

# Localization of Cellular *src* mRNA During Development and in the Differentiated Bipolar Neurons of the Adult Neural Retina in *Xiphophorus*

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The expression of the *c-src* gene in embryonic and adult tissue of the teleost fish *Xiphophorus helleri* was analyzed by in-situ hybridization. The highly conserved fish *c-src* gene was found to be expressed at high levels in midterm embryos, where *c-src* mRNA was localized in developing neurons of the sensory layer of the differentiating retina and in the developing brain. In adult tissues the expression of *c-src* was found to persist in certain cell types of the brain and the neural retina, especially in the bipolar cells of the inner nuclear layer, which are postmitotic, fully differentiated mature neurons. Thus *c-src* in *Xiphophorus* appears to be a developmentally regulated proto-oncogene which is important for neuronal differentiation during organogenesis, but whose persistence of expression in certain terminally differentiated neurons strongly suggests a particular maintenance function for *c-src* in these cells as well.

KEYWORDS: *c-src*, in-situ hybridization, neural retina, *Xiphophorus*, teleost fish

## INTRODUCTION

The proto-oncogene *c-src* was the first cellular homolog of a retroviral transforming gene detected (Stehelin et al., 1976). It has been found in all vertebrates (Spector et al., 1978; Shalloway et al., 1981; Takeya et al., 1981; Scharf and Barnekow, 1984), and cloning of various *c-src* genes revealed a high evolutionary conservation of sequence, e.g., in human (Anderson et al., 1985), mouse (Martinez et al., 1987), chicken (Takeya and Hanafusa, 1983), and fish (S. M. Robertson et al., unpublished observations), as well as in invertebrate species, including the fruit fly (Hoffman et al., 1983; Simon et al., 1985). *c-src* has also been shown to be present in the most primitive multicellular animal, the sponge (Barnekow and Scharf, 1984). The *c-src* gene product is a 60 kD phosphoprotein, pp60<sup>*c-src*</sup>, which is a protein tyrosine kinase (Collett et al., 1979; Hunter and Sefton, 1980). Despite this salient feature the

normal physiological role of *c-src* remains unknown. The pattern of gene expression in normal and transformed vertebrate tissues is the basis for obtaining insights into the function of *c-src*. Several immunohistochemical studies of pp60<sup>*c-src*</sup> expression in various adult tissues and in developing embryos have been carried out, revealing approximately 10-fold higher protein levels and specific tyrosine kinase activity in brain and related neural tissues compared to the levels observed in nonneural tissues of adults or chicken embryo fibroblasts (Cotton and Brugge, 1983; Scharf and Barnekow, 1984; Fults et al., 1985; Maness, 1986). At the RNA level our knowledge is much more incomplete, with the exception of *Drosophila* (Simon et al., 1985). For vertebrates there exist only some *c-src* mRNA data (Gonda et al., 1982; Gessler and Barnekow, 1984; Vardimon et al., 1986; Wang et al., 1987), as well as a regional mapping of *c-src* mRNA to distinct areas of rat brain by in-situ hybridization (Ross et al., 1988). Detailed in-situ investigations during development and at the cellular level in brain and other tissues are lacking, mainly due to the low abundance of *c-src* transcripts

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### Northern Blot Analysis

Northern analysis of RNA prepared from various adult tissues showed that expression of the *Xiphophorus c-src* gene was found predominantly in neuronal tissue. Under conditions of high hybridization stringency the *c-src*-specific riboprobe detected two transcripts of 3.7 and 3.4 kb. Both transcripts could be detected in brain. In eye, the 3.7 kb message was predominantly found. In muscle, only barely detectable amounts of *c-src* mRNA were observed (Fig. 2, lanes 1-4).

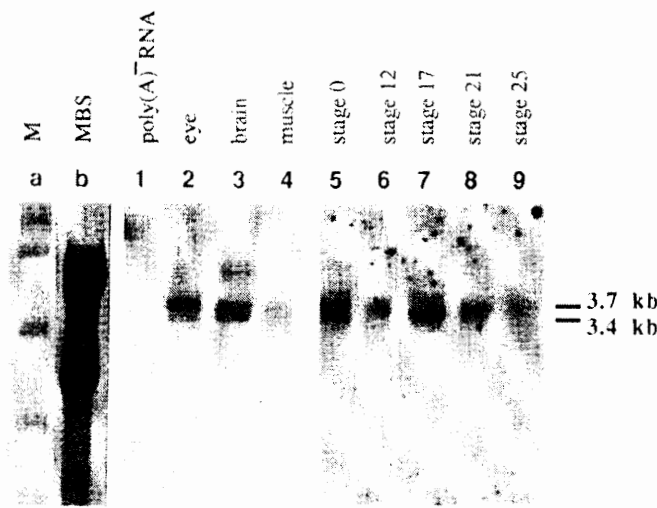


FIGURE 2. Developmental and adult expression of *c-src*. Northern blot analysis: (1) 20  $\mu$ g poly(A<sup>+</sup>) RNA as control; (2-5) 10  $\mu$ g poly(A<sup>+</sup>) RNA each of adult eye (2), brain (3), and muscle (4). (5-9) 20  $\mu$ g total RNA of unfertilized eggs (5), embryos stages 12 (6), 17 (7), 21 (8), and 25 (9). Besides the major two *src* transcripts of 3.7 and 3.4 kb in lanes 3-5, one larger and some smaller minor transcripts are slightly visible. The larger transcript is thought to be probably an unprocessed *c-src* form from hnRNA (total RNA loaded) and the smaller forms could be degradation products. For size determination and quantification of filter-bound RNA, filters were stained with methylene blue (MBS): (a) RNA marker (BRL-ladder) with 9.5/7.5 kb, 4.4 kb, 2.4 kb, and 1.4 kb. (b) 20  $\mu$ g total RNA with 28S and 18S rRNA.

During embryogenesis both transcripts were detected at levels that varied independently from one another. In unfertilized mature ova and in embryos of early stages, approximately similar relative amounts of the 3.7 and 3.4 kb transcripts were found (Fig. 2, lanes 5 and 6). During late organogenesis (6-7 days after fertilization; stages 15-17 according to Tavolga, 1949), an elevation in level of the smaller transcript was apparent, while the longer transcript level decreased compared to earlier stages. In late embryogenesis, in neonates and in young fish, *c-src* expression had returned to a low basal level (Fig. 2, lanes 7-9).

### Localization of *c-src* mRNA

During middle and late organogenesis, the *c-src* expression in embryonic tissue of *Xiphophorus* is restricted to neuronal tissue. In embryos of stage 19, when elevated *src* expression was detected by Northern analysis, in-situ hybridization of whole embryo mounts showed that the sensory layer of the developing eye and the brain were strongly labeled (Fig. 3A, B). No labeling was observed in any other organ or tissue of the embryo. In younger embryos (stage 13), distinct labeling, although considerably lower, of both developing organs was found (Fig. 3C), based upon hybridizations carried out in parallel with the later stage embryonic sections.

To determine which cell type(s) in the adult retina synthesize *c-src* mRNA, we performed in-situ hybridization of <sup>35</sup>S-labeled riboprobes to cryo-sections of adult eyes from albino xiphophorine fish. Albino fish were used because the pigment granules in wild-type pigmented tissue are almost indistinguishable from autoradiographic silver grains. In addition, melanin has a high affinity for nucleic acids and gives rise to high background labeling in in-situ hybridizations.

The general morphology of the cell layers in the mature fish retina is outlined in Fig. 4A. The cell bodies of the six major classes of retinal neurons are arranged in three layers: the outer nuclear layer (ONL), containing the perikarya of the photoreceptor cells; the inner nuclear layer (INL), with the perikarya of bipolar cells, horizontal cells, interplexiform cells, and amacrine cells; and the ganglion cell layer (GC). These cell layers alternate with layers of synapses: the outer plexiform layer (OPL), with the processes of the receptor, bipolar, interplexiform and horizontal cells, and the inner plexiform layer (IPL), containing the processes of the bipolar, interplexiform, amacrine and ganglion cells.

In-situ hybridization revealed a predominant labeling of the inner nuclear layer (Fig. 4B, C). Low-level labeling was seen in the ganglion cell layer and in the outer nuclear layer, and only background hybridization was found in the plexiform layers of the neural retina. No detectable *c-src* expression was observed in the retinal and choroidal pigment epithelia of albinotic fish. Control hybridization with the corresponding sense riboprobe did not show any autoradiographic signal above background (Fig. 4D), nor did sections that were treated with RNase prior to hybridization with the antisense riboprobe (Fig. 4E).

Whereas in embryonic brain (Fig. 3A, B) no differential expression of *c-src* was observed, the adult brain displayed a *src* expression pattern showing regional quantitative differences. For example, the

ganglion cell layer of the mesencephalon (Fig. 5) and the granule cell layer of the cerebellum (data not shown) were intensely labeled. No detectable autoradiographic signal above background was found, however, in cells of the molecular layer of the cerebellum or in the white matter of the tectum opticum.

## DISCUSSION

We have used in-situ hybridization to analyze the localization of *c-src* mRNA in embryonic and adult tissue of *Xiphophorus*. Knowledge of the cell types that express the *c-src* gene and of when during development expression occurs could give important clues to the function of its protein product, pp60<sup>*c-src*</sup>.

Northern blot analyses showed a preferential expression of *c-src* in neural tissue, in agreement with earlier findings in other systems at the RNA level (Gonda et al., 1982; Gessler and Barnekow, 1984) and protein level (Cotton and Brugge, 1983; Scharl and Barnekow, 1984; Maness, 1986). Other tissues of *Xiphophorus* showed low amounts of *c-src* mRNA—e.g., heart, head-nephros, and spleen, or barely detectable amounts—e.g., muscle, liver, and testes (Mäueler et al., 1988). Under the conditions of high hybridization stringency used, two specific *c-src* transcripts of different size were observed. These transcripts might be considered as the consequence of differential splicing or of the presence of multiple transcription start and/or termination sites in the *Xiphophorus c-src* gene, which has been shown to be a single copy gene in the genome of *Xiphophorus* (S. M. Robertson et al., unpublished observations). It is interesting to note in this regard that the *Drosophila c-src* gene *Drsrc64B* gives rise to three transcripts (Simon et al., 1985), while the chicken and human *c-src* genes generate either a single transcript or two transcripts of nearly identical size (Gonda et al., 1982; Gessler and Barnekow, 1984; Tatosyan et al., 1985). A cell type-specific differential splicing event resulting in two mRNA species giving rise to distinct neuronal and fibroblast forms of pp60<sup>*c-src*</sup> has recently been shown in chicken (Brugge et al., 1985, 1987), and in mouse (Martinez et al., 1987). In the fish the situation might be similar and one of the two messages could be that of the neuronal form *src+*. It should be noted that, in *Xiphophorus* adult brain, both *c-src* mRNA species are present, whereas in adult eye predominantly the longer transcript is found.

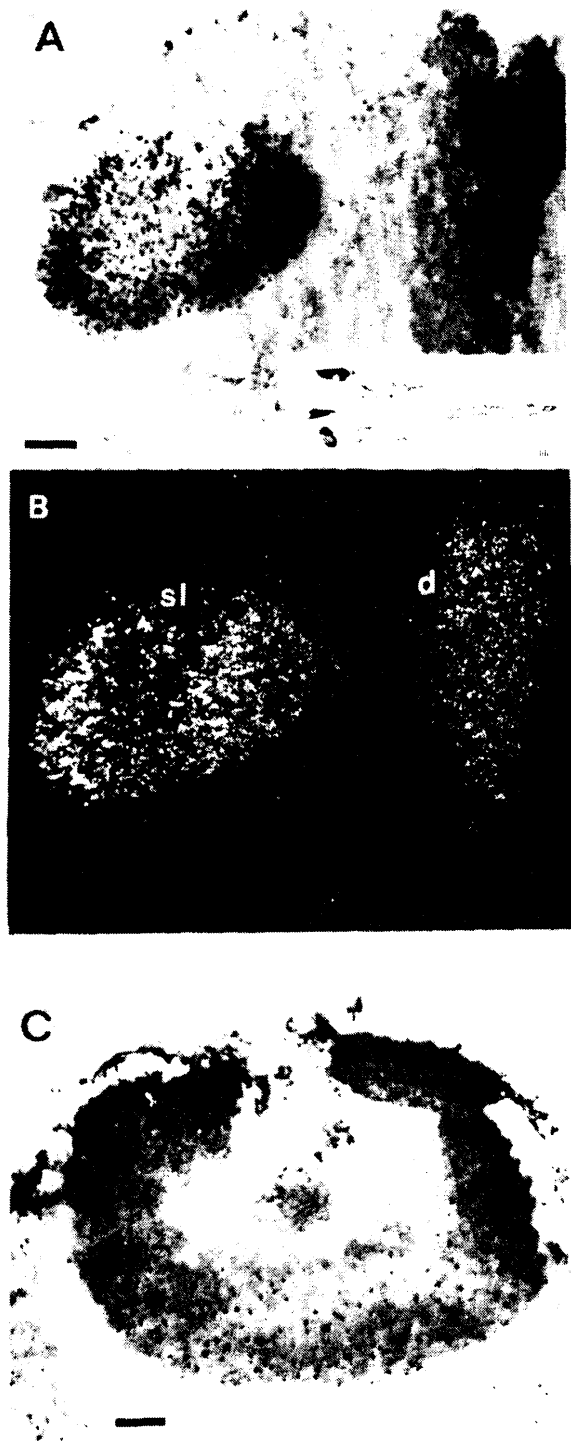


FIGURE 3. Localization of *c-src* mRNA in *Xiphophorus* embryos during organogenesis. Autoradiography of *c-src* in-situ hybridization of albino embryos from *Xiphophorus*. Cephalic region of stage 19 embryo: (A) bright-field view; (B) dark-field view. The small insert in (A) shows the whole embryo section (length: 3.2 mm). (C) Eye region of stage 13 embryo, bright-field view. Counterstaining with toluidine blue. Bar = 50  $\mu$ m.

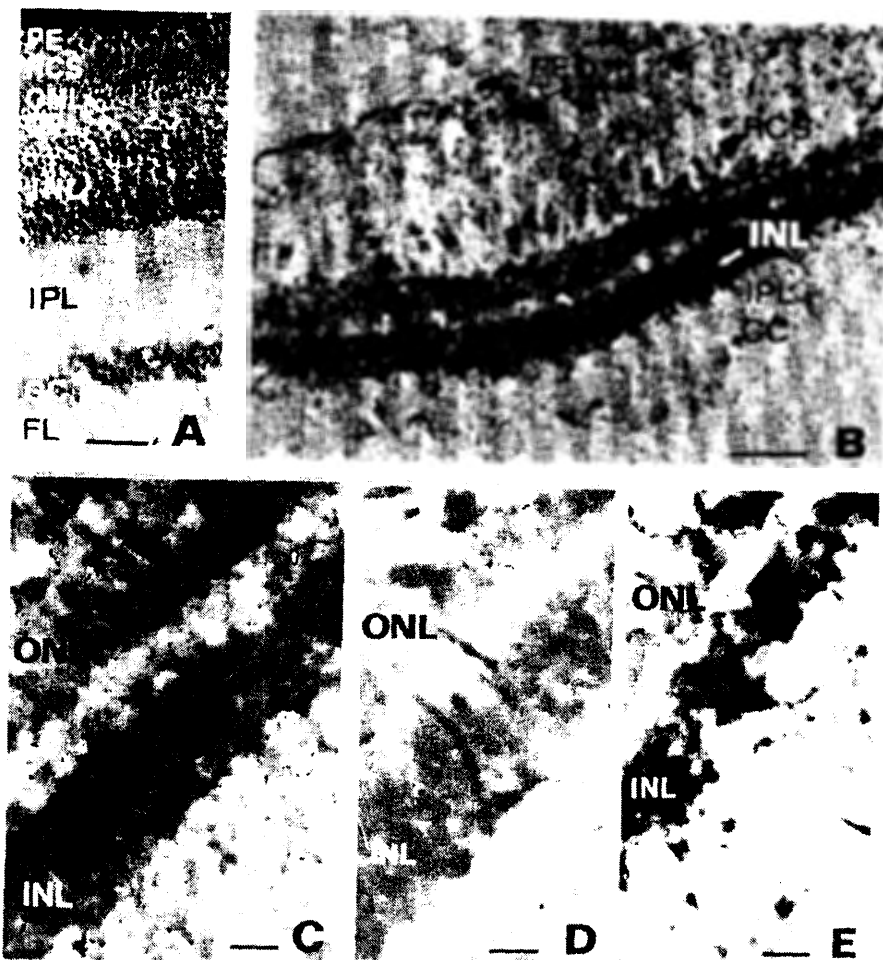


FIGURE 4. Localization of *c-src* mRNA in adult neural retina. (A) Paraffin cross section of adult *Xiphophorus* retina; azan staining. PE, pigment epithelium; RCS, rods and cones segment; ONL, outer nuclear layer; OPL, outer plexiform layer; INL, inner nuclear layer; IPL, inner plexiform layer; GC, ganglion cell layer; FL, nerve fiber layer. (B, C) Autoradiograph of a cryo-section of an adult albino *Xiphophorus* eye after in-situ hybridization with *c-src* anti-sense riboprobe. (D) Control hybridization with sense RNA probe. (E) Hybridization with anti-sense RNA probe following pretreatment of the section with RNase. Hybridization was performed in the presence of 10 mM ribovanadyl complex. Bar = 40 μm (A), 100 μm (B), 20 μm (C-E).

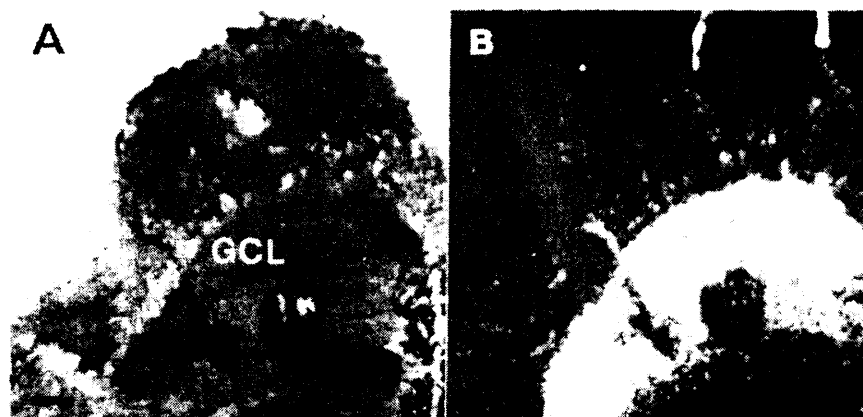


FIGURE 5. In-situ hybridization of *c-src* anti-sense riboprobe to adult *Xiphophorus* brain cross section, mesencephalic region. GCL, ganglion cell layer. (A) Bright-field view. (B) dark-field view. Bar = 100 μm.

During development both fish *c-src* transcripts are abundant in oocytes and early embryonic stages. However, in immunochemical analysis no or only a minimal amount of protein kinase activity could be detected at these stages (Schartl and Barnekow, 1984). But a small amount of pp60<sup>*c-src*</sup>-like immunoreactivity was observed by antibody staining during chicken gastrulation and neurulation (Maness et al., 1986). This points to the possibility that the

maternal *c-src* RNA gives rise to an enzymatically inactive *src* protein, which may play a role in early developmental processes. Alternatively, the vast majority of the maternal *c-src* transcripts may remain untranslated at early stages as a masked message, as shown for maternal mRNA in the sea urchin (Grainger and Winkler, 1987).

An increase of the *c-src* mRNA level in *Xiphophorus* embryos occurred about 7 days after fertiliza-

tion (stage 16/17). This corroborates earlier findings at the enzyme activity level (Schartl and Barnekow, 1984). The peak of expression coincides with the appearance of stellate epineural and cutaneous melanophores and, among other events, with the development of the mesencephalon. This strengthens the hypothesis that *c-src* is involved in differentiation processes of neuroectodermal cells. Indeed, in-situ hybridization to embryos of the corresponding stage of development revealed an exclusive labeling of neuronal tissue, especially brain and the sensory layer of the developing eye, confirming earlier findings at the protein level (Sudol et al., 1988).

In an attempt to avoid proliferative and/or differentiative effects which may influence or override *src*-specific functions, we focused our studies on *c-src* expression of adult neuronal tissues with mainly terminally differentiated cell populations. In adult brain we could detect differential *src* expression in several brain subregions, in agreement with Ross et al. (1988), showing that some neuron classes express *src*, whereas other neuron types either do not at all or do so at very low levels. Due to the immense complexity of neuronal and nonneuronal cell types in the brain, as compounded by the presence of two *c-src* mRNA transcripts of different size, an exact determination of the cell type(s) which express *c-src* was not possible.

An alternative to the brain for these sorts of studies is the neural retina, where certain fully differentiated neuron types arrange in several distinguishable layers, and where only one *src* transcript is predominant. In-situ hybridization of adult neural retina sections with a *c-src* antisense riboprobe revealed labeling predominantly over the inner nuclear layer. Acridine orange staining showed no substantial difference in the RNA content of the outer and inner nuclear layers (data not shown), so that the substantial difference between hybridization signals of both nuclear layers is not due simply to a quantitative RNA effect. The uniform labeling of the inner nuclear layer (Fig. 3B) strongly supports the interpretation that the bipolar neurons of the retina are predominantly responsible for the hybridization signals. The possibility that Müller glia cells could contain *c-src* transcripts, which would give rise to a similar distribution of hybridization signals seems to us to be unlikely, because it has been clearly demonstrated that *c-src* mRNA is neuron-specific in the neural retina (Vardimon et al., 1986). From a comparison of our data at the transcript level to

immunochemical staining in chicken (Sorge et al., 1984), where immunoreactivity in the retina was mainly localized to both plexiform layers, consisting nearly exclusively of nerve processes, only bipolar and interplexiform cells would be able to give the observed protein localization pattern, because only these cells extend processes into both plexiform layers. However, the perikarya of the interplexiform cells lie in the proximal portion of the inner nuclear layer, and only bipolar neurons spread throughout the inner nuclear layer. Amacrine cells may also contribute to the hybridization signals, although they are only arranged along the proximal border of the inner nuclear layer. In contrast to the abundant *c-src* mRNA in the inner nuclear layer, low levels were observed in the ganglion cell layer, and in the outer nuclear layer.

An alternative explanation for the localization of the pp60<sup>*c-src*</sup> protein and its mRNA to different layers of the retina would be that the transcript is localized to the perikarya of all neuronal cells of both nuclear layers and the ganglion cell layer, and that, following translation, the protein product is transported into the axons. This would then suggest a general function for the *src* protein within a unidirectional signal transduction pathway in the nerve cells, as outlined below. However, this explanation is inconsistent with the marked quantitative differences in *c-src* mRNA content of the inner nuclear layer, as compared to the outer nuclear and the ganglion cell layers.

The *c-src* mRNA content of bipolar neurons could be a special feature of this particular type of retinal neuron, in that these neurons are fully differentiated and postmitotic cells. Additional effects of proliferating and/or differentiating cells can be excluded, because rare cell proliferation leading to the formation of new neurons of the inner nuclear layer is clearly restricted to the retinal margin at the ora terminalis, as observed in the adult goldfish eye (Johns, 1983). The persistence of *c-src* expression in certain terminally differentiated neuron types suggests a function for *c-src* in the maintenance of particular mature phenotypes, probably as part of a complex cascade of intra- and intercellular signal transduction. It was recently shown that pp60<sup>*c-src*</sup> may be involved in gap-junctional cell-cell communication (Azarnia et al., 1988), and other data also suggest a role in neurocrine function (Mellström et al., 1987). In all cases, however, the mechanism of the *src* involvement and the cell type specificity of certain modes of *src* action remain to be determined.

## MATERIALS AND METHODS

### Isolation and characterization of *Xiphophorus c-src* Probe

A genomic library of *Xiphophorus maculatus* DNA in EMBL 4 was screened with the nick-translated PstI fragment F of Rous sarcoma virus (SRA-2; DeLorbe et al., 1980) under moderate stringency conditions (S. M. Robertson et al., unpublished observations). The insert of the strongest hybridizing clone ( $\lambda$ 19-4) was subcloned and was shown by sequencing of both DNA strands to contain the fish sequences homologous to chicken, mouse and human *c-src*. The PstI/SphI fragment utilized in this study was subcloned into Bluescribe (Stratagene Cloning Systems, San Diego). For in vitro transcription, the linearized plasmid was incubated in the presence of [ $\alpha$ - $^{32}$ P] or [ $\alpha$ - $^{35}$ S]UTP (New England Nuclear) with T3 or T7 RNA polymerase (Genofit, Heidelberg) according to the supplier's recommendations. Transcripts of specific activity  $1-2 \times 10^9$  dpm/ $\mu$ g were finally purified by NENSORB20 chromatography (New England Nuclear, Dreieich).

### RNA Isolation and Northern Blot Analysis

Total cellular RNA isolation following the LiCl/urea procedure and poly(A)<sup>+</sup>-RNA selection was as described previously (Mäueler et al., 1988). RNA was separated by electrophoresis on 1.2% agarose/2.2 M formaldehyde gels, and electrotransferred to GeneScreen nylon membranes according to the supplier's protocol (New England Nuclear). RNA standards (RNA ladder; BRL) were run in parallel for sizing. After UV fixation, the membrane-bound RNA was stained with methylene blue for quantitation (Khandjian, 1986). Filters were prehybridized overnight at 58°C in 50% formamide, 0.6 M NaCl, 50 mM Tris-HCl (pH 7.5), 1% SDS, 10×Denhardt's solution, and 250  $\mu$ g/ml yeast total RNA. Hybridization was carried out at 58°C for at least 12 hr in the same buffer containing  $5 \times 10^6$  cpm/ml of  $^{32}$ P-labeled cRNA probe. Filters were then washed for 1 hr in three changes of 0.1×SSC, 1% SDS at 68°C, then for 5 min in 0.1×SSC at room temperature, and examined by autoradiography (Kodak X-Omat AR film).

### In-situ Hybridization Histochemistry

Pretreatment of glass microscope slides with 3-aminopropyltriethoxysilane (Fluka, Buchs, Switzerland) was according to Rentrop et al. (1986).

### Preparation of Tissue Sections

Albino *Xiphophorus helleri* were sacrificed by decapitation. Embryos were staged according to Tavolga (1949). Tissues and whole embryos were immediately embedded in Tissue-Tek (Miles Scientific), frozen on powdered dry ice and stored at  $-80^\circ\text{C}$ . Cryostat sections 6  $\mu$ m in thickness were thaw-mounted on pretreated glass slides, dried over desiccant and fixed in 4% paraformaldehyde in phosphate-buffered saline (PBS) with 5 mM MgCl<sub>2</sub> for 15 min. After three washes in PBS, sections were transferred through 30%, 50%, and 70% ethanol for 5 min each and stored in 70% ethanol at 4°C until used.

### Pretreatment of Sections

Prior to hybridization, sections were warmed to room temperature and treated with 120 mM HCl in 70% ethanol for 10 min. Sections were then transferred through 70%, 50% and 25% ethanol for 5 min each, washed twice with PBS, and incubated with 10  $\mu$ g/ml nuclease-free pronase (Calbiochem) in 50 mM Tris-HCl (pH 7.5), 5 mM EDTA for 10 min at 40°C. The pronase digestion was terminated by incubating the sections in 0.1 M glycine in PBS for 10 min, followed by a wash in PBS. At this point controls were treated with 100  $\mu$ g/ml RNase A and 5  $\mu$ g/ml RNase T<sub>1</sub> for 4 hr at 37°C. All slides were post-fixed in 4% paraformaldehyde/PBS for 5 min at room temperature, followed by a wash in PBS. The sections were finally acetylated by immersing in 0.5% acetic anhydride in 0.1 M triethanolamine-Cl, pH 8.0, for 10 min. Thereafter they were washed for 5 min each in PBS, 70% and 95% ethanol, and air dried. Staining with acridine orange according to Hafen et al. (1983) was done to monitor RNA retention during the different pretreatment steps.

### Hybridization

Without prehybridization, the dry prewarmed slides were covered with prewarmed hybridization solution ( $\sim 250$   $\mu$ l/slide) containing 50% formamide, 10% dextran sulfate, 1 M NaCl, 10 mM Tris-HCl (pH 7.6), 10 mM DTT, 1×Denhardt's solution (=0.02% Ficoll, 0.02% polyvinylpyrrolidone, 0.02% bovine serum albumin), 25  $\mu$ M UTP $\alpha$ S (non-labeled thionucleotide, New England Nuclear), 100  $\mu$ g/ml *E. coli* total RNA, and  $5 \times 10^6$  cpm/ml, i.e.,  $\sim 2$  ng/ml, cRNA probe. Hybridization was carried out in small humid chambers at 42°C for 12 hr.

## Washing

After hybridization the slides were rinsed with prewarmed 50% formamide, 2×SSC, 10 mM DTT, and subsequently washed in the same solution at 46°C for 12–16 hr followed by two washes in 2×SSC, 10 mM DTT, and a RNase A digestion (10 μg/ml in 0.5 M NaCl, 10 mM Tris-HCl [pH 8.0], 10 mM DTT) at 37°C for 30 min. Finally, slides were washed twice in 2×SSC, 10 mM DTT, room temperature for 10 min, followed by dehydration with increasing concentrations of ethanol, and air dried.

## Autoradiography

Slides were dipped in K.5 nuclear research emulsion (Ilford) diluted 1:1 with water, air-dried for 2 hr, exposed for periods of 2–5 weeks at 4°C, developed in Kodak D-19, fixed in Ilford Hypam 1+4, and counterstained with 0.02% toluidine blue after development. Silver grains were visualized by bright- and dark-field microscopy.

## Sequence Analysis

DNA sequences were analyzed with the aid of the sequence analysis software package of the University of Wisconsin Genetics Computer Group.

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