likely to have been derived from circular plasmids contaminating the preparation.

From these experiments we conclude that the *ospA* and *ospB* genes of *B. burgdorferi* are arrayed on linear plasmids and that the linear plasmids have covalently closed termini. The first finding, while not unexpected [genes encoding outer membrane proteins of *B. hermsii* had been found on plasmids of that species (*3*)], further demonstrates the novel arrangement of DNA in this group of bacteria. The second finding is perhaps of greater significance: only among eukaryotic organisms and their viruses have covalently closed ends of DNA been found. Vaccinia virus has been most extensively studied (*17*). Bacteriophages can have linear DNA, too, but known double-stranded forms with free ends, as found in phage λ, or protein-bound 5′ ends, as found in Phi29, are separable along their entire lengths (*18*). It is conceivable, though, that linear plasmids of *Borrelia* species do derive from bacteriophages since viruses have been observed in both *B. burgdorferi* and *B. hermsii* (*2, 19*).

Recognizing the similarity between pox viral termini and the ends of chromosomes, some investigators have referred to the hairpins of poxviruses as telomeres (*20*). This term cannot be applied to ends of the linear molecules found in *Borrelia* species; palindromic sequences conducive to hairpin formation would have to be demonstrated. Nevertheless, the present study reveals in a bacterium a form of DNA that was thought to be unique to eukaryotes.

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8. The method for DNA extraction was adapted from A. J. Robinson, H. B. Younghusband, A. J. D. Bellett, *Virology* **56**, 54 (1973); and *Bethesda Research Laboratories Bulletin* (Bethesda Research Laboratories, Gaithersburg, MD, 1984). A harvest of $5 \times 10^{10}$ cells was suspended in 2.4 ml of TES (50 m*M* tris, *p*H 8.0; 50 m*M* EDTA; 15% w/v sucrose). To the suspension was added 1.2 mg of lysozyme in 0.6 ml of water. After 15 minutes of incubation at 4°C, the following reagents were added to the sample: 3 ml of 1% sodium deoxycholate in TES and 70 μl of diethyl pyrocarbonate (DEP; Sigma). The tube was shaken for 10 minutes. To the lysate was added 2.5 ml of 7.5*M* ammonium acetate, and the resultant precipitate was centrifuged (10,000*g* for 20 minutes at 20°C). The pellet (chromosome-rich fraction) was sequentially treated with ribonuclease A, proteinase K in the presence of SDS, and phenol and chloroform (*3*). The supernatant (plasmid-rich fraction) was filtered through a Millex-GV 0.2-μm filter (Millipore). The nucleic acids in the supernatant were precipitated with isopropanol. The precipitate was suspended in 3 ml of TE (10 m*M* tris, *p*H 7.8; 1 m*M* EDTA) and then treated with ribonuclease A at a final concentration of 0.1 mg/ml (37°C for 30 minutes). DEP (11 μl) was added, and the mixture was shaken for 5 minutes. Residual proteins were removed by precipitation with 7.5*M* ammonium acetate (0.5 ml) and centrifugation (5 minutes in a Beckman Microfuge B). The plasmid-rich DNA in supernatant was recovered by ethanol precipitation and then fractionated by ethidium bromide and cesium chloride gradient centrifugation (70,000 rev/min for 16 hours in a Beckman VTi80 rotor).
9. The DNA was mounted for electron microscopy by means of the Kleinschmidt aqueous technique (*10*). Adenovirus 2 DNA (Bethesda Research Laboratories) was the standard used to calibrate contour-length measurements. Grids were examined in a JEOL 100B electron microscope at 40-kV accelerating voltage. Electron micrographs were taken on Kodak Electron Image plates at a magnification of ×7000. The magnification was calibrated for each set of plates with a grating replica (E. F. Fullam), and contour lengths were measured with a Numonics Graphics calculator interfaced with a Tektronik 4052A computer. The mean plus standard deviation of the measured lengths are given.
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11. DNA molecules of greater than 40-kb apparent size were recovered from agarose gels by electroelution into a salt sink of 3*M* sodium acetate in an IBI model UEA electroeluter. The buffer was 20 m*M* tris (*p*H 8.0), 5 m*M* NaCl, and 0.2 m*M* EDTA, and the elution was carried out for 2 hours at 100 V. DNA to be used in electron microscopy and denaturation studies was not stained with ethidium bromide prior to electroelution. Pipette tips with large bores at their ends were used in all DNA manipulations.
12. Electroeluted 49-kb plasmid DNA at 0.1 mg/ml was incubated with proteinase K (Boehringer Mannheim) at 0.5 mg/ml in TE containing 1% SDS for 30 minutes at 65°C. The mixtures were extracted with a mixture of phenol and chloroform before being precipitated with ethanol. Control samples were treated in the same way except for the absence of protease.
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# Neuronal pp60$^{c\text{-}src}$ Contains a Six–Amino Acid Insertion Relative to Its Non-Neuronal Counterpart


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**Neuronal cells express a pp60$^{c\text{-}src}$ variant that displays an altered electrophoretic mobility and a different V8 peptide pattern relative to pp60$^{c\text{-}src}$ expressed in tissues of non-neuronal origin. To determine whether the neuronal form of pp60$^{c\text{-}src}$ is encoded by a brain-specific messenger RNA, a mouse brain complementary DNA (cDNA) library was screened with a chicken c-*src* probe and a 3.8-kilobase c-*src* cDNA clone was isolated. This clone encodes a 60-kilodalton protein that differs from chicken or human pp60$^{c\text{-}src}$ primarily in having six extra amino acids (Arg-Lys-Val-Asp-Val-Arg) within the $NH_2$-terminal 16 kilodaltons of the molecule. S1 nuclease protection analysis confirmed that brain c-*src* RNA contains an 18-nucleotide insertion at the position of the extra six amino acids. This insertion occurs at a position that corresponds to a splice junction in the chicken and human c-*src* genes. The isolated c-*src* cDNA clone encodes a protein that displays an identical V8 peptide pattern to that observed in pp60$^{c\text{-}src}$ isolated from tissues of neuronal origin.**


THE PROTO-ONCOGENE C-*src* ENcodes a 60-kD tyrosine-specific protein kinase (pp60$^{c\text{-}src}$). Recently, Brugge *et al.* (*1, 2*) showed that neuronal cells express an altered form of pp60$^{c\text{-}src}$ which exhibits a slightly slower electrophoretic migration on sodium dodecyl sulfate (SDS)–polyacrylamide gels relative to pp60$^{c\text{-}src}$ isolated from non-neuronal tissues. They mapped this alteration in a 16-kD $NH_2$-terminal peptide obtained after V8 protease digestion and postulated that this structural alteration resides in the primary amino acid sequence of pp60$^{c\text{-}src}$ (*2*). To examine whether the neuronal form of pp60$^{c\text{-}src}$ [pp60$^{c\text{-}src(+)}$] is encoded by a brain-specific messenger RNA (mRNA), a size-selected


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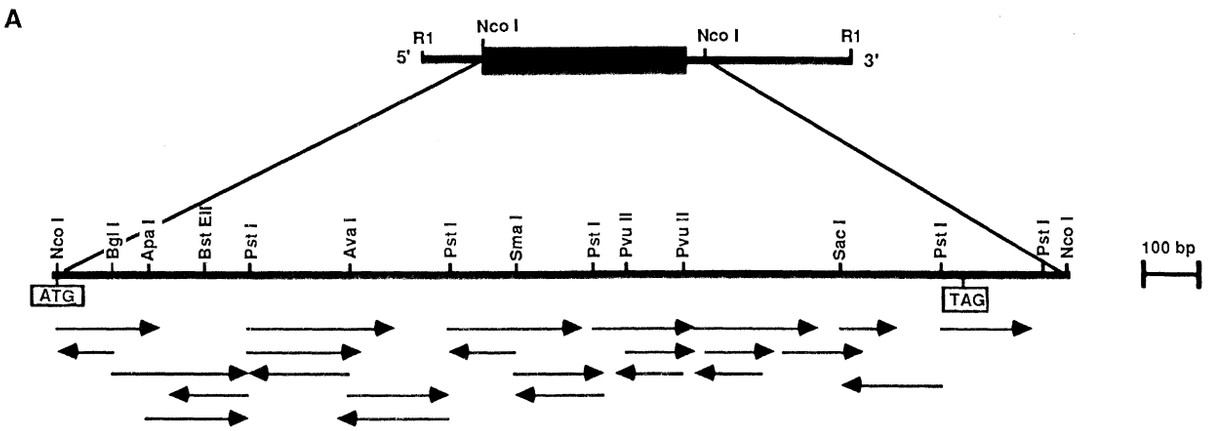
(>2.0 kb) mouse (BALB/c) brain complementary DNA (cDNA) library was screened for c-*src* sequences by means of a chicken c-*src* probe. A screen of $1.5 \times 10^6$ λgt11 recombinants gave 400 positive clones. A clone containing a 3.8-kb insert was selected for further characterization. On the basis of the size of the c-*src* mRNA (*3*), this clone is full-length or nearly full-length. The 3.8-kb Eco RI insert was subcloned into pUC18 and examined by Southern blot hybridization with probes corresponding to the 5′ and 3′ coding region of chicken c-*src*. This analysis revealed that the coding sequence of the 3.8-kb cDNA clone is contained within a 1.8-kb Nco I–Nco I fragment flanked by 0.6 and 1.4 kb of 5′ and 3′ untranslated sequences, respectively (Fig. 1A). The 1.8-kb Nco I–Nco I fragment was subcloned into M13 vectors and sequenced by the chain termination procedure (Fig. 1A). The nucleotide and deduced amino acid sequences are shown in Fig. 1B.

The sequence of mouse pp60$^{c\text{-}src(+)}$ is




B

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    ATG GGC AGC AAC AAG AGC AAG CCC AAG GAC GCC AGC CAG CGG CGC CGC AGC CTG GAG CCC
1   Met Gly Ser Asn Lys Ser Lys Pro Lys Asp Ala Ser Gln Arg Arg Arg Ser Leu Glu Pro

    TCG GAA AAC GTG CAC GGG GCA GGG GGC GCC TTC CCG GCC TCA CAG ACA CCG AGC AAG CCC
21  Ser Glu Asn Val His Gly Ala Gly Gly Ala Phe Pro Ala Ser Gln Thr Pro Ser Lys Pro

    GCC TCC GCC GAC GGC CAC CGC GGG CCC AGC GCC GCC TTC GTG CCG CCC GCG GCC GAG CCC
41  Ala Ser Ala Asp Gly His Arg Gly Pro Ser Ala Ala Phe Val Pro Pro Ala Ala Glu Pro

    AAG CTC TTC GGA GGC TTC AAC TCC TCG GAC ACC GTC ACC TCC CCG CAG AGG GCG GGC GCT
61  Lys Leu Phe Gly Gly Phe Asn Ser Ser Asp Thr Val Thr Ser Pro Gln Arg Ala Gly Ala

    CTG GCA GGT GGG GTG ACC ACC TTT GTG GCC CTC TAT GAC TAT GAG TCA CGG ACA GAG ACT
81  Leu Ala Gly Gly Val Thr Thr Phe Val Ala Leu Tyr Asp Tyr Glu Ser Arg Thr Glu Thr

    GAC CTG TCC TTC AAG AAA GGG GAG CGG CTG CAG ATT GTC AAT AAC ACG AGG AAG GTG GAT
101 Asp Leu Ser Phe Lys Lys Gly Glu Arg Leu Gln Ile Val Asn Asn Thr Arg Lys Val Asp

    GTC AGA GAG GGA GAC TGG TGG CTG GCA CAC TCG CTG AGC ACG GGA CAG ACC GGT TAC ATC
121 Val Arg Glu Gly Asp Trp Trp Leu Ala His Ser Leu Ser Thr Gly Gln Thr Gly Tyr Ile

    CCC AGC AAC TAT GTG GCG CCC TCC GAC TCC ATC CAG GCT GAG GAG TGG TAC TTT GGC AAG
141 Pro Ser Asn Tyr Val Ala Pro Ser Asp Ser Ile Gln Ala Glu Glu Trp Tyr Phe Gly Lys

    ATC ACT AGA CGG GAA TCA GAG CGG CTG CTG CTC AAC GCC GAG AAC CCG AGA GGG ACC TTC
161 Ile Thr Arg Arg Glu Ser Glu Arg Leu Leu Leu Asn Ala Glu Asn Pro Arg Gly Thr Phe

    CTC GTG AGG GAG AGT GAG ACC ACA AAA GGT GCC TAC TGC CTC TCT GTA TCC GAC TTC GAC
181 Leu Val Arg Glu Ser Glu Thr Thr Lys Gly Ala Tyr Cys Leu Ser Val Ser Asp Phe Asp

    AAT GCC AAG GGC CTA AAT GTG AAA CAC TAC AAG ATC CGC AAG CTG GAC AGC GGC GGT TTC
201 Asn Ala Lys Gly Leu Asn Val Lys His Tyr Lys Ile Arg Lys Leu Asp Ser Gly Gly Phe

    TAC ATC ACC TCC CGC ACC CAG TTC AAC AGC CTG CAG CAG CTC GTG GCT TAC TAC TCC AAA
221 Tyr Ile Thr Ser Arg Thr Gln Phe Asn Ser Leu Gln Gln Leu Val Ala Tyr Tyr Ser Lys

    CAT GCT GAT GGC CTG TGT CAC CGC CTC ACT ACC GTA TGT CCC ACA TCC AAG CCT CAG ACC
241 His Ala Asp Gly Leu Cys His Arg Leu Thr Thr Val Cys Pro Thr Ser Lys pro Gln Thr

    CAG GGA TTG GCC AAG GAT GCG TGG GAG ATC CCC CGG GAG TCC CTG CGG CTG GAG GTC AAG
261 Gln Gly Leu Ala Lys Asp Ala Trp Glu Ile Pro Arg Glu Ser Leu Arg Leu Glu Val Lys

    CTG GGC CAG GGT TGC TTC GGA GAG GTG TGG ATG GGG ACC TGG AAC GGC ACC ACG AGG GTT
281 Leu Gly Gln Gly Cys Phe Gly Glu Val Trp Met Gly Thr Trp Asn Gly Thr Thr Arg Val

    GCC ATC AAA ACT CTG AAG CCA GGC ACC ATG TCC CCA GAG GCC TTC CTG CAG GAG GCC CAA
301 Ala Ile Lys Thr Leu Lys Pro Gly Thr Met Ser Pro Glu Ala Phe Leu Gln Glu Ala Gln

    GTC ATG AAG AAA CTG AGG CAC GAG AAA CTG GTG CAG CTG TAT GCT GTG GTG TCG GAA GAA
321 Val Met Lys Lys Leu Arg His Glu Lys Leu Val Gln Leu Tyr Ala Val Val Ser Glu Glu

    CCC ATT TAC ATT GTG ACA GAG TAC ATG AAC AAG GGG AGT CTG CTG GAC TTT CTC AAG GGG
341 Pro Ile Tyr Ile Val Thr Glu Tyr Met Asn Lys Gly Ser Leu Leu Asp Phe Leu Lys Gly

    GAA ACG GGC AAA TAT TTG CGG CTA CCC CAG CTG GTG GAC ATG TCT GCT CAG ATC GCT TCA
361 Glu Thr Gly Lys Tyr Leu Arg Leu Pro Gln Leu Val Asp Met Ser Ala Gln Ile Ala Ser

    GGC ATG GCC TAT GTG GAG CGG ATG AAC TAT GTG CAC CGG GAC CTT CGA GCC GCC AAT ATC
381 Gly Met Ala Tyr Val Glu Arg Met Asn Tyr Val His Arg Asp Leu Arg Ala Ala Asn Ile

    CTA GTA GGG GAG AAC CTG GTG TGC AAA GTG GCC GAC TTT GGG TTG GCC CGG CTC ATA GAA
401 Leu Val Gly Glu Asn Leu Val Cys Lys Val Ala Asp Phe Gly Leu Ala Arg Leu Ile Glu

    GAC AAC GAA TAC ACA GCC CGG CAA GGT GCC AAA TTC CCC ATC AAG TGG ACC GCC CCT GAA
421 Asp Asn Glu Tyr Thr Ala Arg Gln Gly Ala Lys Phe Pro Ile Lys Trp Thr Ala Pro Glu

    GCT GCT CTG TAC GGC AGG TTC ACC ATC AAG TCG GAT GTG TGG TCC TTT GGG ATT CTG CTG
441 Ala Ala Leu Tyr Gly Arg Phe Thr Ile Lys Ser Asp Val Trp Ser Phe Gly Ile Leu Leu

    ACC GAG CTC ACC ACT AAG GGA AGA GTG CCC TAT CCT GGG ATG GTG AAC CGT GAG GTT CTG
461 Thr Glu Leu Thr Thr Lys Gly Arg Val Pro Tyr Pro Gly Met Val Asn Arg Glu Val Leu

    GAC CAG GTG GAG CGG GGC TAC CGG ATG CCT TGT CCC CCC GAG TGC CCC GAG TCC CTG CAT
481 Asp Gln Val Glu Arg Gly Tyr Arg Met Pro Cys Pro Pro Glu Cys Pro Glu Ser Leu His

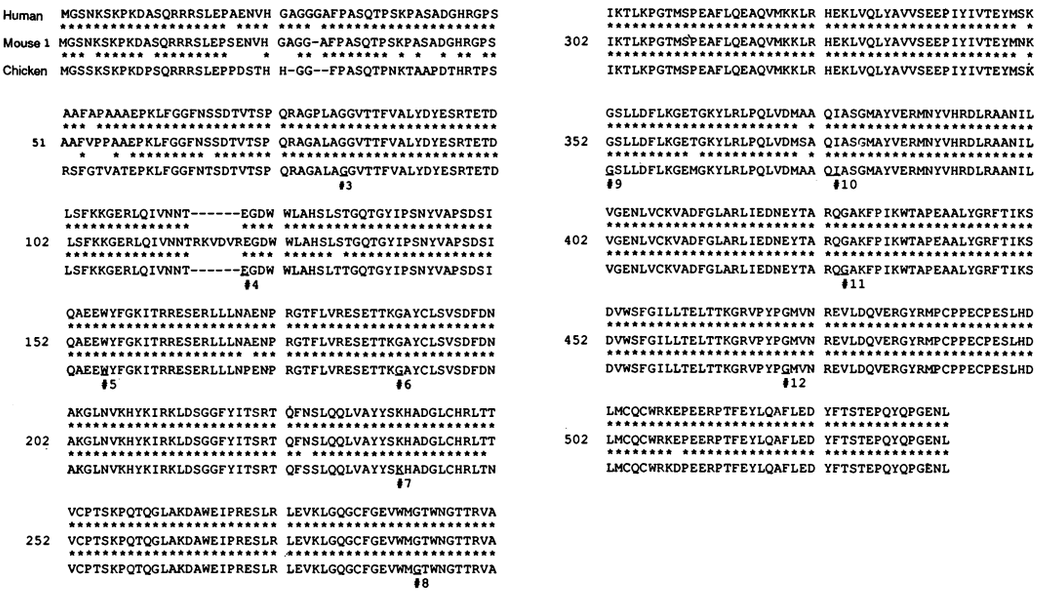
    GAC CTT ATG TGC CAG TGC TGG CGG AAG GAG CCC GAG GAG CGG CCC ACC TTC GAG TAC CTG
501 Asp Leu Met Cys Gln Cys Trp Arg Lys Glu Pro Glu Glu Arg Pro Thr Phe Glu Tyr Leu

    CAG GCC TTC CTG GAA GAC TAC TTT ACG TCC ACT GAG CCA CAG TAC CAG CCC GGG GAG AAC
521 Gln Ala Phe Leu Glu Asp Tyr Phe Thr Ser Thr Glu Pro Gln Tyr Gln Pro Gly Glu Asn

    CTA TAG
541 Leu TER
```

**Fig. 1.** Restriction map, nucleotide sequence, and deduced amino acid sequence of the mouse brain c-*src* cDNA clone. (**A**) Restriction map of 3.8-kb λgt11 c-*src* Eco RI insert and 1.8-kb Nco I–Nco I fragment containing the coding region (dark box). Sequencing strategy with M13 vectors is depicted by arrows. The mouse brain λgt11 cDNA library used in this work was constructed in a fashion similar to that described (*15*). Recombinant phage was plated at a density of 50,000 plaques per 150-mm dish with Y1088 as a host bacterium. Screening was performed as described (*16*) with an oligolabeled DNA probe containing the entire coding sequence of chicken c-*src* [Nco I–Bgl II fragment from p5H (*17*)]. Oligolabeling was performed as described in (*18*). Excess probe was removed by washing filters as follows: once in 2× SSC (0.1% SDS) at room temperature; three times in 2× SSC (0.1% SDS) at 68°C; once in 1× SSC (0.1% SDS) at 68°C and once in 0.2× SSC (0.1% SDS) at 68°C. Filters were exposed on Kodak XAR film with intensifying screens at −70°C. Phage isolated from positive plaques was plaque-purified and phage DNA was prepared as described (*16*). Insert size was determined by Southern blot hybridization of Eco RI–digested λgt11 recombinant phage DNA with chicken c-*src* as a probe. Selected inserts were subcloned into pUC18 for further characterization. The 3.8-kb insert was digested with Nco I and mapped by Southern blot hybridization with probes derived from the 5′ and 3′ ends of the coding region of chicken c-*src* [5′ probe; Nco I–Stu I fragment from p5H (*17*); 3′ probe: Stu I–Bgl II fragment from p5H (*17*)]. Restriction enzyme fragments derived from the 1.8-kb Nco I–Nco I fragment were subcloned into the M13 vectors mp18 and mp19 and sequenced by the chain termination procedure (*19*) with the strategy depicted by the arrows. (**B**) Nucleotide sequence and translation product of mouse brain c-*src*. Insertion in mouse pp60$^{c\text{-}src(+)}$ sequence relative to chicken or human pp60$^{c\text{-}src}$ sequences is underlined.

**Fig. 2.** Comparison of the deduced amino acid sequences of mouse pp60$^{c\text{-}src(+)}$ and chicken and human pp60$^{c\text{-}src}$. Chicken pp60$^{c\text{-}src}$ sequence was taken from Takeya and Hanafusa (*5*). Human pp60$^{c\text{-}src}$ was from Tanaka *et al.* (*6*). Numbers underneath the chicken pp60$^{c\text{-}src}$ sequence indicate exon junctions on the chicken or human c-*src* sequences. Asterisks denote positions of amino acid identity.




nearly identical to that of chicken or human pp60$^{c\text{-}src}$ except for a six–amino acid insertion (Arg-Lys-Val-Asp-Val-Arg) corresponding to residues 117 to 122 in the mouse pp60$^{c\text{-}src(+)}$ sequence (Fig. 2). In addition, mouse pp60$^{c\text{-}src(+)}$ and chicken pp60$^{c\text{-}src}$ show some amino acid differences in the region corresponding to the second exon of chicken c-*src*. These latter sequence differences are most likely species-specific. Mouse pp60$^{c\text{-}src(+)}$ is two amino acids longer than chicken pp60$^{c\text{-}src}$ and one amino acid shorter than human pp60$^{c\text{-}src}$. Alignment of the mouse pp60$^{c\text{-}src(+)}$ with the chicken or human pp60$^{c\text{-}src}$ sequences reveals that the six–amino acid insertion in mouse pp60$^{c\text{-}src(+)}$ occurs precisely at the junction of exons 3 and 4 of the chicken or human pp60$^{c\text{-}src}$ sequences. The possibility that this insertion represents a tissue-specific as opposed to a species-specific difference was next examined.

A DNA probe spanning the sequence that codes for the six–amino acid insertion of mouse pp60$^{c\text{-}src(+)}$ (Fig. 3A, probe 1) was end-labeled and hybridized to total RNA isolated from mouse brain and lung. The hybrid was digested with S1 nuclease and the digestion products were resolved on a sequencing gel. To differentiate the fully protected fragment from the probe, the probe used in this experiment contained pUC18 polylinker sequences at the unlabeled end. Brain RNA gave rise to a fully protected fragment of 197 nucleotides and a partially protected fragment of 158 nucleotides (Fig. 3B). A DNA sequence ladder generated by Maxam-Gilbert sequencing of probe 1 established that the 158-nucleotide-long fragment is protected against S1 nuclease digestion precisely up to the point where the 18-bp insertion in mouse c-*src* ends (*3*). RNA extracted from lung gave rise exclusively to the 158-nucleotide-long partially protected fragment (Fig. 3B). Longer exposure times failed to reveal the fully protected fragment in the lane where lung RNA was analyzed. RNA isolated from other non-brain tissues (kidney, spleen, heart, and testis) gave identical results to those observed with lung RNA (*3*).

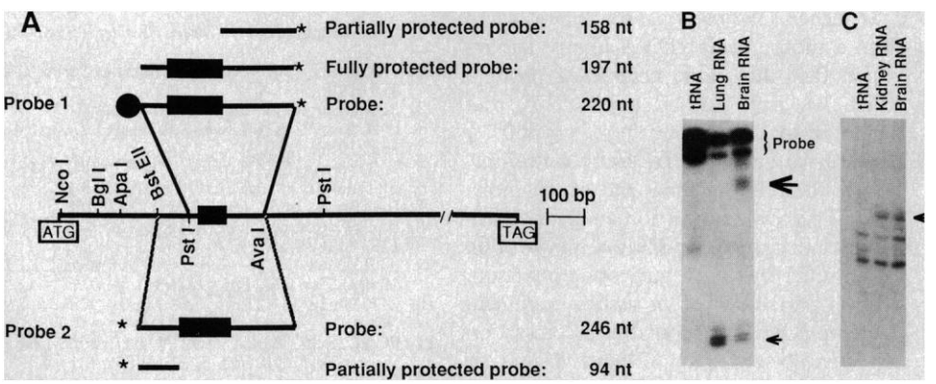



**Fig. 3.** S1 nuclease protection analysis of RNAs from several mouse tissues with brain c-*src* cDNA probes. (**A**) Size and alignment with respect to c-*src* cDNA clone of S1 probes and protected fragments. The insertion sequence, which is represented by the dark box, is not drawn to scale. Solid circle next to probe 1 indicates the position of the polylinker sequence. Asterisk denotes the labeling site; nt, nucleotides. (**B**) Mapping of the 3′ border of the insertion in brain c-*src* mRNA with probe 1 (*20*). Lanes 1 through 3 contain probe hybridized to 30 μg of transfer RNA (lane 1); 30 μg of lung RNA (lane 2), and 30 μg of RNA isolated from brain (lane 3). (**C**) Mapping the 5′ border of the insertion in brain c-*src* mRNA. The protocol was similar to the one described (*20*), except that the probe used was a Bst EII–Ava I fragment from brain c-*src* [probe 2 in (A)]. This probe was 3′ end-labeled at the Bst EII site with the Klenow fragment of DNA polymerase. The hybridization temperature for this probe was 52°C. Lanes 1 through 3 contain probe hybridized to 30 μg of transfer RNA (lane 1), 30 μg of kidney RNA (lane 2) and 30 μg of brain RNA (lane 3). Probes 1 and 2 were sequenced according to the procedure of Maxam and Gilbert (*21*). Arrows on (B and C) point to the position of the fully protected fragment (large arrow) and the partially protected fragments (small arrows). Fragment sizes were determined with end-labeled Hae III–digested ϕX174 DNA.

To define the 5′ boundary of the insertion, a second DNA probe spanning the insertion sequence (Fig. 3A, probe 2) was end-labeled and hybridized to total RNA isolated from mouse brain or kidney. The hybrids were incubated with S1 nuclease and the protected fragments were separated on a sequencing gel. A partially protected 94-nucleotide-long fragment was observed with both types of RNA (Fig. 3C). The fully protected fragment expected with brain RNA would migrate with the full-length probe in this case and thus could not be quantitated. A Maxam-Gilbert sequence ladder derived from probe 2 established that the partial protection of this probe by mouse brain or kidney RNA occurred exactly up to the nucleotide where the insertion starts in mouse c-*src* (*3*).

We next asked whether additional tissue-specific sequence differences existed up-

stream of the 18-nucleotide-long insertion. A probe containing the 250-bp Nco I–Bst EII region from the mouse brain c-*src* clone and upstream polylinker sequences (Fig. 3A) was end-labeled, hybridized to brain or nonbrain RNAs, digested with S1 nuclease and examined on a sequencing gel. Full protection of the *src*-specific sequence of this probe was observed with all RNA species tested, indicating that there is no detectable RNA sequence heterogeneity upstream of the insertion (*3*).

The S1 nuclease data presented above demonstrates that mouse brain has at least two classes of c-*src* RNA. As mentioned earlier, two forms of $pp60^{c\text{-}src}$ have been shown to be expressed in a tissue-specific manner (*1, 2*). To examine which form was encoded by the 3.8-kb mouse brain c-*src* cDNA clone, the 1.8-kb Nco I–Nco I fragment from this clone was subcloned into a retroviral expression vector (see legend to Fig. 4). The resulting construct, pVN1.8, was co-transfected into NIH/3T3 cells in the presence of pSV2Neo. G418-resistant colonies were isolated and tested for the overexpression of $pp60^{c\text{-}src(+)}$. A positive clone was expanded, and cells were labeled with [$^{32}$P]orthophosphate and lysed in detergent-containing buffer. The $pp60^{c\text{-}src(+)}$ was immunoprecipitated from the cell lysates with a monoclonal antibody raised against chicken $pp60^{v\text{-}src}$ (Mab 327) (*4*), and separated on an SDS-polyacrylamide gel. The 60-kD phosphoprotein was detected by autoradiography, excised from the gel, partially digested with V8 protease, and resolved by electrophoresis through an SDS-polyacrylamide gel. Limited V8 proteolysis of $pp60^{c\text{-}src}$ should give rise to four peptides, V1 to V4 (Fig. 4, top). V3 and V4 are known to be derived from V1 (*1, 2*). The bottom part of Fig. 4 shows that peptides V1, V3, and V4 generated by the overproduced $pp60^{c\text{-}src(+)}$ migrated more slowly than the equivalent peptides derived from endogenous $pp60^{c\text{-}src}$ present either in the over-expressing NIH/3T3 cell line (Fig. 4, lane B) or in the nontransfected NIH/3T3 control culture (Fig. 4, lane A). In addition to peptides V1 to V4, $pp60^{c\text{-}src(+)}$ gave rise to at least three low molecular weight V8 peptides which migrated ahead of V4 and were previously observed in V8 digests of $pp60^{c\text{-}src}$ isolated from neuronal cultures (*1, 2*).

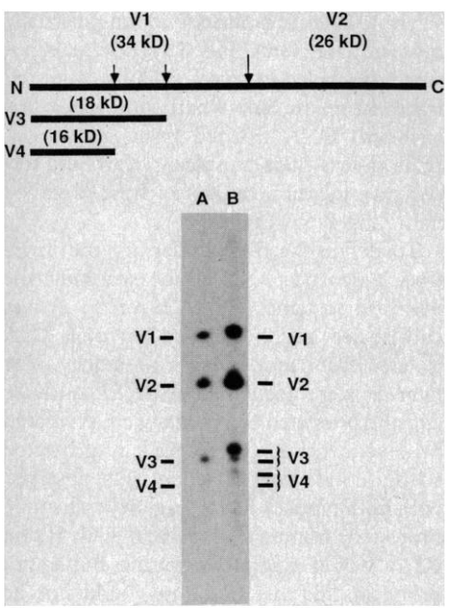



**Fig. 4.** V8 proteolytic mapping of $pp60^{c\text{-}src}$ immunoprecipitated from NIH/3T3 cells and from NIH/pVN1.8-p (*22*). Lane A, V8 peptides derived from $pp60^{c\text{-}src}$ from NIH/3T3 cells (V1 to V4). Lane B, V8 peptides derived from $pp60^{c\text{-}src(+)}$ overexpressed in NIH/pVN1.8-p cells. Note that peptides derived from the endogenous NIH/3T3 $pp60^{c\text{-}src}$ are also observed on this lane.

The pattern of electrophoretic migration of peptides V3 and V4 derived from mouse $pp60^{c\text{-}src(+)}$ is remarkably similar to that generated by the equivalent peptides derived from neuronal $pp60^{c\text{-}src}$ observed by Brugge *et al.* (*1, 2*). The increase in molecular mass contributed by the six–amino acid insertion present in mouse $pp60^{c\text{-}src(+)}$ (753 daltons) is consistent with the apparent increase in mass observed by SDS–polyacrylamide gel electrophoresis of peptides V3 and V4 derived from neuronal $pp60^{c\text{-}src}$ (*1, 2*). This observation strongly suggests that the altered peptide pattern characteristic of neuronal $pp60^{c\text{-}src}$ is a direct consequence of the six–amino acid insertion found in brain $pp60^{c\text{-}src(+)}$.

Sequence analysis of a c-*src* clone isolated from a mouse brain cDNA library has revealed that this clone encodes a protein, which we have termed $pp60^{c\text{-}src(+)}$, that differs from chicken or human $pp60^{c\text{-}src}$ primarily in having a six–amino acid insertion in the $NH_2$-terminal end of the molecule. S1 nuclease protection analyses identified two species of c-*src* RNA in mouse brain which differ by an 18-nucleotide insertion. From a partial survey of tissues examined, the c-*src* RNA that contains the insertion appears to be restricted to brain. Brain c-*src* RNA lacking the insertion may be contributed by non-neuronal tissue (such as glial cells) present in brain.

The mechanism by which the two species of c-*src* RNA are generated is not clear. Because the insertion sequence maps precisely at a splice junction [on the basis of the chicken and human c-*src* exon/intron structure (*5, 6*)], it appears likely that the two RNA species arise by alternative splicing of a single transcriptional unit. We cannot rule out, however, that these RNAs are derived from two separate, albeit highly conserved transcriptional units. Preliminary Southern analyses in which mouse genomic DNA was probed with either chicken c-*src* or mouse brain c-*src* cDNA coding sequences make this last hypothesis unlikely (*3*).

The $pp60^{c\text{-}src(+)}$, at least based on V8 proteolytic mapping, appears to be structurally identical to the neuronal form of $pp60^{c\text{-}src}$ previously described by Brugge *et al.* (*1, 2*). It is not known what effect the inserted sequence found in $pp60^{c\text{-}src(+)}$ might have on the biochemical properties of $pp60^{c\text{-}src}$. Relative to its non-neuronal counterpart, the neuronal form of $pp60^{c\text{-}src}$ displays an increased tyrosine kinase activity as well as a novel serine phosphorylation (*7*). It is conceivable that these biochemical properties are mediated by the inserted sequence present in $pp60^{c\text{-}src(+)}$.

Finally, an issue that was raised by previously reported work (*1, 2*), as well as our own, relates to the biological function of $pp60^{c\text{-}src(+)}$. It is well documented that the most active phase of c-*src* expression occurs in the developing nervous system during active neuronal cell differentiation as well as in fully differentiated postmitotic neurons (*8–14*). Since neurons express predominantly $pp60^{c\text{-}src(+)}$ as opposed to $pp60^{c\text{-}src}$ (*1, 2*), it is conceivable that $pp60^{c\text{-}src(+)}$ is important for induction and/or maintenance of the neuronal differentiated state. Expression of $pp60^{c\text{-}src(+)}$ in cell lines capable of undergoing neuronal differentiation should make these questions amenable to experimental test.

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20. Mapping of the 3′ border of the insertion was accomplished with probe 1, a 220-bp fragment that runs from the Ava I site downstream of the brain-specific insertion to the Bam HI site in the pUC18

polylinker. This probe contains 197 bp of *src*-specific sequences that correspond to the Pst I–Ava I fragment shown on Fig. 3A. In the preparation of this probe, a slightly shorter fragment than the 220-bp-long Bam HI–Ava I probe was invariably observed. This fragment is most likely generated by Bam HI* activity. The probe was 5′ end-labeled at the Ava I site with T4 polynucleotide kinase and hybridized (20,000 count/min) for 16 hours at 56°C in 30 μl of S1 hybridization buffer (40m*M* Pipes, *p*H 6.4, 1 m*M* EDTA, 0.4*M* NaCl, and 50% formamide) [A. J. Berk and P. Sharp, *Cell* **12**, 1721 (1977)] to 30 μg of total RNA isolated from various mouse tissues. After the hybridization, 0.3 ml of ice-cold S1 buffer (0.25*M* NaCl, 0.05*M* Na acetate, *p*H 4.6, 4.5 m*M* $ZnSO_4$ and single-stranded carrier DNA at 20 μg/μl) containing 400 units of S1 nuclease/ml was added and single-stranded nucleic acids were digested for 90 minutes at 34°C. The S1 digestion was stopped by adding 50 μl of 4.0*M* ammonium acetate and 0.1*M* EDTA followed by a phenol/chloroform extraction, addition of 20 μg of transfer RNA, and isopropanol precipitation. The precipitate was dissolved in 0.2 ml of Na acetate (*p*H 5.6) and reprecipitated with ethanol. Finally the precipitate was dissolved in 6 μl of sequence loading buffer, boiled and size-separated on a 5% acrylamide, 7*M* urea sequencing gel.

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22. The 3.8-kb λgt11 cDNA clone was cut with Nco I and the 1.8-kb fragment was isolated. The ends were filled in by means of the Klenow fragment of DNA polymerase and the fragment was blunt end–ligated to pUC18 previously digested with Bam HI and blunted with the Klenow fragment of DNA polymerase. This ligation recreates both the Bam HI site of pUC18 and the Nco I sites of the fragment. The resulting plasmid, pN1.8, was digested with Nco I and Sac I and the 1.4-kb c-*src*–containing fragment was isolated. In addition, pN1.8 was cut with Sac I and Bam HI and the 0.4-kb c-*src*–containing fragment was isolated. These fragments were ligated to a murine expression vector, pV5H, cut with Nco I and Bgl II to generate pVN1.8. pV5H consists of a Moloney murine leukemia virus backbone in which the chicken c-*src* coding sequence of p5H was cloned (B. Mathey-Prevot, unpublished). Digestion of pV5H with Nco I and Bgl II liberates the whole coding sequence of chicken c-*src* (see map of p5H) (*17*). pVN1.8 DNA was co-transfected with pSV2-Neo DNA [P. J. Southern and P. Berg, *J. Mol. Appl. Genet.* **1**, 327 (1982)] into NIH/3T3 cells. Colonies were selected in the presence of G418 (1 mg/ml). Clones expressing $pp60^{c\text{-}src}$ were identified by immunoprecipitating cell lysates with the anti-$pp60^{v\text{-}src}$ monoclonal antibody Mab 327 (*4*). A positive clone, NIH/pVN1.8-p, was chosen for further studies. V8 mapping: NIH/3T3 and NIH/pVN1.8-p cultures were labeled with [$^{32}$P]orthophosphate (1 mCi/ml) for 4 hours. Cells were washed and lysed. Extracts were immunoprecipitated with 1 μl of Mab 327 and immunoprecipitates were electrophoresed on a 8.5% Laemmli SDS-polyacrylamide gel [U. K. Laemmli, *Nature (London)* **227**, 680 (1970)]. The $pp60^{c\text{-}src}$ bands were excised, treated with 100 ng of *Staphylococcus aureus* V8 protease as described [D. W. Cleveland *et al.*, *J. Biol. Chem.* **252**, 1102 (1977)], and separated on a 12.5% SDS-polyacrylamide gel. Following electrophoresis, the gel was dried and autoradiographed on XAR (Kodak) film with a lightning-plus screen.
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# The Molecular Basis of the Sparse Fur Mouse Mutation

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**The ornithine transcarbamylase–deficient sparse fur mouse is an excellent model to study the most common human urea cycle disorder. The mutation has been well characterized by both biochemical and enzymological methods, but its exact nature has not been revealed. A single base substitution in the complementary DNA for ornithine transcarbamylase from the sparse fur mouse has been identified by means of a combination of two recently described techniques for rapid mutational analysis. This strategy is simpler than conventional complementary DNA library construction, screening, and sequencing, which has often been used to find a new mutation. The ornithine transcarbamylase gene in the sparse fur mouse contains a C to A transversion that alters a histidine residue to an asparagine residue at amino acid 117.**

ORNITHINE TRANSCARBAMYLASE (OTC) is a mitochondrial matrix enzyme that catalyzes the conversion of ornithine and carbamyl phosphate to citrulline in the mammalian urea cycle (*1*). The enzyme is encoded by a single X-linked gene and is expressed in only two tissues, liver and intestine (*2*). Human OTC deficiency causes severe ammonia intoxication with accompanying metabolic disorders and neurological symptoms (*3*). Two mouse strains with an OTC mutation have been described—sparse fur (*spf*) (*4*) and sparse fur with abnormal skin and hair ($spf^{ash}$) (*5*). The *spf* mouse has an OTC with an overall decrease in activity, altered substrate affinity, a change in *p*H optima, and an increased amount of material that cross-reacts immunologically with an antibody to OTC (CRM) (*4, 6*).

Here we describe the molecular cloning and sequencing of the *spf* mouse OTC complementary DNA (cDNA) by the application of two recently described techniques—ribonuclease A (RNase A) cleavage (*7*) and the polymerase chain reaction (PCR) method for amplification of specific nucleotide sequences (*8*). In the past, the analysis of such a mutation would have required a substantial effort in both library construction and screening. However, the application of RNase A cleavage to localize the mutation followed by PCR amplification of the mutated site has greatly simplified this procedure.

To study the *spf* mutation, we first isolated the wild-type mouse OTC complementary DNA (cDNA) from a λgt11 library that was generated (*9*) from C57BL/6 mouse liver polyadenylated [poly(A)$^+$] RNA and screened with a partial rat OTC cDNA probe (*10*). The nucleotide sequence of the entire coding region and the 3′ untranslated region of the murine OTC cDNA are shown in Fig. 1. The protein coding regions of the mouse, rat, and human cDNA clones are the same length (1062 bases) and the murine gene is 96 and 88% homologous to the rat and human genes in this region, respectively. Most of the nucleotide substitutions are silent. The homology within the 3′ untranslated region of the murine gene is decreased to 80 and 62% of that of the rat and human sequences, respectively. Northern blot analysis indicated that the mouse OTC messenger RNA (mRNA) length is 1650 bases, which corresponds well with the rat and human (*11*) OTC mRNA size.

It was predicted from previous biochemical and enzymological studies that the *spf* mouse mutation could be a single point mutation (*6*), which was consistent with our observation that no differences in mRNA size could be seen between normal and *spf* mRNA by Northern blot analysis. To localize the *spf* mutation, we used RNase A cleavage (*7*) to characterize the OTC *m*RNA, thus avoiding the inherent problems of analyzing the large gene (approximately 60 kb) (*12*). A radiolabeled, antisense RNA probe was synthesized in vitro from the normal mouse OTC cDNA and then annealed to total RNA isolated from wild-type and *spf* mice. The samples were treated with RNase A to digest single-stranded RNA and to cleave any mismatches between the wild-type probe and the *spf* OTC mRNA. Wild-type RNA protected an approximately 1270-base probe fragment, which was the same length as the predicted length of homology with the OTC mRNA.

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