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Evolution of the Neuron-Specific Alternative Splicing Product of the c-*src* Proto-Oncogene

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The observation of a slower migrating form of pp60$^{c\text{-}src}$ in neural tissue of chicken and mouse has recently been shown to be due to an alternative transcript form of the c-*src* gene (Martinez et al.: *Science* 237:411–415, 1987; Levy et al.: Mol Cell Biol 7:4142–4145, 1987). An insertion of 18 basepairs between exons 3 and 4, presumed to be due to alternative splicing of a mini-exon, gives rise to six amino acid residues not found in the non-neuronal (termed fibroblastic) form of pp60$^{c\text{-}src}$.

We have addressed the question of the evolutionary origin of the c-*src* neuronal insert and its functional significance regarding neural-specific expression of the c-*src* gene. To this end we have investigated whether the c-*src* gene of a lower vertebrate (the teleost fish *Xiphophorus*) gives rise to a neural-specific transcript in an analogous manner. We could show that the fish c-*src* gene does encode for a "fibroblastic" and a "neuronal" form of transcript and that the neuronal transcript does indeed arise by way of alternative splicing of a mini-exon. The mini-exon is also 18 basepairs long and we could demonstrate directly that this exon lies within the intron separating exons 3 and 4.

For comparative purposes we have examined whether the fish c-*yes* gene, the member of the *src* gene family most closely related to c-*src*, also encodes a neural tissue-specific transcript. No evidence for a second transcript form in brain was obtained. This result suggests that the mini-exon arose within the c-*src* gene lineage sometime between the *src*/*yes* gene duplication event and the divergence of the evolutionary lineage giving rise to the teleost fish. Published genomic sequence of *src*-related genes in *Drosophila* and our own results with *Hydra* demonstrate no intron in these species at the analogous location, consistent with first appearance of this mini-exon sometime between 550 and 400 million years ago.

Key words: *Xiphophorus*, teleost fish, polymerase chain reaction, RT-PCR, mini-exon, pp60$^{c\text{-}src}$

INTRODUCTION

Proto-oncogenes, the cellular homologs of retroviral transforming genes, have been implicated in the regulation of normal cellular processes such as proliferation and differentiation. The c-*src* gene was the first cellular proto-oncogene detected (Stehelin et al., 1976). It encodes a phosphoprotein of 60,000 daltons (pp60$^{c\text{-}src}$) and possesses, like its viral counterpart, a tyrosine-specific kinase activity (for review see Hunter and Cooper, 1985). Expression studies on the pp60$^{c\text{-}src}$ protein and c-*src* mRNA have revealed that cells of the neural cell lineage preferentially express the c-*src* gene although it has also been found that differentiating monomyelocytic cells in vitro (Barnekow and Gessler, 1986; Gee et al., 1986) and blood platelets (Golden et al., 1986) express the gene at elevated levels. Neural tissues contain high amounts of pp60$^{c\text{-}src}$, of its kinase activity (Cotton and Brugge, 1983; Schartl and Barnekow, 1984; Levy et al., 1984; Fults et al., 1985; Maness, 1986), and of c-*src* mRNA (Gessler and Barnekow, 1984; Simon et al., 1985; Vardimon et al., 1986; Mäueler et al., 1988a). Low levels of c-*src* mRNA have been detected in several other tissues, including heart, muscle, and liver (Gessler and Barnekow, 1984; Mäueler et al., 1988a).

Investigations on the phylogenetic distribution of the c-*src* gene and its evolutionary precedents, respectively, have indicated that this gene, appearing first during phylogenesis in the most primitive multicellular animals, the sponges (Barnekow and Schartl, 1984), might therefore be as old as 1.5 billion years. The sequence of the c-*src* gene has been found to be highly conserved in human (Anderson et al., 1985), mouse (Martinez et al., 1987), chicken (Takeya and Hanafusa, 1983), fish (Rob-

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ertson et al., submitted), fruit fly (Hoffmann et al., 1983; Simon et al., 1985), and sponge (Ottilie and Schartl, unpublished). In addition, the enzymatic properties of pp60$^{c\text{-}src}$ have been shown to be comparable in all metazoans tested so far and the preferential expression in neural tissues was also found to be conserved during evolution (Schartl and Barnekow, 1982; Barnekow and Schartl, 1987). This evolutionary conservation already points to a crucial function for this gene in multicellular animals. During ontogenesis it has been demonstrated in various species that pp60$^{c\text{-}src}$ kinase activity is found preferentially in post-mitotic neurons. In the chicken during embryonal development pp60$^{c\text{-}src}$ is detected in the cerebellum and the developing neural retina during terminal differentiation of neurons (Maness, 1986). In the adult chicken brain highest levels of c-*src* gene expression are found associated with membranes in the molecular layer of the cerebellum and to a lesser extent in the granular layer (Sudol et al., 1988). In the central nervous system of the rat all regions were shown to contain detectable levels of pp60$^{c\text{-}src}$, with areas characterized by a high content of grey matter and an elevated density of synaptosomes displaying the highest amounts of the kinase (Walaas et al., 1988). Localization of c-*src* mRNA by in situ histochemistry (Raulf et al., 1989) compared with the distribution of pp60$^{c\text{-}src}$ (Maness et al., 1988; Sorge et al., 1984) indicated translocation of the c-*src*-encoded protein product to the processes of nerve cells.

Based on these studies it was deduced that the c-*src* gene product primarily plays a role in differentiation or maintenance of the neuronal cell phenotype. Recently, a slower-migrating form of pp60$^{c\text{-}src}$ (termed pp60$^{c\text{-}src+}$) has been found primarily in the central nervous system of higher vertebrates (Brugge et al., 1985, 1987; Lynch et al., 1986; LeBeau et al., 1987). pp60$^{c\text{-}src+}$ is encoded by a unique mRNA that contains in contrast to the previously identified protein from non-neuronal cells a six amino acid insertion (Martinez et al., 1987; Levy et al., 1987). pp60$^{c\text{-}src+}$ is preferentially detected in membranes of neuronal growth cones and nerve processes, leading to the suggestion of its possible role in growth cone-mediated neurite extension (Maness et al., 1988).

We have addressed the question of the evolutionary origin of the c-*src* neuronal insert. For this purpose we have investigated whether this insert can be detected in lower vertebrates, e.g., fish. We also looked for a comparable "neuronal" form of the c-*yes* gene in these animals. The c-*yes* gene has arisen due to a gene duplication event involving c-*src* prior to evolution of modern-day teleost fish (Robertson et al., submitted). Although this gene shows a high degree of structural similarity and a comparable preferential expression in neural tissues of chicken (Sudol et al., 1988), it is not known if a specific neuronal form exists. In addition, we examined whether *Hydra*, the first organism with a defined nervous system and also demonstrating a neural-specific *src* expression (Schartl et al., submitted), could generate a neuron-specific form of pp60$^{c\text{-}src}$ in an analogous manner. To elucidate the mechanism by which such altered transcripts arise, two possibilities were investigated; firstly, that the neuronal insert is contained as a "mini-exon" in the intron between exons 3 and 4 and is inserted by an alternative splicing event, as has been suggested (Martinez et al., 1987; Levy et al., 1987); or secondly, that a neuron-specific genomic rearrangement of the c-*src* locus occurs.

MATERIALS AND METHODS

Experimental Animals

The fish used in this study were bred under standard conditions (see Kallman, 1975) in the aquarium of the Genecenter. The following species were included in our analyses: *Xiphophorus helleri* (stock "Rio Lancetilla"), and *X.maculatus* (stock "Rio Usumacinta"),*X. xiphidium* (stock "Rio Purification"), and *X. helleri* backcross hybrids bearing the *Tu-Sd* oncogene locus from *X. maculatus* (stock "Rio Jamapa") responsible for the spontaneous development of melanomas (see Schartl et al., 1989a).

The *Hydra* used in this study *(Hydra viridissima)* were maintained under standard conditions and obtained from T. Holstein (Zoology Institute, Ludwig-Maximilians-University, Munich, F.R.G.).

Cell Lines

Cell lines derived either from spontaneous hereditary melanoma of *Xiphophorus* hybrids, PSM cells (Wakamatsu et al., 1984), or from non-tumorous embryos of wildtype *X. xiphidium*, A2 cells (Kuhn et al., 1979). Cells were cultured in F12 medium (Biochrom, Berlin, F.R.G.) containing 10% fetal calf serum and 1.25g $NaHCO_3$/L at 28°C under 5% CO_2.

Isolation of Genomic DNA

Preparation of high molecular weight genomic DNA was according to Blin and Stafford (1976). Prior to PCR the genomic DNAs were treated with RNase A (100 μg/ml) at 37°C for 1 hr.

Restriction enzyme digestions (enzymes from Boehringer Mannheim, Mannheim, F.R.G.) and standard molecular biology techniques were carried out essentially as described (Maniatis, 1982).

Isolation of RNA and Reverse Transcription

Total cellular RNAs from cell cultures were isolated as described previously (Mäueler et al., 1988a).

For RNA preparation from fish organs a microscale GuaSCN/CsCl procedure (Chirgwin et al., 1979) was used; e.g., 1–3 freshly dissected fish brains (approx. 15–30 mg) were homogenized for 1 min at 25,000 rpm with an Ultra-turrax T5 (Janke & Kunkel, Staufen, F.R.G.) in 600 μl GuaSCN solution (6 M guanidine thiocyanate, 10 mM EDTA, 4% N-laurylsarcosine, 1% 2-mercaptoethanol, 200 μg/ml *E. coli* RNA). RNA was purified by centrifugation through 5.7 M CsCl, 100 mM EDTA pH 8.0 for 2 hr at 18°C at 80,000 rpm (TLA100.2 rotor, TL-100 ultracentrifuge, Beckman, Palo Alto, CA), solubilized in 300 μl 4 M NH_4Ac, and precipitated with ethanol.

For reverse transcription oligo$(dT)_{15}$ primer (Boehringer Mannheim, Mannheim, F.R.G.) was annealed to 1–4 μg total cellular RNA and incubated with 50 U AMV reverse transcriptase (Life Sciences, St. Petersburg, FL) according to the supplier's recommendations for 1 hr at 42°C.

Polymerase Chain Reaction

For RT-PCR 0.1 volume of the first-strand cDNA synthesis was directly introduced into the polymerase chain reaction. Conventional PCR (Saiki et al., 1988) was performed by using about 1 ng genomic DNA. The reaction mix contained further two primers (1 μM each), all four dNTPs (200 μM each), 50 mM KCl, 10 mM Tris-Cl pH 8.3 (at room temperature), 1.5 mM $MgCl_2$, 0.01% (w/v) gelatin, and 2.5 U *Taq* DNA polymerase (Perkin Elmer Cetus, Norwalk, CT) in a volume of 50 μl overlaid with mineral oil. A self-made computer-controlled PCR-machine using a Peltier element as thermoelectric heat pump (Wittbrodt and Erhardt, 1989) was used for 45 cycles of 1 min at 92°C, 1.5 min at 60°C, and 2 min at 72°C. Following termination of the reaction the samples were kept at 4°C; 20% aliquots of each reaction were analyzed by agarose gel electrophoresis, and the remaining volume was subjected to direct sequencing of the PCR product.

Oligonucleotides

Oligonucleotides were synthesized on a 380A DNA Synthesizer from Applied Biosystems (Foster City, CA).

#S95 5'AAG AGA CTA AAT CTC CAG GGT CTT GGC CAT 3'(30mer),

#S777R 5'GGC GTG CTT GCC GTA TGA TTG CAC GAG CTG 3'(30mer),

#S372 5'TAC GAC TAC GAG TCT CGA A 3'(19mer),

#S515R 5'CCA CAT AAT TGC TGG GGA T 3'(19mer),

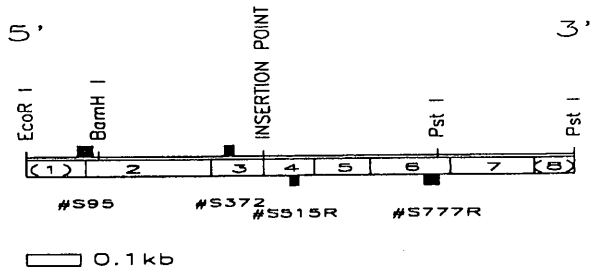

Fig. 1. Partial map of *Xiphophorus* c-*src* cDNA from A2 cells (fibroblast form, 1,029bp EcoR I/Pst I—fragment comprising exons 2–7) with the positions marked (filled-in rectangles) of the *src*-specific oligonucleotides used in this study in comparison to the exon arrangement. Those oligonucleotides above the bar are in sense orientation, whereas those below the bar are anti-sense. The start of translation is in exon 2 at position 122.

#SNR 5'CCC TGC AGT TTA TCT TCC TC 3'(20mer),

Direct Sequencing of PCR Products

A 0.1 volume of 2 M NaOH, 2 mM EDTA was added to the PCR mix and incubated for 10 min at room temperature. After addition of 0.1 volume 3 M NaAc pH 5.2 an ethanol precipitation was performed. The salt-free washed and dried DNA pellet was subjected to DNA sequencing by using a ^{32}P-endlabeled oligonucleotide as primer and the T7 DNA polymerase kit according to the suppliers recommendations (Pharmacia,Freiburg, F.R.G.).

Isotachophoresis

Cut out DNA fragments were recoverd from agarose gels by isotachophoresis essentially as described (Öfverstedt et al., 1984).

Sequence Analysis

Sequence comparisons were carried out by using the sequence analysis software package (GCG) of the University of Wisconsin Genetics Computer Group (Devereux et al., 1984).

RESULTS

Xiphophorus Brain Contains Two Forms of src mRNA

To investigate if a neuronal insert of the c-*src* proto-oncogene is already present in lower vertebrates, we used the polymerase chain reaction (PCR) to look for a neuronal c-*src* form in the teleost fish *Xiphophorus*. For this purpose oligonucleotide primers were designed based upon a previously cloned and sequenced *Xipho-*

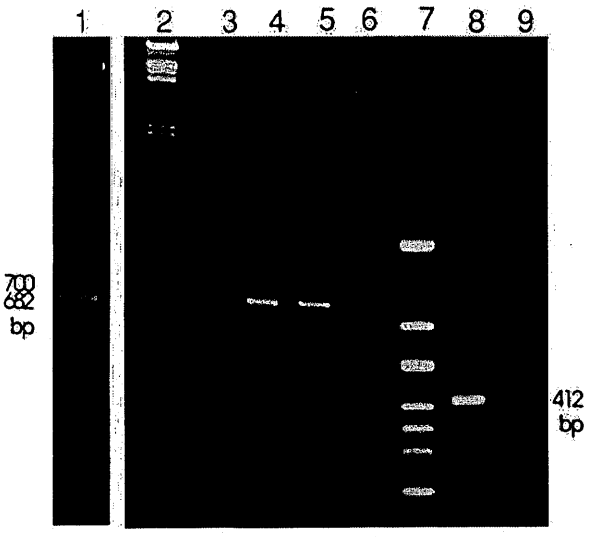

Fig. 2. RT-PCR of total RNA from *Xiphophorus* tissues and cell lines. **Lanes 1,3–6:** Polymerase chain reaction with c-*src* primers #S95 and #S777R. (1) *X. xiphidium* brain, (3) *X. helleri* brain, (4) A2 cells, (5) PSM cells, (6) a control experiment with water instead of template. **Lanes 8 and 9:** Polymerase chain reaction with c-*yes* primers (see text). (8) *X. helleri* brain, (9) *X. helleri* liver. Size of PCR products as marked (in basepairs). **Lane 2:** Molecular weight marker λ/EcoR I + Hind III. **Lane 7:** Molecular weight marker pBR322/EcoR I + Hinf I.

phorus c-*src* cDNA (Raulf, unpublished). This cDNA clone was derived from an embryonal cell line (A2) and represents the fibroblast form of c-*src* (Fig. 1). To analyze the c-*src* mRNA the primer set #S95 and #S777R were designed as 30mers spanning 682bp between exon 2 and exon 5 including the expected (i.e., by analogy to the mouse and chicken) insertion point at the border of exons 3 and 4.

PCR after first-strand reverse transcription (RT-PCR) revealed two distinct fragments from total brain RNA preparations of *X. xiphidium* and *X. helleri* (Fig. 2, lanes 1 and 3). To test the fidelity of the reaction total RNA from A2 cells was used in parallel. This reaction yielded only the 680bp fragment, representing the fibroblast form as expected from the cDNA data (Fig. 2, lane 4). The faster-migrating fragment of the brain PCR product comigrates with the A2 PCR product and therefore is regarded to represent the amplification product of the fibroblast form. The additional, about 20bp longer, PCR product was presumed to represent the neuronal form of fish c-*src*. Both bands hybridized in Southern blot analysis to the ^{32}P-labeled exon 3–specific oligonucleotide #S372 which was designed 73bp upstream from the expected insertion (data not shown). This result confirmed that both amplification products derived from the *Xiphophorus* c-*src* locus.

To address the question of whether c-*src* in other cells of the neural cell lineage can also give rise to a transcript with a neuronal insert, RNA from a *Xiphophorus* melanoma cell line (PSM) was analyzed. This cell line, composed of transformed melanocytes, has been shown to contain high levels of c-*src* mRNA and pp60$^{c\text{-}src}$ kinase activity (Mäueler et al., 1988b). The resulting single PCR band had the size of the fibroblast form (Fig. 2, lane 5).

Length and Location of the Neuronal Insert Are Conserved in Fish

For sequence analysis both RT-PCR bands from brain were separated by gel electrophoresis on a 1.8% agarose gel. The bands were cut out, purified by isotachophoresis, and sequenced by using ^{32}P-labeled oligonucleotide #S372. In parallel, unpurified PCR product from A2 cells and PSM cells were subjected to sequence analysis. In addition, the higher molecular weight band from brain was subcloned and sequenced by standard plasmid ^{35}S sequencing. These sequence analyses revealed that the higher molecular weight band from brain contained, in comparison to the A2 and PSM bands and the lower molecular weight band from brain, an 18bp insertion exactly at the exon 3/4 border (Fig. 3), completely analogous to the situation in mouse (Martinez et al., 1987) and chicken (Levy et al., 1987). The neuronal insert sequence itself when compared to the corresponding mouse sequence is less conserved (61% on nucleic acid, 50% on amino acid level) in contrast to the situation found for exons 3 (78.4% NA, 90.6% AA) and exons 4 (85.9% NA, 87.9% AA). The most striking difference is the exchange of valine for cysteine in the fifth amino acid position of the neuronal insert (Fig. 3). This results in a new Pst I site, which was used as a diagnostic marker site for the neuronal fish c-*src* form. Comparison of the neuronal insert sequence from fish and chicken gives the same values as the fish-mouse comparison above.

The Neuronal Insert Is a Mini-Exon Which Is Subjected to Alternative Splicing

To elucidate the location of the neuronal insert of c-*src* at the genomic DNA level we designed a pair of oligonucleotide primers flanking the intron between exons 3 and 4. Genomic DNA either from pooled fish organs (testis, liver, brain) or from fish total brain was used and the resulting PCR product was in every case a single band of 1.6kb (Fig. 4, lanes 2–4). This band was subjected to an analytical Pst I restriction enzyme digestion which revealed three bands of 0.95kb, 0.4kb, and 0.25kb (Fig. 4, lane 5). Due to the second Pst I site in the

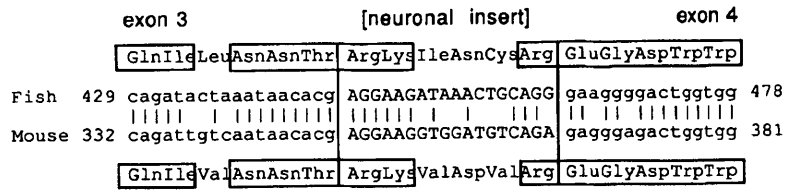

Fig. 3. Sequence comparison between fish and mouse c-*src* neuronal insert and flanking regions of exons 3 and 4. Identical amino acids are boxed. Mouse c-*src* sequence was taken from Martinez et al. (1987). The amino acid sequence of the chicken neuronal insert (Levy et al., 1987) is identical to that of mouse.

region between exons 3 and 4 an exact location of the neuronal insert was not possible. Therefore an oligonucleotide primer of the neuronal insert sequence (#SNR) was designed. A PCR with genomic DNA from *X. helleri* and primers #S372 and #SNR yielded a fragment of about 1.2kb (Fig. 4, lane 8). The generation of only one PCR product confirmed the presence of the insert between exons 3 and 4 in the genomic c-*src* locus. The size of this PCR product localized the neuronal insert about 1.13kb downstream from the end of exon 3 and about 0.33kb upstream from the beginning of exon 4 (after accounting for the exon 3 and exon 4 sequences present in the PCR product).

Neuronal and Non-Neuronal Forms Are Characteristic for c-*src* But Are Not Found for c-*yes*

To answer the question of whether c-*yes* exhibits the same polymorphism of a neuronal and nonneuronal form, we used a similar approach to that for c-*src*. From a cloned cDNA of *X. helleri* c-*yes* (Hannig, Ottilie, and Schartl, submitted) we designed a pair of oligonucleotide primers for PCR, encompassing 412bp around the border of exons 3 and 4 where an c-*src* homologous neuronal insert would be expected. RT-PCR of *X. helleri* brain and liver RNA preparations revealed only one band with the expected fragment length (Fig. 2, lanes 8 and 9), but no additional amplification product of larger size, which would be indicative of a neuronal form of c-*yes*.

Hydra c-*src* Does Not Encode an Analogous Neuronal Form

Based upon the cDNA sequence of the *H. attenuata src*-related tyrosine-kinase gene *stk* (Bosch, T.C.G., unpublished), a pair of 22mer oligonucleotides was prepared which span 113bp of the region analogous to the exon 3/4 boundary of the vertebrate c-*src* gene. PCR amplification was performed with these two oligonucleotides by using either *Hydra* genomic DNA or *stk* cDNA as templates. In both cases an amplification product of 157bp was observed (data not shown), demonstrating that no analogous intron exists in the *Hydra* genomic locus.

DISCUSSION

The experiments reported here show that a lower vertebrate (the teleost fish *Xiphophorus*) possesses a neural form of c-*src* mRNA. Analogous to the situation in chicken (Levy et al., 1987) and in mouse (Martinez et al, 1987), this form arises due to the presence of an additional 18 basepair insertion between exons 3 and 4. Differential splicing of this mini-exon specifically within certain cells of neural origin gives rise to this alternative transcript form. Our data represent the first direct demonstration that this mini-exon does indeed reside within the intron separating exons 3 and 4. Our results are inconsistent with a rearrangement of the genomic c-*src* locus specifically within neural tissue, which had remained a theoretical possibility. The fish c-*yes* gene does not give rise to two forms of mRNA in brain. This is interesting because the c-*yes* gene is the member of the *src* gene family of tyrosine kinases most closely related to c-*src* and has also been shown to be expressed in neural tissue (Sudol et al., 1988). The question arises as to whether a neuronal c-*yes* transcript form could be present, but in such a small relative amount so as to be

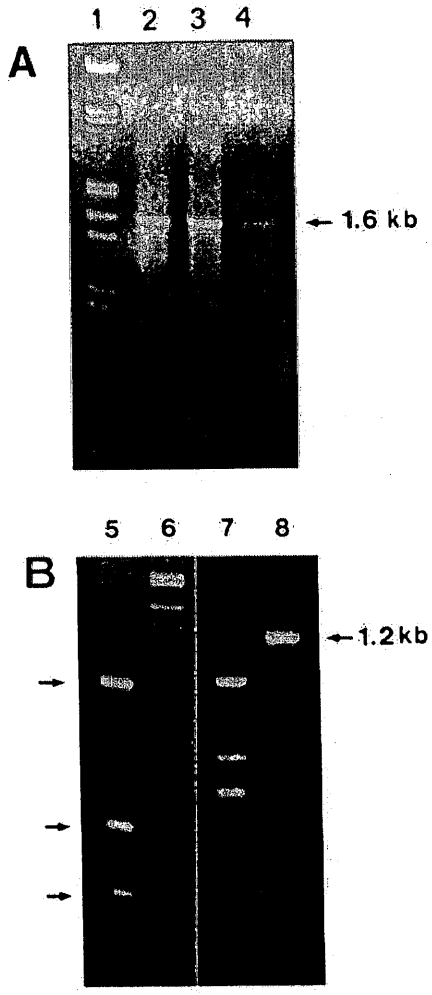

Fig. 4. PCR of genomic DNA from *Xiphophorus*. **A:** 1% agarose gel with PCR products of reactions performed with *src* primers #S372 and #S515R. **(2)** *X. helleri* DNA from pooled organs, **(3)** *X.maculatus* DNA from pooled organs, **(4)** *X. helleri*$^{Tu\text{-}Sd}$ DNA from brain (approx. one-fifth the template amount as in lanes 2 and 3). **(1)** Molecular weight marker λ/EcoR I + Hind III. **B:** 1.5% agarose gel. **(5)** PCR product as in A lane 2 after digestion with Pst I. **(6)** molecular weight marker λ/EcoR I + Hind III. **(7)** Molecular weight marker pBR322/EcoR I + Hinf I. **(8)** PCR product of *X. helleri* DNA (pooled organs) and *src* primers #S372 and #SNR.

undetectable via this sort of RT-PCR analysis; 4 μg brain total RNA was used for the RT-PCR amplification, which represents approximately 0.03 fmol or 10^7 copies of the c-*yes* transcript (F. Raulf, unpublished). Control experiments of ours under very similar conditions to those employed here (S. Robertson and J. Wittbrodt, unpublished) have shown successful PCR amplification from as few as 10^4 target sequences. Therefore, if a neuronal form was present at a relative level as low as 0.1% of the non-neuronal form, a visible band would have been expected.

The sequence of the fish neuronal insert, when compared to that of the mouse (Martinez et al., 1987) and chicken (Levy et al., 1987), has several interesting features. First, the percentage identity at both the nucleic acid and amino acid level would appear to be lower than in the immediately adjacent regions of exons 3 and 4 (Fig. 3). Of the six amino acid residues, three are identical and two are conservative changes (Ile→ Val;Asn→ Asp), with the three changes occurring in the middle of the neuronal insert. Second, a preponderance of polar sidegroups in the mouse, chicken, and fish neuronal inserts leads to a hydrophilic stretch of amino acid residues with a significant probability for exposure at the surface of the protein. This conservation of the hydrophilic nature of the neuronal insert suggests that this feature may play an important role in the tissue-specific function of pp60$^{c\text{-}src+}$.

We have shown that the fish c-*src* gene encodes, by way of alternative splicing of a mini-exon located between exons 3 and 4, a neuronal-specific mRNA form whereas the fish c-*yes* gene apparently does not. This would suggest that the neuron-specific sequence was not present prior to the *src/yes* gene duplication event, calculated to have occurred approximately 550 million years (Myr) ago (Robertson et al., submitted). This raises two possibilities pertaining to the evolutionary origin of this particular sequence; one, that a sequence containing the 18bp mini-exon inserted into the c-*src* intron separating exons 3 and 4 sometime between the emergence of the protochordates (i.e., approximately 550 Myr ago) and the divergence of the teleost fish lineage approximately 400 Myr ago; or two, that this mini-exon evolved specifically within the c-*src* gene lineage from a pre-existing sequence within this intron found in the common progenitor of the c-*src* and c-*yes* genes. Direct sequence analysis of the respective c-*src* and c-*yes* introns may help to distinguish between these two possibilities.

If the proposed timing for the appearance of this neuronal mini-exon is correct, then no neuronal insertion should be detected in c-*src* sequences from evolutionarily more primitive organisms. In *Drosophila*, several *src*-related sequences have been characterized, none of which have introns between their exon 3 and 4 analogous sequences (Hoffmann et al., 1983; Gregory et al., 1987). Therefore, a corresponding mini-exon in the analogous position is not found for the *Drosophila src*-related loci. In *Hydra*, we report here that no analogous intron exists between the sequences of the *src*-related locus corresponding to the vertebrate c-*src* exons 3 and 4. This again discounts the possibility of an alternative splicing

mechanism in *Hydra* analogous to that seen in vertebrates.

The presence of the neuronal mini-exon only in the vertebrate c-*src* gene must be reconciled with two sets of observations: first, neuronal-specific expression of the c-*src* gene has been observed well prior to the emergence of the protochordates, e.g., *Drosophila* (Simon et al., 1985), and *Hydra* (Schartl et al., 1989b); and second, the c-*yes* gene, a very closely related member of the *src* family of tyrosine kinases which, however, lacks a neuronal mini-exon, is also expressed in neural tissue (Sudol et al., 1988). Therefore the neuronal mini-exon is not the basis for this tissue specificity of expression, suggesting that it is possibly involved in a vertebrate-specific neuronal function of the c-*src* gene.

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