MOLECULAR AND CELLULAR BIOLOGY, May 1990, p. 2035–2040
0270-7306/90/052035-06$02.00/0

Vol. 10, No. 5

Identification of a Novel Neuronal C-*SRC* Exon Expressed in Human Brain

JOANNA M. PYPER* AND JOSEPH B. BOLEN

Laboratory of Tumor Virus Biology, National Cancer Institute, Bethesda, Maryland 20892

Received 7 November 1989/Accepted 20 January 1990

Neuronal cells are known to express at least two different forms of the C-*SRC* proto-oncogene as a consequence of alternative splicing events which add an 18-nucleotide exon (the NI exon) between C-*SRC* exons 3 and 4. Here we report that a second neuronal exon of C-*SRC* is also present between C-*SRC* exons 3 and 4. This neuronal exon (the NII exon) of C-*SRC* was isolated from human adult and fetal brain-derived cDNAs and contains 33 nucleotides capable of encoding 11 amino acids (Gln-Thr-Trp-Phe-Thr-Phe-Arg-Trp-Leu-Gln-Arg). The human NI exon was located approximately 390 nucleotides from the end of C-*SRC* exon 3, whereas the NII exon was approximately 1,000 nucleotides from the beginning of C-*SRC* exon 4. Analysis of human brain RNA revealed that the NII exon is utilized primarily in conjunction with the NI exon to yield transcripts capable of encoding C-*SRC* products possessing 17 additional amino acids. These splicing events, which occur between the NI and NII exons, are predicted to alter the sixth amino acid encoded by the NI exon from an arginine to a serine residue, producing a potentially novel phosphorylation site. Analysis of the different C-*SRC* RNA transcripts revealed that the level of C-*SRC* RNA containing both NI and NII exons is similar in adult and fetal brain tissue, whereas the level of C-*SRC* RNA containing only the NI exon or the nonneuronal form of C-*SRC* RNAs is significantly higher in fetal brain tissues. These results indicate that the expression and splicing pattern of the C-*SRC* gene are developmentally regulated in the human brain.

The membrane-associated tyrosine protein kinase, $pp60^{c\text{-}src}$, is encoded by the C-*SRC* gene. This gene is classified as a proto-oncogene based upon the evidence that mutations can generate c-*src* proteins competent for oncogenic cellular transformation (34; J. A. Cooper, *in* B. Kemp and P. F. Alewood ed., *Protein and Peptide Phosphorylation*, in press). Although the normal function of $pp60^{c\text{-}src}$ is not yet known, an increasing number of observations suggest that this enzyme may play a role in events associated with neuronal differentiation and maintenance of mature neuronal cell functions (5–7, 11, 15, 19, 26, 28–30). In addition to high levels of $pp60^{c\text{-}src}$ expression in neuronal tissues, it has been found that the majority of the *src* protein in neuronal tissues is a neuron-specific isoform. This isoform has been identified in normal avian and rodent neurons (5–7, 38) and in some human neuronal tumors (3, 22, 23, 36, 39). c-*src* cDNA clones isolated from mouse and chicken brains contain an 18-nucleotide exon inserted at the splice junction of exons 3 and 4 (17, 21). These neuron-specific exons encode the same amino acid sequence for both species.

c-*src* exons 3 and 4 encode part of the domain believed to be potentially involved in substrate interactions and regulation of the activity of the c-*src* gene product. Mutations within exon 3 have been identified which activate the transforming potential and the kinase activity of the c-*src* gene product (13, 24). Additionally, Levy and Brugge (16) have shown that the chicken neuronal $pp60^{c\text{-}src}$ has both elevated tyrosine kinase activity and altered transforming activity relative to the nonneuronal $pp60^{c\text{-}src}$. These observations indicate that modifications within the exon 3/4 region may profoundly affect $pp60^{c\text{-}src}$ functions.

Here we describe the identification of a second C-*SRC* neuron-specific exon (NII) which appears to be utilized mainly in conjunction with the previously described neuron-specific exon (NI) (25). The splice between exons NI and NII is predicted to alter the coding for the sixth amino acid of NI from an arginine residue to a serine residue, thus creating a potentially novel phosphorylation site within the putative regulatory domain. Analyses of RNA from human fetal and adult brain tissue indicate that the expression and splicing of the C-*SRC* gene are developmentally regulated in the human brain.

MATERIALS AND METHODS

RNA and DNA samples. Poly(A)$^+$ RNA was selected by oligo(dT)-cellulose chromatography from RNA purified by the guanidine isothiocyanate method (8). Human adult brain tissue was obtained at autopsy and frozen prior to RNA isolation. Human fetal brain tissue was obtained from a spontaneous abortion and frozen prior to RNA extraction. Human genomic placental DNA was isolated from fresh tissue by the method of Blin and Stafford (2).

cDNA synthesis and PCR. The oligonucleotide primers used for the first-strand cDNA synthesis and for the polymerase chain reactions (PCR) contained 20 bases complementary (for cDNA synthesis) or homologous to the predicted c-*src* RNA and a 6-base restriction site (either *Hin*dIII or *Bam*HI) flanked by 3 bases to facilitate restriction digestion. The sequences of the primers were 5′-ATTAAAGCTT TAGGACGGAGACAGACCTGT-3′ (5′ primer) and 5′-AT AGGATCCGGAGTCGGAGGGCGCCACGT-3′ (3′ primer). For synthesis of the first strand of cDNA, 0.5 μg of poly(A)$^+$ RNA was annealed to 0.1 μg of the 3′ oligonucleotide in 100 mM NaCl–20 mM Tris hydrochloride (pH 8.3)–0.1 mM EDTA in a volume of 10 μl. The mixture was heated to 90°C for 3 min, incubated at 55°C for 10 min, and then allowed to cool slowly to room temperature. The annealed RNA template was then reverse transcribed at 37°C for 1 h in a reaction containing 50 mM Tris hydrochloride (pH 8.3), 75 mM KCl, 3 mM $MgCl_2$, 10 mM dithiothreitol, 1 mM dATP, 1 mM dCTP, 1 mM dGTP, 1 mM dTTP, 200 U of cloned Moloney murine leukemia virus reverse transcriptase (Be-

* Corresponding author.

thesda Research Laboratories, Inc.), and 40 U of RNasin (Promega Biotec) in a final volume of 20 μl. Following heat inactivation at 95°C for 5 min, the reaction mixtures were prepared for PCR by following the guidelines included with the kit (Perkin Elmer Cetus Gene Amp DNA Amplification Kit) and contained 0.5 μg each of the appropriate 5′ and 3′ oligonucleotide primers. A Perkin Elmer DNA Thermal Cycler was used for 30 DNA amplification cycles, with a denaturation step at 94°C for 1 min, an annealing step at 55°C for 2 min, and an extension step at 70°C for 2 min. Five percent of the amplified DNA was analyzed by electrophoresis in a 4% Nusieve (FMC Corp.) gel. Under these conditions, fragments could be distinguished that differed by less than 20 base pairs.

PCR amplification of genomic DNA. The PCR amplifications of 1 μg of human placental DNA used conditions recommended by the manufacturer (Cetus), except that annealing was carried out at 65°C and the extension step at 72°C was performed for 1 min. Pairs of oligonucleotide primers were used to amplify different portions of the exon 3/4 region of the C-*SRC* gene. These primers contained sequences from C-*SRC* exons 3, NI, NII, or 4 in the appropriate orientations. At the end of each primer a *Bam*HI or *Hin*dIII site was added plus three additional nucleotides to facilitate cleavage by the restriction enzymes. Three oligonucleotides were used for priming from 5′ ends: oligonucleotide 28 (from exon 3), 5′-ATAAAGCTTTAGGACGGAGACAGACCTGT-3′; oligonucleotide 39 (from exon NI), 5′-ATAAAGCTTGAGGAAGGTGGATGTCAG-3′; and oligonucleotide 44 (from exon NII), 5′-ATAAAGCTTCCAGACCTGGTTCACATTCA-3′. Three oligonucleotides were used for priming from 3′ ends: oligonucleotide 27 (from exon 4), 5′-ATAGGATCCGGAGTCGGAGGGCGCCACGT-3′; oligonucleotide 38 (from exon NI), 5′-ATAGGATCCCTGACATCCACCTTCCTC-3′; and oligonucleotide 40 (from exon NII), 5′-ATAGGATCCTGAATGTGAACCAGGTCTG-3′. The following combinations of primers were used in amplifying sequences between the indicated exons: oligonucleotides 28 and 38 (exons 3 and NI), oligonucleotides 39 and 40 (exons NI and NII), oligonucleotides 27 and 44 (exons NII and 4), oligonucleotides 28 and 40 (exons 3 and NII), oligonucleotides 27 and 39 (exons NI and 4), and oligonucleotides 27 and 28 (exons 3 and 4). Five percent of each reaction was analyzed on a 1.5% agarose gel.

Molecular cloning and sequence analysis of the PCR products. The PCR products were cloned into the *Bam*HI-*Hin*dIII sites of pT7T3 18U (Pharmacia, Inc.) or pGEM4Z (Promega Biotec) by standard methods (20). Purified plasmid DNAs were sequenced by using Sequenase (U.S. Biochemical Corp.) and the conditions recommended for sequencing double-stranded DNA.

RNase protection assay. RNase protection assays used conditions described by Veillette et al. (35). For each assay, 0.5 μg of poly(A)$^+$ RNA was used. C-*SRC* [α-^{32}P]UTP (Du Pont, NEN Research Products)-labeled antisense transcripts were generated from the PCR products cloned into pGEM4Z (Promega Biotec). Plasmid DNA was linearized with *Hin*dIII, and the antisense RNA was transcribed with SP6 polymerase. Each transcript contained 60 nucleotides from the 3′ end of exon 3 and 85 nucleotides from the 5′ end of exon 4; exons NI-NII (transcript 1) or exon NI (transcript 2) was inserted at the junction between exons 3 and 4. Transcript 3 contained sequences only from exons 3 and 4. Each antisense transcript contained 28 nucleotides derived from the vector sequence in addition to the indicated C-*SRC* sequences; the vector sequences are not protected from RNase A digestion in the RNase protection assay. Labeled size markers were generated by filling in the recessed ends of *Hpa*II-digested pUC19 DNA with the Klenow fragment of DNA polymerase I in the presence of [α-^{32}P]dCTP.

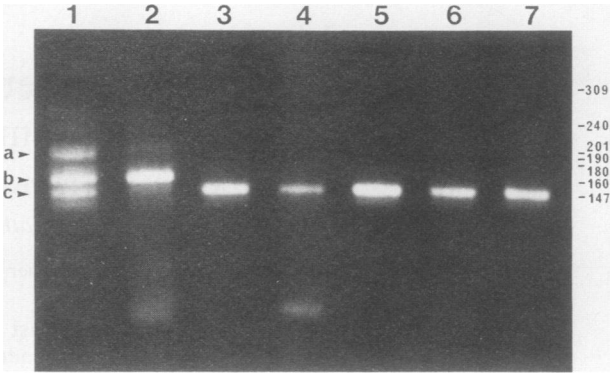

FIG. 1. PCR amplifications of adult and fetal human C-*SRC* cDNAs generated from RNA isolated from various human tissues. The arrows marked a, b, and c mark the positions of the three major PCR products. Lanes contain 5% of the amplification reaction with RNA extracted from the following sources: lane 1, adult brain; 2, fetal brain; 3, adult lung; 4, fetal lung; 5, adult liver; 6, fetal liver; 7, placenta. Locations of markers are indicated on the right.

RESULTS

Identification of human neuron-specific C-*SRC* exons. During the course of experiments which confirmed the presence of a neuron-specific alternatively spliced human C-*SRC* mRNA in human brain (25), analogous to the avian and murine neuronal c-*src* cDNAs previously described (17, 21), we observed species of C-*SRC*-related PCR products in human brain-derived cDNAs which were not present in cDNAs derived from human lung, liver, or placental tissues (Fig. 1, bands a and b). Analysis of the PCR products from adult brain tissue (Fig. 1, lane 1) revealed the presence of other faint bands in addition to the major products represented by bands a, b, and c; it is not clear whether these represent additional rare transcripts. However, similar analyses of PCR products derived from human fetal brain tissue (lane 2) and from murine adult brain tissue (data not shown) failed to reveal similar higher-molecular-weight bands.

For these experiments C-*SRC* cDNAs were PCR amplified in the region spanning exons 3 and 4 from human brain, liver, lung, and placenta RNA and the cDNAs were molecularly cloned. Initial screening of cDNA clones from adult brain tissue showed that 14 contained a ca. 200-nucleotide (nt) insert, 11 contained a ca. 170-nt insert, and 13 contained a ca. 150-nt insert. Molecular clones have not been isolated from products represented by the fainter bands seen in Fig. 1, lane 1. Nucleotide sequences were determined for three clones from each size class. Screening of insert-containing cDNA clones from fetal brain revealed 6 containing a ca. 200-nt insert, 34 containing a ca. 170-nt insert, and 13 containing a ca. 150-nt insert. Nucleotide sequences were determined for two clones containing the ca. 200-nt insert and for one clone from each of the other two size classes. Clones containing the ca. 150-nt insert contained sequences identical to the previously reported human nonneuronal or fibroblast C-*SRC* sequences (33), a second species was found to harbor an 18-nt insertion (the NI exon) with the capacity to encode the same six amino acids as the avian and murine neuronal c-*src* cDNAs previously characterized (17,

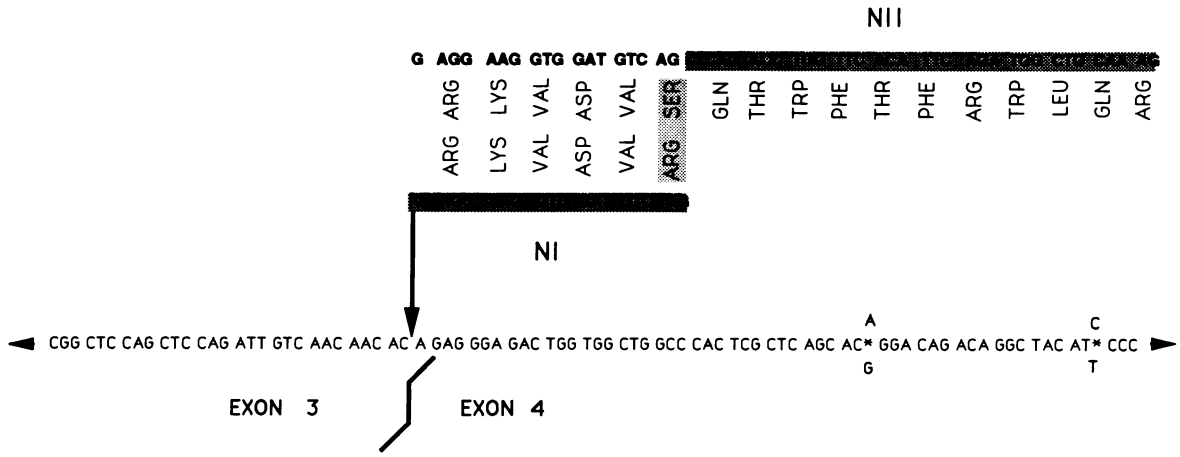

FIG. 2. Sequences of the human neuronal C-*SRC* exons. The DNA sequence and predicted amino acid sequence are presented for the two types of neuron-specific cDNA clones isolated in these experiments. The top line shows the neuron-specific sequence of molecular clones containing the NI and NII exons; the NII exon is shaded. The corresponding amino acids are indicated below the nucleotide sequence. The middle line shows the neuron-specific nucleotide sequence present in molecular clones containing the NI exon (shaded), and above it is shown the corresponding amino acid sequence. The shaded amino acids (Arg and Ser) indicate the alternative codons created by the presence or absence of the splice junction between exons NI and NII. The bottom line shows part of the nucleotide sequence from exons 3 and 4 and also indicates the site of insertion of the neuron-specific exons at the exon 3/4 junction. Within exon 4 are two sites showing nucleotide polymorphism among individuals; the amino acid sequence is unaffected by these differences.

21), and the third species harbored a 51-nt insertion between the nucleotides corresponding to C-*SRC* exons 3 and 4. This 51-nt sequence was determined to contain the 18-nt NI exon coupled to an additional 33-nt sequence which represents a previously unknown C-*SRC* exon (the NII exon) (Fig. 2). Interestingly, the splicing event which joins the NI and NII exons produces a change in codon 6 of the NI exon from AGA (arginine) to AGC (serine), thereby creating a potentially unique serine phosphorylation site (Fig. 2). Analysis of the PCR products from fetal brain tissue revealed that cDNA clones possessing the NI exon were most abundant, followed by clones representing the nonneuronal splice and less frequently clones containing the NI-plus-NII 51-nt insertion. Clones isolated from PCR-amplified cDNAs derived from fetal or adult lung, liver, and placenta tissue were all found to represent exclusively the nonneuronal species of C-*SRC* transcripts.

Expression of C-*SRC* RNA in adult and fetal human brain tissue. To compare relative levels of expression of the various C-*SRC* transcripts, we performed RNase protection assays (Fig. 3). [α-^{32}P]UTP was incorporated into in vitro-transcribed antisense RNAs generated from the three major cloned cDNAs, the NI-plus-NII clone, the NI clone, and the C-*SRC* clone (Fig. 3). These antisense transcripts were hybridized to fetal and adult liver and brain poly(A)$^+$ RNAs, the hybrid molecules were digested with RNase A, and the RNase A-resistant fragments were resolved on a denaturing gel. Resistant fragments were detected for all three antisense transcripts in poly(A)$^+$ RNA from both adult and fetal brain tissue, whereas protected fragments were detected only for the C-*SRC* antisense transcript in the liver poly(A)$^+$ RNAs. Analysis of the relative intensities shows that transcripts containing the NI-plus-NII exons are expressed at similar levels in adult and fetal brain tissue, whereas the C-*SRC* transcripts and transcripts containing the NI exon are expressed at significantly higher levels in fetal brain. In addition to the elevated levels of C-*SRC* transcripts in fetal brain tissue, expression of the nonneuronal form of C-*SRC* RNA was also higher in fetal liver than in adult liver tissue. These analyses of RNA expression indicate that there is developmental regulation of both neuronal and nonneuronal forms of C-*SRC* RNA.

Extended exposure of the autoradiogram from the RNase protection experiment (data not shown) revealed the presence of an additional RNase-resistant fragment resulting from digestion of hybrid molecules formed between fetal brain RNA (but not adult brain RNA) and the antisense transcript containing both the NI and NII exons. This 118-nt protected RNA species may represent a transcript containing the NII but not the NI exon, which would be expected if exon NII (33 nt) and exon 4 (85 nt) were protected as a single fragment while exon 3 was not. However, analysis of 30 NII-containing molecular clones from fetal brain tissue failed to identify any which contained the NII exon exclusively. This result suggests that the exon 3-exon NII splicing event is rare and that the 118-nt protected fragment may represent RNA which utilizes a splice donor other than that in exon 3. Such a splice is predicted to generate defective C-*SRC* RNAs, since an exon 2-exon NII splice would result in frameshifting and exon 1 represents a noncoding exon (32, 37). However, it is possible that exons upstream of exon 1 are spliced to the NII exon to generate a functional transcript (37).

Localization of the NI and NII exons between C-*SRC* exons 3 and 4. Within human genomic DNA, the intron between exons 3 and 4 is approximately 8,000 nt (33). The relative locations of the NI and NII exons within this region were determined by using PCR amplification of genomic DNA isolated from placenta. Oligonucleotide primers representing sequences from exons 3, 4, NI, and NII were synthesized and used in the following pairs for PCR amplification of genomic DNA: exons 3 and 4, exons 3 and NI, exons NI and NII, exons NII and 4, exons 3 and NII, and exons NI and 4. Products were detected and cloned only from the reactions with oligonucleotide primers from exons 3 and NI (a ca. 390-nt fragment) and from exons NII and 4 (a ca. 1,000-nt fragment) (Fig. 4). Other combinations of primers yielded no specific PCR products. Sequence analysis of these cloned fragments confirmed the presence of exon sequences and consensus boundary sequences. The splice junctions are as

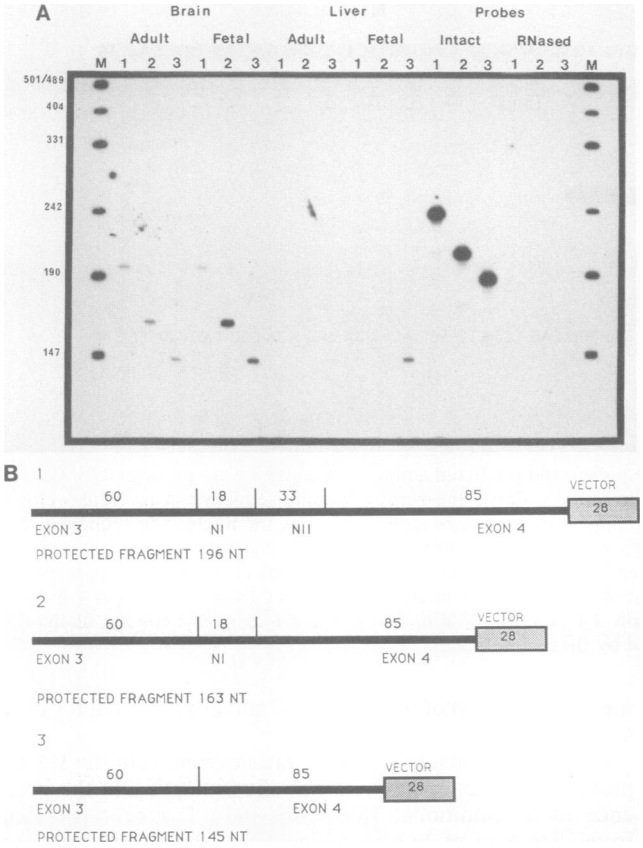

FIG. 3. Analysis of C-*SRC* RNA expression by RNase protection assays. For each assay, 0.5 μg of adult or fetal poly(A)$^+$ RNA from brain or liver tissue was hybridized to C-*SRC* [α-^{32}P]UTP-labeled antisense transcripts. The hybrid molecules were subjected to RNase A digestion, and the protected fragments were resolved on a denaturing gel. Antisense transcript 1 contains exons NI and NII and portions of exons 3 and 4; antisense transcript 2 contains exon NI and portions of exons 3 and 4; antisense transcript 3 contains portions of exons 3 and 4 (see panel B). (A) RNase protection assay. Lanes: 1, antisense transcript 1; 2, antisense transcript 2; 3, antisense transcript 3. The sources of the RNA used for each hybridization are indicated above each panel. The panel labeled Probes contains intact antisense transcripts and RNase A-digested antisense transcripts. M, Marker lanes containing pUC19 DNA digested with *Hpa*II and the ends filled in with [α-^{32}P]dCTP by using the Klenow fragment of DNA polymerase I. (B) Schematic diagram of the antisense transcripts 1, 2, and 3 used for the RNase protection assays. Each antisense transcript contains the indicated exon sequences in addition to 28 nt of vector sequence; the vector sequences are not protected from RNase A digestion in the RNase protection assay.

follows: exon 3-intron 3, ...CAACAACAC/GTGAGT...; intron 3-exon NI, ...CCTTAG/GAGGAA...; exon NII-intron NII, ...CTGCAAAG/GTAC...; intron NII-exon 4, ...TGCT CAG/AGAGGG.... On the basis of these results, a model can be derived for the genetic organization of the C-*SRC* gene in the region between exons 3 and 4: the NI exon lies approximately 390 nt downstream from exon 3, whereas the NII exon is located approximately 1,000 nucleotides upstream from exon 4, with about 6,500 nucleotides between the NI and NII exons (Fig. 4).

DISCUSSION

The results presented in this paper demonstrate the presence of a second neuron-specific C-*SRC* exon (NII) in RNA from adult and fetal human brain tissue. Splicing of this NII exon to the previously described NI exon leads to alteration in the codon for the sixth amino acid in exon NI from one coding for arginine to one coding for serine. Analyses of the C-*SRC* transcripts showed that RNA species containing both NI and NII exons are expressed at similar levels in adult and fetal brain tissue, whereas the nonneuronal form of C-*SRC* RNA and the neuronal form containing only the NI exon are expressed at significantly higher levels in fetal brain tissue.

To extend these observations to other species, we have recently analyzed both mouse and chicken c-*src* exon 3/4 regions by using both brain-derived poly(A)$^+$ RNA and genomic DNA. From mouse brain tissue, three types of cDNA clones have been isolated: a nonneuronal type, one containing the NI exon, and one containing the NI and NII exons (data not shown). These murine neuron-specific exons are identical in nucleotide sequence to the human exons. Hence, the splicing event joining exons NI and NII again creates a codon for a novel serine residue from one encoding arginine. The conservation of this splicing pattern between two species suggests that this alternatively spliced RNA is functionally important in neuronal physiology. Additionally, preliminary results from PCR mapping experiments of the genomic DNA indicate that the relative locations of the NI and NII exons within the exon 3/4 region are similar to those determined for the human gene. The NI exon is ca. 300 nt downstream from exon 3, and the NII exon is ca. 900 nt upstream from exon 4.

In contrast, only two types of avian cDNA clones have been isolated from 86 clones which have been analyzed; these correspond to the nonneuronal form and to one containing the NI exon. The entire exon 3/4 region has been amplified by PCR from genomic chicken DNA, but the product has not yet been analyzed. Sequence analysis is needed to determine whether NII sequences are present in chicken DNA or whether the NII exon is present only in mammalian species. Comparison of the intron sequences among all three species may reveal conserved regions important in neuronal alternative splicing of c-*src* transcripts.

Two human retinoblastoma lines, Y79 and WERI, also produce only the nonneuronal and the exon NI-containing forms of C-*SRC* RNA (data not shown). Thus, it is possible that expression of the NII exon is confined to cells with a more neuronally differentiated phenotype. The murine embryonal carcinoma cell line p19S1801A1 can be induced by retinoic acid to undergo neuronal differentiation (10, 18) and is presently being analyzed to determine whether the state of differentiation affects the types of c-*src* expressed.

Different alternative splicing patterns have been described for a variety of genes, including those which are neuron specific (1, 4, 14, 31). Alternative splicing is a method of generating protein isoform diversity at the posttranscriptional level. Although examples of alternative splicing are present in many cell types, it appears to be particularly advantageous as a method of isoform switching in terminally differentiated cells, such as neurons, which no longer replicate their DNA. Such cells may be constrained in promoter usage because of DNA methylation and chromatin conformation.

Within the *SRC* family, examples of alternative splicing have been documented within the region encoding the catalytic domain (9) and within the region believed to be involved in substrate interactions (17, 21, 25). *FYN*, which is closely related to *SRC*, displays tissue-specific mutually exclusive splicing of alternative seventh exons. Thymocytes, splenocytes, and some hematolymphoid cell lines use a different

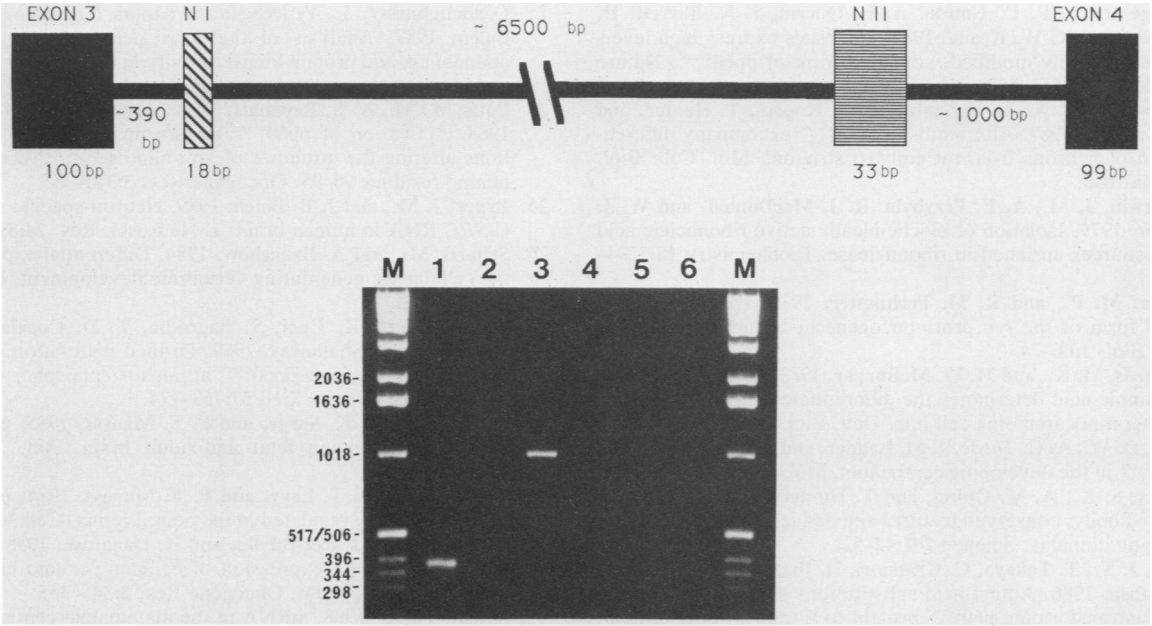

FIG. 4. C-*SRC* neuronal exons in human genomic DNA. At the top is a model of the exon 3-exon 4 region of the C-*SRC* gene in genomic human DNA. The model is based on the sizes of the fragments generated in PCR amplification of human placental DNA. The NI exon is ca. 390 nt from the end of exon 3, and the NII exon is ca. 1,000 nt from exon 4, with ca. 6,500 nt between exons NI and NII. Below the diagram is the PCR amplification of genomic DNA, using pairs of oligonucleotide primers representative of C-*SRC* exons 3, NI, NII, and 4. Lanes 1 to 6 contain amplification reaction products resulting from priming the reactions with pairs of oligonucleotides representing sequences from the following exons: lane 1, exons 3 and NI; lane 2, exons NI and NII; lane 3, exons NII and 4; lane 4, exons 3 and NII; lane 5, exons NI and 4; lane 6, exons 3 and 4. M, Marker lanes containing the 1-kb ladder (Bethesda Research Laboratories, Inc.). Reaction products from lanes 1 and 3 were molecularly cloned for nucleotide sequence analysis (data not shown).

exon 7 than do other tested cell types (9). Exon 7 of $p60^{fyn}$ encodes the ATP-binding site. The hematopoietic form of $p60^{fyn}$ contains an ATP-binding site which differs from the consensus sequence present in the tyrosine protein kinases; however, this alternative sequence is present in another protein kinase, the yeast protein serine kinase Kin 28 (12). This suggests that the hematopoietic isoform has kinase activity but that its activity may be significantly altered. Other members of the *SRC* family have not yet been examined for similar alternative isoforms affecting the catalytic domain.

The regulatory domain of members of the *src* family includes regions encoded by exons 3 and 4. Previous examination of this region of C-*SRC*, *FYN*, and C-*YES* revealed that only C-*SRC* transcripts contained neuron-specific alternative exons, although the three genes are highly homologous within the exon 3/4 region and all three are expressed at significant levels in neural tissues (25). That changes within this region modulate the regulation of *src* activity has been shown by the effects of insertion of the NI exon into avian c-*src* (16) and by the effects of mutations within exon 3 (13, 24).

In contrast to the mutually exclusive splicing pattern observed for exon 7 of $p60^{fyn}$, the *src* splicing pattern can be described as incremental combinatorial splicing. In neural tissues, neuron-specific *src* transcripts contain the NI exon, which may or may not be spliced to the NII exon. From our data it appears that NII is rarely if ever used in the absence of NI. The reason for this is not clear, since an exon 3-exon NII splice would not alter the reading frame for C-*SRC*, although it would result in insertion of an 11-amino-acid peptide which would be preceded by threonine 117 (encoded by exon 3) instead of a serine residue (encoded by the codon created by the NI-NII splice). No detectable phosphorylated threonine 117 residues have been observed in either resting or mitotic fibroblasts (27). Further investigations are necessary to evaluate the sequence requirements of this preferential splicing pattern observed in neuronal C-*SRC* RNA.

ACKNOWLEDGMENTS

This study was supported by a fellowship from the Leukemia Society of America (to J.P.).

We thank our colleagues in the Laboratory of Tumor Virus Biology for critical review of this manuscript and J. Byrne for preparation of the oligonucleotides.

ADDENDUM IN PROOF

The nucleotide sequence of exons NI and NII has been submitted to GenBank.

LITERATURE CITED

1. **Andreadis, A., M. E. Gallego, and B. Nadal-Ginard.** 1987. Generation of protein isoform diversity by alternative splicing: mechanistic and biological implications. Annu. Rev. Cell Biol. **3**:207–242.
2. **Blin, N., and D. W. Stafford.** 1976. A general method for isolation of high molecular weight DNA from eukaryotes. Nucleic Acids Res. **3**:2303–2309.
3. **Bolen, J. B., N. Rosen, and M. A. Israel.** 1985. Increased $pp60^{c\text{-}src}$ tyrosyl kinase activity in human neuroblastomas is associated with amino-terminal tyrosine phosphorylation of the *src* gene product. Proc. Natl. Acad. Sci. USA **82**:7275–7279.
4. **Breitbart, R. E., A. Andreadis, and B. Nadal-Ginard.** 1987. Alternative splicing: a ubiquitous mechanism for the generation of multiple protein isoforms from single genes. Annu. Rev. Biochem. **56**:467–495.
5. **Brugge, J. S., P. Cotton, A. Lustig, W. Yonemoto, L. Lipsich, P. Coussens, J. N. Barrett, D. Nonner, and R. W. Keane.** 1987. Characterization of the altered form of the c-*src* gene product in neuronal cells. Genes Dev. **1**:287–296.

6. **Brugge, J. S., P. C. Cotton, A. E. Queral, J. N. Barrett, D. Nonner, and R. W. Keane.** 1985. Neurones express high levels of a structurally modified, activated form of pp60$^{c\text{-}src}$. Nature (London) **316**:554–557.
7. **Cartwright, C. A., R. Simantov, P. L. Kaplan, T. Hunter, and W. Eckhart.** 1987. Alterations in pp60$^{c\text{-}src}$ accompany differentiation of neurons from rat embryo striatum. Mol. Cell. Biol. **7**:1830–1840.
8. **Chirgwin, J. M., A. E. Przybyla, R. J. MacDonald, and W. J. Rutter.** 1979. Isolation of biochemically active ribonucleic acid from sources enriched in ribonuclease. Biochemistry **18**:5294–5299.
9. **Cooke, M. P., and R. M. Perlmutter.** 1989. Expression of a novel form of the *fyn* proto-oncogene in hematopoietic cells. New Biol. **1**:66–74.
10. **Edwards, M. K., and M. W. McBurney.** 1983. The concentration of retinoic acid determines the differentiated cell types formed by a teratinocarcinoma cell line. Dev. Biol. **98**:187–191.
11. **Fults, D. W., A. C. Towle, J. M. Lauder, and P. F. Maness.** 1985. pp60$^{c\text{-}src}$ in the developing cerebellum. Mol. Cell. Biol. **5**:27–32.
12. **Hanks, S. K., A. M. Quinn, and T. Hunter.** 1988. The protein kinase family: conserved features and deduced phylogeny of the catalytic domains. Science **241**:42–52.
13. **Kato, J. Y., T. Takeya, C. Grandori, H. Iba, J. B. Levy, and H. Hanafusa.** 1986. Amino acid substitutions sufficient to convert the nontransforming p60$^{c\text{-}src}$ protein to a transforming protein. Mol. Cell. Biol. **6**:4155–4160.
14. **Leff, S. E., M. G. Rosenfeld, and R. M. Evans.** 1986. Complex transcriptional units: diversity in gene expression by alternative RNA processing. Annu. Rev. Biochem. **55**:1091–1117.
15. **Levy, B. T., L. K. Sorge, A. Meymandi, and P. F. Maness.** 1984. pp60$^{c\text{-}src}$ kinase is in chick and human embryonic tissues. Dev. Biol. **104**:9–17.
16. **Levy, J. B., and J. S. Brugge.** 1989. Biological and biochemical properties of the c-*src*$^{+}$ gene product overexpressed in chicken embryo fibroblasts. Mol. Cell. Biol. **9**:3332–3341.
17. **Levy, J. B., T. Dorai, L. H. Wang, and J. S. Brugge.** 1987. The structurally distinct form of pp60$^{c\text{-}src}$ detected in neuronal cells is encoded by a unique c-*src* mRNA. Mol. Cell. Biol. **7**: 4142–4145.
18. **Lynch, S. A., J. S. Brugge, and J. M. Levine.** 1986. Induction of altered c-*src* product during neural differentiation of embryonal carcinoma cells. Science **234**:873–876.
19. **Maness, P. F., M. Aubry, C. G. Shores, L. Frame, and K. H. Pfenninger.** 1988. C-*src* gene product in developing rat brain is enriched in nerve growth cone membranes. Proc. Natl. Acad. Sci. USA **85**:5001–5005.
20. **Maniatis, T., E. F. Fritsch, and J. Sambrook.** 1982. Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
21. **Martinez, R., B. Mathey-Prevot, A. Bernards, and D. Baltimore.** 1987. Neuronal pp60$^{c\text{-}src}$ contains a six-amino acid insertion relative to its non-neuronal counterpart. Science **237**:411–415.
22. **Mellstrom, K., C. Bjelfman, U. Hammerling, and S. Pahlman.** 1987. Expression of c-*src* in cultured human neuroblastoma and small-cell lung carcinoma cell lines correlates with neurocrine differentiation. Mol. Cell. Biol. **7**:4178–4184.
23. **O'Shaughnessy, J., V. DeSeau, S. Amini, N. Rosen, and J. B. Bolen.** 1987. Analysis of the c-*src* gene product structure, abundance, and protein kinase activity in human neuroblastoma and glioblastoma cells. Oncogene Res. **2**:1–18.
24. **Potts, W. M., A. B. Reynolds, T. J. Lansing, and J. T. Parsons.** 1988. Activation of pp60$^{c\text{-}src}$ transforming potential by mutations altering the structure of an amino terminal domain containing residues 90–95. Oncogene Res. **3**:343–355.
25. **Pyper, J. M., and J. B. Bolen.** 1989. Neuron-specific splicing of C-*SRC* RNA in human brain. J. Neurosci. Res. **24**:89–96.
26. **Schartl, M., and A. Barnekow.** 1984. Differential expression of the cellular *src* gene during vertebrate development. Dev. Biol. **105**:415–422.
27. **Shenoy, S., J. K. Choi, S. Bagrodia, T. D. Copeland, J. L. Maller, and D. Shalloway.** 1989. Purified maturation promoting factor phosphorylates pp60$^{c\text{-}src}$ at the sites phosphorylated during fibroblast mitosis. Cell **57**:763–774.
28. **Sorge, J. P., L. K. Sorge, and P. F. Maness.** 1985. pp60$^{c\text{-}src}$ is expressed in human fetal and adult brain. Am. J. Pathol. **119**:151–157.
29. **Sorge, L. K., B. T. Levy, and P. F. Maness.** 1984. pp60$^{c\text{-}src}$ is developmentally regulated in the neural retina. Cell **36**:249–257.
30. **Sudol, M., A. Alvarez-Buylla, and H. Hanafusa.** 1988. Differential developmental expression of cellular *yes* and cellular *src* proteins in cerebellum. Oncogene Res. **2**:345–355.
31. **Sutcliffe, J. G.** 1988. mRNA in the mammalian central nervous system. Annu. Rev. Neurosci. **11**:157–198.
32. **Takeya, T., and H. Hanafusa.** 1983. Structure and sequence of the cellular gene homologous to the RSV *src* gene and the mechanism for generating the transforming virus. Cell **32**: 881–890.
33. **Tanaka, A., C. P. Gibbs, R. R. Arthur, S. K. Anderson, H. J. Kung, and D. J. Fujita.** 1987. DNA sequence encoding the amino-terminal region of the human c-*src* protein: implications of sequence divergence among *src*-type kinase oncogenes. Mol. Cell. Biol. **7**:1978–1983.
34. **Veillette, A., and J. B. Bolen.** 1989. *Src*-related protein tyrosine kinases, p. 121–142. *In* C. Benz and E. Liu (ed.), Oncogenes. Kluwer Academic Publishers, Norwell, Mass.
35. **Veillette, A., F. Foss, E. Sausville, J. B. Bolen, and N. Rosen.** 1987. Expression of the *Ick* tyrosine kinase gene in human colon carcinoma and other non-lymphoid tumor cell lines. Oncogene Res. **1**:357–374.
36. **Veillette, A., J. O'Shaughnessy, I. D. Horak, M. A. Israel, D. Yee, N. Rosen, D. J. Fujita, H. J. Kung, J. L. Biedler, and J. B. Bolen.** 1989. Coordinate alteration of pp60$^{c\text{-}src}$ abundance and c-*src* RNA expression in human neuroblastoma variants. Oncogene **4**:421–427.
37. **Wang, L. H., S. Iijima, T. Dorai, and B. Lin.** 1987. Regulation of the expression of proto-oncogene c-*src* by alternative RNA splicing in chicken skeletal muscle. Oncogene Res. **1**:43–59.
38. **Wiestler, O. D., and G. Walter.** 1988. Developmental expression of two forms of pp60$^{c\text{-}src}$ in mouse brain. Mol. Cell. Biol. **8**:502–504.
39. **Yang, X., and G. Walter.** 1988. Specific kinase activity and phosphorylation state of pp60$^{c\text{-}src}$ from neuroblastomas and fibroblasts. Oncogene **3**:237–244.