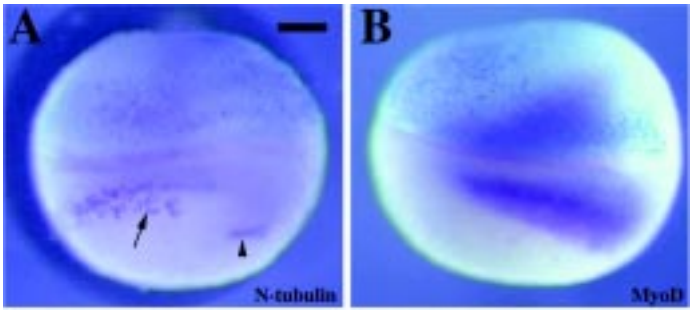

Fig. 4. Effects of *elrB* misexpression on *N-tubulin* and *MyoD* expression at the neurula stage. *Dorsal views of embryos injected with elrB+LacZ RNAs at the 4-cell stage. Expression of N-tubulin and MyoD was visualized by whole-mount in situ hybridization (dark blue staining) after detection of β-galactosidase activity (light blue staining) revealing the injected side.* **(A)** *N-tubulin expression in a representative embryo, showing on the injected side a strong reduction of N-tubulin expression in the medial and lateral bands (arrow on the control side), and the absence of labeling in the trigeminal placode (arrowhead on the control side).* **(B)** *MyoD expression in a representative embryo, showing no differences between the injected and the uninjected side. Bar, 300 μm.*

endogenous cross-regulation of these genes, as suggested by the differential expression of ELGs in different subsets of neurons in mouse and *Xenopus* brain (Okano and Darnell, 1997; Perron *et al.*, 1999). Another explanation for the down regulation of *elrC* and *elrD* in *elrB* injected embryos is that their expression would be perturbed as that of other neural markers such as *N-tubulin*, by unknown mechanisms.

Function of ELRB in the control of proliferation and/or differentiation of neural cells

Histological analysis of tailbud stage embryos shows that the structure of the neural tube is abnormal. The organization of cell layers in the brain, the prospective retina and all along the spinal cord on the injected side is modified. This is mainly visible at the level of the proliferative luminal epithelium. Massive loss of cells on the injected side of tailbud embryos (stage 38) is observed only in the nervous system, indicating that the cell-loss phenotype requires specific factors present in the neural lineage. However, the shape of epithelial cells expressing *elrB* is altered, indicating possible interference of ectopic *elrB* expression with the differentiation program outside of the nervous system.

We showed that the loss of cells correlates with both an early arrest of cell division as detected by the reduction of the number of BrdU incorporating cells, and the induction of chromosomal fragmentation as detected by TUNEL. The latter result indicates that the loss of cells is not due to a direct toxicity of the injected RNA but rather to the induction of programmed cell death, which is in good accordance with the nuclear fragmentation characteristic of apoptotic cells also observed in tailbud. Both cell proliferation arrest and apoptosis under *elrB* misexpression are highly specific and dependent on neural commitment.

Apoptosis is a necessary process for modeling of the nervous system during normal development. Park *et al.* (1997, 1998a) showed that several apoptosis pathways co-exist in a given neural lineage, acting at the level of the cell cycle or at the level of neurotrophic factors dependency. Conflicting proliferation or differentiation signals, incompatible with a particular cellular or environmental context may thus lead to apoptosis. Since a decrease in BrdU positive cells is detected as early as stage 24, when no apoptosis is detected, the reduction in cell number may first be due to an early arrest in cell division. Then, cell loss would be amplified by the induction of cell death by *elrB* misexpression. However, these two events might be linked since a proliferation arrest is often a first step of apoptosis (King and Cidlowski, 1998). We found that the loss of function of ELAV in *Drosophila* leads to ectopic programmed cell death in the nervous system (M.-P.F., unpublished results). By analogy with this result in *Drosophila*, and in accordance with the down regulation of *elrC* and *elrD* in *Xenopus*, a plausible explanation for *elrB* induction of apoptosis is that apoptosis results from the loss of function of one of the ELGs, most likely that of the earliest expressed, *elrC*. However, down regulation of *elrC* after *elrB* misexpression leads to the same phenotype as that resulting from overexpression of *elrC*, i.e., down regulation of *N-tubulin*, nuclear fragmentation at the tailbud stage accompanying disorganization of the neural tube on the injected side. We therefore hypothesize that overexpression of a single neural specific ELG produces an autoregulatory dominant negative effect. Such effect of the overdose of a wild type protein has already been described for instance for *Pax6* (Kirby *et al.*, 1998) and *polyhomeotic* (Fauvarque *et al.*, 1995). In both of these cases however, negative transcriptional regulation is thought to be responsible for the effect, whereas for ELGs a dominant negative effect would be exerted through the regulation of RNA stability. If this were the case, the resulting loss of function of ELGs may prevent committed neural cells to differentiate, and cell death would result from this incapacity of neural cells to follow the neural differentiation pathway, thereby preventing expression of neural markers.

In normal development, *elrB* is a marker for differentiating post-mitotic neurons as well as for mature neurons (Perron *et al.*, 1995). Therefore its expression might be necessary for cell cycle arrest preceding differentiation in normal development. In fact, mammalian ELAV/Hu proteins were shown to bind the mRNA for $p21^{waf1}$, a protein that induces cell cycle arrest at G_1/S preceding cell differentiation (Joseph *et al.*, 1998). *elrB* may similarly control the cell cycle in *Xenopus* by stabilizing a $p21^{waf1}$ homolog. Apoptosis may thus result from blockage of the cell cycle without concomitant support of other factors necessary for the expression of a differentiation pathway. Alternatively, *elrB* expression might be necessary for the correct expression of the differentiation and maintenance programs after cell cycle exit in post-mitotic cells. Along the same lines, Wakamatsu and Weston (1997) reported that apoptosis following overexpression of HuD in cultured quail neural crest cells is rescued when neurotrophic factor NT-3 is added to the culture, thereby suggesting that cell death possibly occurs by an increased dependency on neurotrophic factor due to heterochronic differentiation. Apoptosis observed when *elrB* is misexpressed is in accordance with these data. In contrast, the early decrease in neural marker expression also observed in our experiments does not favor heterochronic neural differentiation of cells misexpressing e*lrB*. Taken together, our results and those reported above indicate different pathways leading to cell death in *elav*/Hu misexpression experiments.

The perturbation in neural cell differentiation shown here by misexpressing RNA-binding proteins highlights the importance of post-transcriptional regulation during development. Proteins from

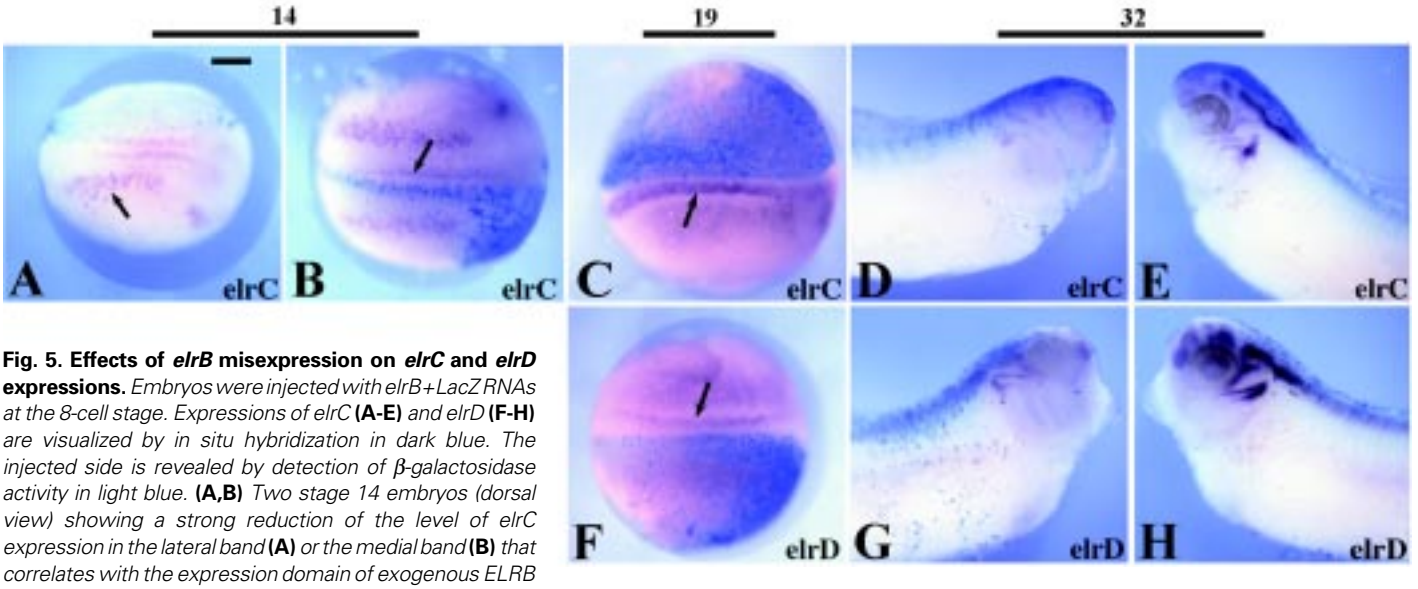

Fig. 5. Effects of *elrB* misexpression on *elrC* and *elrD* expressions. *Embryos were injected with elrB+LacZ RNAs at the 8-cell stage. Expressions of elrC* **(A-E)** *and elrD* **(F-H)** *are visualized by in situ hybridization in dark blue. The injected side is revealed by detection of β-galactosidase activity in light blue.* **(A,B)** *Two stage 14 embryos (dorsal view) showing a strong reduction of the level of elrC expression in the lateral band* **(A)** *or the medial band* **(B)** *that correlates with the expression domain of exogenous ELRB as revealed by β-galactosidase activity. The normal expression in these regions is indicated on the control side (arrows).* **(C,F)** *Stage 19 embryos (dorsal view). A strong reduction of the expression level of elrC* **(C)** *and elrD* **(F)** *is observed on the injected side in the neural tube. The normal expression in the corresponding region is indicated on the control side (arrows).* **(D,E,G,H)** *Stage 32 embryos (lateral view). A strong reduction of the expression level of both elrC* **(D)** *and elrD* **(G)** *is observed on the injected side when compared to that on the control side of the same embryo* ***(E, H****, respectively****)****. Bar, 300 μm.*

the same family play various functions, an early one restricted to ELRA in cytoplasmic polyadenylation of mRNAs, distinct from later functions played by the three other ELGs in neural differentiation. Other nervous system-specific RNA-binding proteins such as *nrp-1* (Richter *et al.*, 1990), *mouse-Musashi-1* (*m-Msi-1*, Sakakibara *et al.*, 1996) and *etr* (elav-type ribonucleoproteins, Knecht *et al.*, 1995) have been characterized in vertebrates. Each is highly specific of a developmental step. For instance *m-Msi-1* is expressed in multipotent glial/neuronal precursors, and remains expressed in the glial lineage, whereas it is shut down in post-mitotic neurons that instead begin to express neural specific ELGs (Sakakibara *et al.*, 1996; Sakakibara and Okano, 1997). Concomitantly, there is a transient decrease in HuA expression (Wakamatsu and Weston, 1997). The combination of RNA-binding proteins expressed at a given step of development may thus control very specifically the stability and translational efficiency of a common panel of mRNA species in neural sub-lineages. This complexity of RNA-binding proteins may help generate the functional complexity that is required for the function of the nervous system.

Materials and Methods

Constructs and in vitro transcription

elrB constructs tagged or not with a MYC epitope in N-terminal region are described in Perron *et al.* (1997) (previously called RN3-*Xel-1*$_{NT}$ and RN3-*Xel-1*, respectively). The β-galactosidase coding sequence containing a nuclear localization signal subcloned in CS2 (a gift from Nancy Papalopulu) was linearized at the *Not*I site. *In vitro* transcription of capped mRNAs was performed with the T3 or SP6 mMessage mMachine *in vitro* transcription kit (Ambion, Cliniscience).

In vitro fertilization, RNA injection and immunodetection

In vitro fertilization was performed as described (Perron *et al.*, 1997). Embryos were staged according to Nieuwkoop and Faber (1967). Fifty-200 pg of synthetic RNA in water was injected with a pressure driven injector (Eppendorf). Embryos were kept in 0.1xNAM, 5% Ficoll, before and during injection and for 12 h following injection, then transferred to 0.1xNAM without Ficoll.

Whole-mount ELRB$_{NT}$ immunodetection and histological sections were performed as described previously (Perron *et al.*, 1997) using anti-MYC antibody (1/10 dilution, gift from A. Prochiantz), peroxydase conjugated anti-mouse antibody (1/200; Sigma) or alkaline-phosphatase conjugated anti-mouse antibody (1/50; Sigma).

β-galactosidase activity was visualized as described previously (Perron *et al.*, 1997). Once stained, embryos were washed in PBS, 20 mM EDTA, then post-fixed for 1 h in 20 mM PIPES, 0.4 mM $MgCl_2$, 0.25 mM EGTA and 4% paraformaldehyde.

BrdU immunodetection

For BrdU labeling, embryos were anaesthesized with 3-aminobenzoic acid ethyl ester, then transferred to 0.1xNAM, 5% Ficoll for injection with undiluted BrdU labeling reagent (Boehringer Mannheim, 5-Bromo-2'-deoxy-uridine labeling and detection kit I). BrdU solution was injected with a pressure driven injector (Eppendorf) in the yolk until visible swelling was observed. Immediately after injection, embryos were transferred to 0.1xNAM for 1 h, then fixed for 2 h in 20 mM PIPES, 0.4 mM $MgCl_2$, 0.25 mM EGTA and 4% paraformaldehyde. After whole-mount ELRB$_{NT}$ immunodetection, both anti-MYC and anti-mouse antibodies were removed by treatment with 0.1 M glycine-HCl (pH 2.2) for 10 min, and five subsequent washes with PBS. BrdU immunodetection was performed on paraffin sections (10 μm). Slides were progressively rehydrated, then rinsed with PBS and incubated for 45 min in HCl 2N and rinsed extensively with PBS. BrdU immunodetection was performed following the indications of the manufacturer. Slides were mounted in Vectashield (Vector Laboratories).

TUNEL

Detection of chromosomal fragmentation was performed using the Apopdetek kit FI/SA (Enzo) according to the manufacturer's instructions, except for proteinase K proteolytic treatment that was done instead by incubation in citrate buffer 0.1 M pH 6, preheated in a micro-wave oven for 2 min at 700 W. Histological sections were transferred from PBS to preheated citrate buffer and heated for 1 min at 700 W in a micro-wave

oven. Histological sections were allowed to cool down at room temperature and rinsed twice with PBS. Before mounting, slides were rinsed twice with "detergent wash" provided with the kit, then with PBS and stained with Hoechst dye (10 μg/ml, Sigma) and mounted in Vectashield.

Whole-mount in situ hybridization

Digoxygenin (DIG)-labeled antisense RNA probes were generated for *elrC* (Good, 1995), *elrD* (Good, 1995), *N-tubulin* (Richter *et al.*, 1988), *MyoD* (Hopwood *et al.*, 1989) and used as described in Harland (1991) except for the following modifications. Before incubation with anti-DIG antibody, embryos were rinsed in MAB (maleic acid 0.1 M, 0.15 M NaCl, pH 7.5), incubated 1 h in MAB/2% BMBR (Boehringer Mannheim blocking reagent), then for 1 h in MAB/2% BMBR/20% normal goat serum. The anti-DIG antibody was incubated overnight at 4°C in MAB/2% BMBR/20% normal goat serum. Embryos were rinsed in five changes of MAB (1 h each). Detection of alkaline-phosphatase activity was revealed using BCIP/NBT (Sigma) according to Harland (1991).

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