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The Structurally Distinct Form of pp60$^{c\text{-}src}$ Detected in Neuronal Cells Is Encoded by a Unique c-*src* mRNA

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A cellular *src* (c-*src*) cDNA clone was isolated from a chicken embryonic brain cDNA library and characterized by DNA sequence analysis. Comparison with the published sequence of a chicken genomic c-*src* clone indicated that the brain cDNA clone contained an 18-base-pair insertion located between exons 3 and 4 of the c-*src* gene. The six amino acids encoded by the insertion caused an alteration in the electrophoretic mobility of the c-*src* gene product similar to that of the structurally distinct form of the *src* protein detected in neuronal cultures.

Cellular proto-oncogenes, the homologs of retroviral transforming genes, have been implicated in the regulation of normal cellular processes such as proliferation and differentiation. One widely studied proto-oncogene, the cellular *src* gene (c-*src*), encodes a phosphoprotein of 60,000 daltons (pp60$^{c\text{-}src}$). This cellular protein, like its viral counterpart, possesses a tyrosine-specific protein kinase activity (for reviews, see reference 2 and A. Golden and J. S. Brugge, *in* E. P. Reddy, T. Curran, and A. Skalka, ed., *The Oncogene Handbook*, in press). The exact function of the c-*src* gene product and its role in cellular regulation, however, remains unclear.

Investigations of the expression of pp60$^{c\text{-}src}$ in tissues from avian and mammalian species have indicated that neural tissues, such as brain and neural retina, contain elevated levels of a structurally distinct form of the c-*src* gene product (denoted pp60$^{c\text{-}src+}$; 5, 9, 14, 22). This unique form of the c-*src* protein displays a retarded electrophoretic mobility compared with the pp60$^{c\text{-}src}$ expressed in other tissues (5). There are several lines of evidence which suggest that this unique form of pp60$^{c\text{-}src}$ is specifically expressed in neuronal cells. (i) High levels of pp60$^{c\text{-}src+}$ are detected in neuronal cultures isolated from embryonic rat brains (5). (ii) Neuron-like cells that differentiate from an embryonal carcinoma cell line on treatment with retinoic acid contain high levels of pp60$^{c\text{-}src+}$ (17). (iii) Several cell lines derived from human neuroblastomas also contain pp60$^{c\text{-}src+}$ (3). (iv) Finally, this variant form of the *src* gene product is not detected in any nonneural tissues, cultured astrocytes, glioblastomas, or other types of cultured cells (3, 5; J. Bolen and N. Rosen; K. Mellstrom and S. Pahlman, personal communications).

The c-*src* gene product expressed in cultured neurons can also be distinguished from the pp60$^{c\text{-}src}$ expressed in cultured astrocytes and nonneural tissues by two other criteria. First, the *src* gene product extracted from neurons displays a higher specific activity in immunocomplex protein kinase assays than the pp60$^{c\text{-}src}$ from astrocytes or fibroblasts (5, 6). Second, neuronal pp60$^{c\text{-}src+}$ contains an additional site of serine phosphorylation within the amino-terminal region of the molecule (5, 6). These qualitative and quantitative differences in the c-*src* gene product expressed in neurons could specifically affect the functional activity of this protein in neuronal cells.

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To understand how structural changes in the *src* protein affect its function in neural cells, it is necessary to determine the nature of the structural variation that causes the retarded electrophoretic mobility of pp60$^{c\text{-}src+}$. This structural difference in neural-specific pp60$^{c\text{-}src+}$ has been mapped to the amino-terminal 16,000 daltons of the protein (5, 6). Previous experiments have shown that posttranslational modifications such as the neuron-specific serine phosphorylation are not responsible for the retarded electrophoretic mobility of pp60$^{c\text{-}src+}$ on polyacrylamide gels (4). However, the evidence that the c-*src* gene product synthesized in vitro by using a rabbit reticulocyte lysate programmed with poly(A)$^+$ RNA from embryonic brain tissue displayed an electrophoretic mobility similar to that of pp60$^{c\text{-}src+}$ suggested that the neural-specific c-*src* protein may be encoded by a uniquely processed c-*src* mRNA (4).

To characterize the c-*src* mRNA transcribed in neural tissues, we constructed a cDNA library using poly(A)$^+$ RNA isolated from the brains of 10-day chicken embryos. Since the c-*src* mRNA in embryonic chick brain tissue is approximately 4 kilobases (kb) in length (19, 25), we first fractionated the poly(A)$^+$ RNA and selected for RNA between 3.5 and 4.5 kb to serve as a template. First-strand cDNA was synthesized by using an avian myeloblastosis virus reverse transcriptase (Life Sciences, Inc., St. Petersburg, Fla.) under conditions recommended by the supplier. The products were converted to double-stranded cDNAs by the method described by Gubler and Hoffman (10). The procedures used to prepare the double-stranded cDNA for ligation to *Eco*RI-digested λgt10 arms (Stratagene Cloning Systems, San Diego, Calif.) have been published by Watson and Jackson (26). Recombinant DNA molecules were packaged into phage particles by using the in vitro packaging extract (Gigapack Gold) provided by Stratagene Cloning Systems. The titer of the library was determined on the host strain BNN102 (28) and was found to contain a total of 3.6×10^7 recombinant plaques.

Phage containing c-*src*-specific cDNA inserts were identified by using the in situ plaque transfer procedures of Benton and Davis (1) and hybridization conditions specified by Thomas (24). The library was screened by using a ^{32}P-labeled, nick-translated *Nco*I-*Bgl*II fragment from the plasmid p5H (15; Fig. 1A). This fragment contains the entire c-*src* coding region, including exons 2 to 12 (without introns). From the initial 600,000 plaques that were screened,

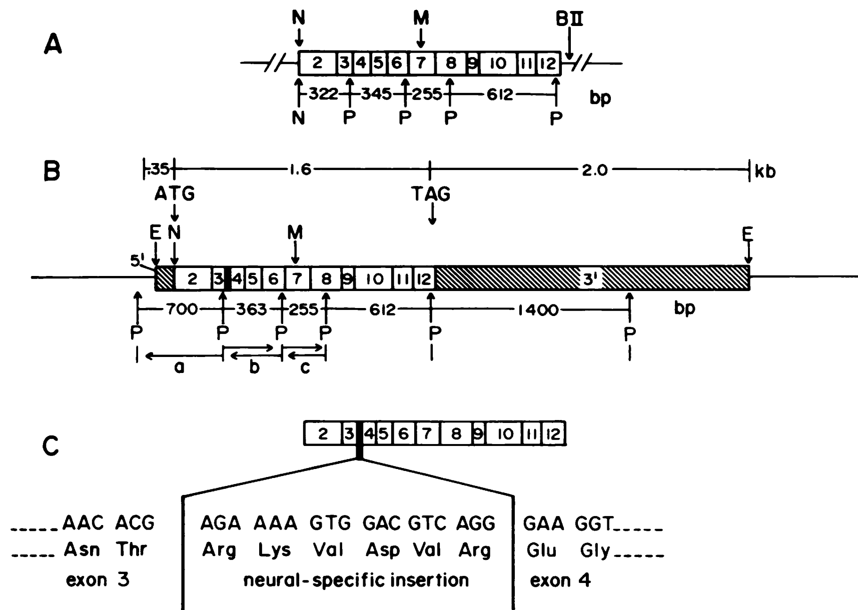

FIG. 1. Characterization of the c-*src* cDNA clone isolated from the chicken cDNA library. (A) Map of the *Nco*I (N) to *Bgl*II (BII) restriction fragment of p5H (15). This fragment, which carries the entire chicken c-*src* coding sequences (exons 2 to 12) was used as a probe to screen the chicken embryonic brain cDNA library (see text for details). (B) Map of the 3.9-kb c-*src* cDNA insert of pB6. The hatched boxes represent 5' and 3' noncoding information, 0.35 and 2.0 kb in length, respectively. The c-*src* coding exons 2 to 12 are shown in the open boxes, with the initiation and termination codons appropriately designated. The solid box represents the 18-bp insertion located between exons 3 and 4. (a, b, and c) *Pst*I (P) fragments of 700, 363, and 255 bp, respectively, that were sequenced (see text for details) according to the strategy indicated by the arrows. (C) Nucleotide sequence of the 18-bp neural-specific insertion located at the junction of exons 3 and 4 of the c-*src* gene (23). The six amino acids encoded by this 18-bp insertion are also designated. Restriction enzyme sites are *Bgl*II (BII), *Eco*RI (E), *Nco*I (N), *Mlu*I (M), and *Pst*I (P).

50 hybridized to the labeled c-*src* probe. The largest cDNA insert was 3.9 kb in length, approximately the same size as the full-length c-*src* mRNA in chicken brain tissue. The *Eco*RI fragment containing the 3.9-kb cDNA was subcloned into the *Eco*RI site in the polylinker region of pUC18 (20). The resulting plasmid, pB6, was further characterized by restriction enzyme analysis. Similar digestions were performed by using the plasmid p5H, which carried a copy of the coding sequence of the c-*src* gene (15). Comparison of the digestion products of pB6 and p5H DNAs revealed that this cDNA clone contained the entire coding region of the c-*src* gene (data not shown), including the *Nco*I site at the initiator codon in exon 2, the first coding exon of c-*src* (23; Fig. 1A and B). An additional 350 base pairs (bp) of 5' noncoding information was contained within this cDNA insert located immediately upstream of the *Nco*I site (Fig. 1B). At the 3' end of the cDNA, a long noncoding region measuring 2.0 kb in length was also detected (Fig. 1B). The presence of the long 3' noncoding sequence in the brain cDNA agrees with the result obtained previously by Northern blot analysis of the 4-kb c-*src* mRNA (25). The only restriction fragment difference between the coding region of the neural cDNA and the p5H c-*src* gene was detected in the *Pst*I fragment b shown in Fig. 1B. The fragment derived from the neural cDNA clone migrated slightly slower than the analogous fragment from p5H, suggesting that additional sequences were present in this region of the cDNA.

Since the structural alteration mapped within the 5' half of the c-*src* gene product (4–6), we sequenced the first 922 nucleotides of our cDNA, which included exons 2 to 7 and a portion of exon 8. Three *Pst*I fragments, labeled a, b, and c in Fig. 1B, were subcloned into the *Pst*I site of M13mp18 (20) and sequenced by the Sanger dideoxy-nucleotide chain termination method (21). When compared with the published sequence of the corresponding region of the chicken c-*src* gene (15, 23), the sequence of the neural cDNA clone was almost identical, except for the presence of an additional 18 bp located between nucleotides 342 and 343 at the junction of exons 3 and 4 of the c-*src* gene (Fig. 1C). This 18-bp insert encodes the six amino acids shown in Fig. 1C.

To determine whether the insert of six amino acids encoded by this cDNA causes a retardation in the electrophoretic mobility of the c-*src* protein similar to that observed for neuronal $pp60^{c\text{-}src+}$, an avian retroviral vector was constructed which carried the 18-bp insertion within the c-*src* gene. The vector used to introduce this 18-bp insertion into the c-*src* gene was the plasmid pRSVc-*src*, which carries the entire Rous sarcoma virus genome with the coding region of the c-*src* gene in place of the viral *src* sequences. An *Nco*I-*Mlu*I fragment containing the first 773 nucleotides of the c-*src* gene was replaced with the corresponding fragment of the neural cDNA clone (see Fig. 1A and B for the location of these sites in the c-*src* gene and the cDNA clone). As described above, this portion of our cDNA clone is identical in sequence to the c-*src* gene except for the 18-bp insertion at nucleotide 342. Transfection of this chimeric construct, designated pRSVc-src^{+}, into chicken embryo fibroblasts (CEF) produces replication-competent virus that spreads through the cultures after transfection. The intact c-*src* protein encoded by the retrovirus is expressed at 15- to 20-fold-higher levels than the endogenous $pp60^{c\text{-}src}$ present in fibroblasts. pRSVc-*src* and pRSVc-src^{+} plasmid DNAs were introduced into CEF by using a calcium phosphate transfection procedure (27). Ten days after transfection, the cultures were labeled with $^{32}P_i$ (1 mCi/ml) for 4 h, and $pp60^{c\text{-}src}$ was immunoprecipitated from cell lysates (16). The

c-*src* gene product produced at high levels in cultures transfected with pRSVc-*src* DNA displayed an electrophoretic mobility similar to that of endogenous pp60$^{c\text{-}src}$ present in nontransfected CEF cultures (Fig. 2A, lanes 1 and 3). However, the abundant c-*src* gene product expressed in pRSVc-*src*$^+$-transfected cultures displayed a retarded electrophoretic mobility similar to the migration of pp60$^{c\text{-}src+}$ detected in neuronal cells (5).

To compare the peptide maps of the *src* proteins from the transfected CEF cultures with pp60$^{c\text{-}src+}$ from neuronal cells, we subjected the proteins shown in Fig. 2A, as well as ^{32}P-labeled pp60$^{c\text{-}src+}$ produced in rat neuronal cultures, to limited digestion with V8 protease by using the method of Cleveland et al. (7). A map showing the location of the four major fragments (labeled V1 to V4) produced in this digestion is shown in Fig. 2B. V1, V3, and V4 are amino-terminal peptides, and the V2 peptide is derived from the carboxy end of the protein (8; Fig. 2B). Figure 2C shows that the V3 and V4 peptides (labeled V3$^+$ and V4$^+$) which are generated by digestion of pp60$^{c\text{-}src}$ produced in pRSVc-*src*$^+$ cultures migrated more slowly than the corresponding peptides derived from pp60 immunoprecipitated from nontransfected cultures or pRSVc-*src*-transfected cultures (compare lanes 1, 2, and 3 in Fig. 2C). The migration of V3$^+$ and V4$^+$ from pRSVc-*src*+-transfected cultures was very similar to the analogous peptides of pp60$^{c\text{-}src+}$ from rat neuronal cells (Fig. 2C, lanes 3 and 4). In addition, a fragment denoted V5, previously detected in V8 protease digestions of neural-specific pp60$^{c\text{-}src+}$ (4) was also present in the *src* protein expressed in pRSVc-*src*+-transfected cultures. These results clearly demonstrate that the extra six amino acids encoded by the neural c-*src* cDNA insert cause an alteration in the mobility of the c-*src* gene product similar to that displayed by pp60$^{c\text{-}src+}$ in neuronal cultures.

The data in this report provide strong evidence that the variant form of pp60$^{c\text{-}src+}$ present in neural tissues is encoded by a unique c-*src* mRNA species. A more definitive conclusion awaits the demonstration that this 18-nucleotide insert is present in c-*src* mRNA from chicken neural tissues. According to the published chicken c-*src* gene sequence, the insertion in the brain c-*src* cDNA is located precisely at a splice junction site. Therefore, it is likely that the neural-specific transcript is the product of an alternately spliced c-*src* mRNA. A rare form of α-A crystallin mRNA has been shown to contain a similar small nucleotide insertion at the junction of two exons (12). The inserted sequences reside within the intron located between the two exons that flank the insertion. If the neuronal c-*src* mRNA employs a similar splicing mechanism to produce pp60$^{c\text{-}src+}$, it is conceivable that intron 3 of the c-*src* gene contains the small 18-bp insertion. Analysis of the nucleotide sequences within 100 nucleotides of the 5′ and 3′ junction regions of intron 3 (kindly provided by H. Hanafusa, The Rockefeller University) did not reveal the presence of this 18-bp insertion. We are currently determining the nucleotide sequence of this 2-kb intron to identify the location of this insert. Recently, it was shown that the c-*src* locus in chick muscle directs the synthesis of an alternately spliced 3-kb c-*src* mRNA that lacks information in the kinase-coding domain of the c-*src* gene (25). Thus, it appears that the c-*src* mRNAs produced in neurons and in muscle have undergone different processing events to produce the two distinct tissue-specific c-*src* transcripts.

A c-*src* cDNA clone isolated from a mouse brain cDNA library was recently shown to contain a similar 18-bp insertion at the junction of exons 3 and 4 (18). By an S_1 nuclease protection assay, it was shown that this insert is specifically

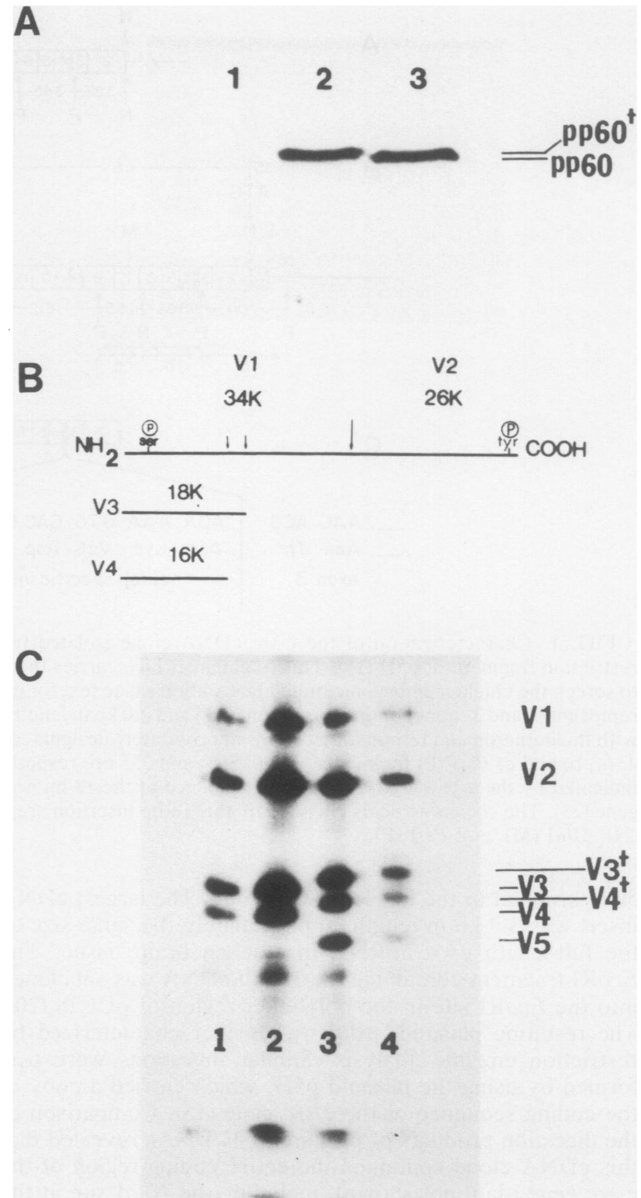

FIG. 2. Analysis of the protein encoded by the c-*src*$^+$ gene. pRSV*src* is a plasmid that carries the entire Rous sarcoma virus genome, including all of the viral structural genes as well as the v-*src* gene (11). The v-*src* gene in pRSV*src* was replaced with the entire coding region of the c-*src* gene of p5H to produce pRSVc-*src* (L. Fox and J. Brugge, unpublished results). pRSVc-*src*$^+$ was constructed by substituting the *Nco*I-*Mlu*I fragment of the c-*src* gene in pRSVc-*src* with the corresponding fragment of pB6 (see text for more details). (A) Analysis of ^{32}P-labeled *src* proteins immunoprecipitated from transfected CEF cultures. ^{32}P-labeled pp60$^{c\text{-}src}$ was immunoprecipitated with monoclonal antibody 327 (16) from mock-transfected (lane 1), pRSVc-*src*$^+$-transfected (lane 2), and pRSVc-*src*-transfected (lane 3) CEF cultures. The immunoprecipitated proteins were analyzed on sodium dodecyl sulfate–7.5% polyacrylamide gels (13) and detected by autoradiography. (B) *Staphylococcus aureus* V8 protease cleavage map of pp60$^{c\text{-}src}$ (8). (C) Partial proteolytic peptide map of ^{32}P-labeled pp60$^{c\text{-}src}$ variants. The pp60$^{c\text{-}src}$ proteins were excised from the gel shown in Fig. 2A and digested with 100 ng of V8 protease (7). A similar digestion was performed on ^{32}P-labeled pp60$^{c\text{-}src+}$ extracted from primary cultures of pure rat neuronal cells, as described previously (5). The products were resolved on sodium dodecyl sulfate–12.5% polyacrylamide gels. Lanes: 1, mock-transfected CEF; 2, pRSVc-*src*-transfected CEF; 3, pRSVc-*src*+-transfected CEF; and 4, rat neuronal cells.

detected in c-*src* mRNA isolated from mouse brain tissue. The insert present in the mouse brain cDNA differs from the chicken brain insert at four nucleotide positions; however, the six amino acids encoded by both the chicken and mouse insertions are identical. Since this structural difference in pp60$^{c\text{-}src+}$ is highly conserved between two divergent species, it would appear to have functional significance. This variation could be important in conferring specificity to the interaction of pp60$^{c\text{-}src+}$ with other neuronal membrane proteins that regulate the tyrosine kinase activity of the enzyme or that function as substrates of pp60$^{c\text{-}src+}$. The expression of high levels of a unique form of the c-*src* tyrosine-specific protein kinase in the postmitotic neuronal cell would suggest a role for this protein in either differentiation or maintenance of the neuronal phenotype. Expression of the neural-specific c-*src* cDNA in primitive neural stem cells will provide valuable information in analyzing the function of this protein in neural tissues.

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