The generally expressed hnRNP F is involved in a neural-specific pre-mRNA splicing event

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The proteins and RNA regulatory elements that control tissue-specific pre-mRNA splicing in mammalian cells are mostly unknown. In this study, a set of proteins is identified that binds to a splicing regulatory element downstream of the neuron specific *c-src* N1 exon. This complex of proteins bound specifically to a short RNA containing the regulatory sequence in neuronal extracts that splice the N1 exon. It was not seen in non-neuronal cell extracts that fail to splice this exon. UV-cross-linking experiments identified a neuron-specific 75-kD protein and several nontissue-specific proteins, including the 53-kD heterogeneous nuclear ribonucleoprotein F (hnRNP F), as components of this complex. Although present in both extracts, hnRNP F binds tightly to the RNA only in the neuronal extracts. A mutation in the regulatory RNA sequence, that inhibits N1 splicing in vivo, abolished formation of the neuron-specific complex and the binding of the neuron-specific 75-kD protein. Competition experiments in the two extracts show that the binding of the neuronal protein complex to the *src* pre-mRNA is required to activate N1 exon splicing in vitro. Antibody inhibition experiments indicate that the hnRNP F protein is a functional part of this complex. The assembly of regulatory complexes from both constitutive and specific proteins is likely to be a general feature of tissue-specific splicing regulation.

[Key Words: Splicing; hnRNP F; neural-specific splicing; RNA regulatory complexes]

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Alternative splicing is a common mechanism of gene regulation in eukaryotic cells (Moore et al. 1993; Rio 1993). A single messenger RNA precursor (pre-mRNA) can be spliced differentially according to sex, tissue, or developmental stage to produce multiple mRNAs and ultimately multiple proteins with varying functions. Our understanding of the regulation of alternative splicing comes primarily from systems in *Drosophila*, where the regulatory genes are known (Rio 1993). One of the best understood examples occurs in the *Drosophila doublesex* (*dsx*) gene (Baker 1989). The acceptor splice site of *dsx* exon 4 has a poor polypyrimidine tract, causing the exon to be skipped in males. In females, the female-specific transformer protein (Tra) and the transformer-2 protein (Tra-2) activate exon 4 splicing by binding to an RNA regulatory element within the exon (Hedley and Maniatis 1991; Hoshijima et al. 1991; Ryner and Baker 1991; Inoue et al. 1992; Tian and Maniatis 1993). The RNA-bound Tra and Tra-2 apparently recruit general splicing factors of the SR family and promote spliceosome assembly at the acceptor splice site upstream (Tian and Maniatis 1992, 1993; Zahler et al. 1992; Wu and Maniatis 1993; Lynch and Maniatis 1995).

In mammalian cells, systems of cell-type specific alternative splicing are not as well characterized as *dsx*. Mammalian *cis*-acting regulatory elements have been identified that can enhance or suppress splicing at specific sites (Guo et al. 1991; Laviguer et al. 1993; Watakabe et al. 1993; Xu et al. 1993; Dirksen et al. 1994; Gooding et al. 1994; Huh and Hynes 1994). These elements, however, often act in all cell types and thus their role in tissue-specific alternative splicing is not clear. Similarly, *trans*-acting protein factors of the SR family have been identified in mammalian cells that can alter splice-site choice (Ge and Manley 1990; Krainer et al. 1990; Fu et al. 1992; Zahler et al. 1993; Horowitz and Krainer 1994). Although these proteins are not highly cell-type specific and seem to also serve roles in the general splicing reaction, their relative amounts can vary between cell types and can control the variation in the splicing pattern of some transcripts (Caceres et al. 1994; Horowitz and Krainer 1994; Yang et al. 1994). By analogy to the *dsx* system, the SR proteins are likely to also serve as targets for more specific mammalian regulatory factors. Another group of potential splicing regulators is the heterogeneous nuclear ribonucleoprotein (hnRNP) family of proteins, which bind to unspliced hnRNA in mammalian cells, but again these proteins are not highly tis-

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sue specific (Dreyfuss et al. 1993). As yet there are no cell type-specific splicing factors identified in mammalian cells, and there are few systems where a regulated splicing event has been reconstructed in vitro (Ge and Manley 1990; Black 1992).

Alternative splicing is especially common in the mammalian nervous system, where it controls the production of a number of proteins important for neuronal development and function (Burke et al. 1992). We are using the mouse *c-src* gene as a model system for studying neural-specific splicing in mammalian cells (Black 1991, 1992). *c-src* is a proto-oncogene encoding a protein tyrosine kinase (Cooper 1990; Bolen 1993). It contains an 18-nucleotide exon, N1, that is inserted between constitutive exons 3 and 4 in neurons (producing *n-src*) but skipped in other cells (Fig. 1A) (Levy et al. 1987; Martinez et al. 1987). A less frequently used exon, N2, is found inserted between exons N1 and 4 in some *src* mRNAs of human brain (Pyper and Bolen 1990). The neural-specific pattern of N1 inclusion has been reconstructed and studied both in vivo and in vitro (Black 1991, 1992). Mutational analyses identified an intronic sequence between 38 and 70 nucleotides downstream of the N1 5' splice site as an essential element to yield normal levels of N1 splicing in vivo. In vitro splicing competition experiments, with an RNA containing nucleotides 38–142 downstream of N1, indicated that required splicing factors bound to this sequence. These studies, however, did not resolve whether the bound factors were of general distribution or whether they included neural-specific regulatory proteins.

In this report we identify a complex of proteins that binds very specifically to this downstream regulatory sequence (between 38 and 70 nucleotides downstream of N1). We show that this complex is neural specific and yet contains the nontissue-specific hnRNP F protein (Dreyfuss et al. 1993; Matunis et al. 1994). We further show that neural-specific factors, as well as the hnRNP F protein that bound to this sequence, are critical to N1 exon splicing in vitro.

Results

A short RNA regulatory sequence binds neural-specific factors necessary for N1 exon splicing in vitro

Previously, in vivo mutagenesis analyses and in vitro splicing experiments identified the mouse sequence 38–142 nucleotides downstream of the N1 exon as required for the proper splicing of N1 into the *src* mRNA in neuronal cells (Fig. 1A) (Black 1992). Within this region, the sequence from 38 to 70 nucleotides downstream of N1 is sufficient in vivo to activate N1 splicing to near normal levels, in the absence of the downstream 71–142 sequence. This 38–70 sequence in the mouse gene contains a short homology (called CH1) to a sequence in the equivalent region of the chicken *src* sequence (Black 1992). An even more striking homology exists between mouse and human sequences in this region (Fig. 1B). Shorter stretches of similarity between the mouse and

Figure 1. (A) A schematic diagram of the mouse *src* gene showing exons 3, N1, and 4 and the introns between them. The exons are indicated by boxes, and the introns by lines. Positions of the N1 downstream nucleotides 38 and 70 are indicated by vertical bars. In neuronal cells, introns A and B are spliced out to produce *n-src* mRNA. In non-neuronal cells, the intron C is spliced out to produce *c-src* mRNA. (B) The sequence from 1 to 142 downstream of the N1 exon in the mouse aligned with the corresponding human sequence. (x) Identical nucleotides. The mouse sequence that makes up the N70 probe and contains the DCS is flanked by the arrows. The large block of conserved nucleotides that make up the DCS are shown in boldface type in both mouse and human sequences. Dashes are used for a better alignment.

human sequences are seen outside of this region, although they do not overlap completely with the CH2 and CH3 sequences defined previously in the chicken. Because the human sequence identifies a larger sequence than CH1 as conserved, we now call the 38–70 sequence the downstream control sequence (DCS).

An RNA containing the sequence from 38 to 142 nucleotides downstream of N1 can inhibit *src* splicing competitively in vitro (Black 1992). It was not clear from previous studies whether the inhibition was attributable to factors binding the DCS (nucleotides 38–70) or to downstream sequences. It was also unclear whether the bound factors were general splicing factors or whether some were neural specific. We set out to determine what factors bound to the DCS and whether they were neural specific.

Neuronal extracts derived from WERI-1 retinoblastoma cells are capable of splicing the N1 exon in vitro, whereas similar extracts from HeLa cervical carcinoma cells are not (Black 1992). Some preparations of the WERI-1 extract were found to be unable to splice the N1

exon by themselves but still induce N1 splicing when added to the HeLa extract (Black 1992; Chan and Black 1995). An RNA splicing substrate was constructed that contained exons N1 and 4, a shortened intron B between them, and the splice acceptor site upstream of N1 (BS-7 Fig. 2A; see Materials and methods; Chan and Black 1995). This substrate was not spliced well either by the HeLa extract or by a preparation of WERI-1 extract (Fig. 2B, lanes 1,2), but when these two extracts were mixed, this substrate was spliced efficiently (Fig. 2B, lane 3). Thus, each extract lacks different factors needed for BS-7 splicing. An adenovirus intron was spliced by either the HeLa or the WERI-1 extract and this activity was not increased by the mixture (data not shown).

To test the functional significance of the DCS specifically instead of the whole 38–142 sequence, splicing competition experiments were performed with the N70W RNA (Fig. 2A). The N70W RNA has the wild-type mouse sequence from 29 to 70 nucleotides downstream of N1. The HeLa/WERI-1 extract mixture was used for *in vitro* splicing assays in the presence of either the N70W RNA or a nonspecific RNA competitor of similar length. BS-7 splicing activity was not affected by 0.08, 0.16, 0.24, or 0.32 μM of the nonspecific RNA competitor (Fig. 2B, lanes 4–7). In contrast, the splicing activity was strongly inhibited by $>0.16 \mu\text{M}$ N70W RNA (Fig. 2B, lanes 8–11). The amount of N70W (0.16–0.24 μM) needed to inhibit BS-7 splicing was comparable to the amount of the longer 38–142 RNA needed to inhibit N1 splicing (0.1–0.2 μM ; Black 1992). Splicing of an adenovirus substrate was not inhibited by 0.40 μM of N70W (data not shown). Thus, factors essential to the splicing of the N1 exon are binding to the DCS.

We next tested whether the N70W RNA inhibits N1 splicing by adsorbing general splicing factors or neural-specific factors. We reasoned that if neuronal proteins must bind to the control sequence to allow N1 splicing, then the preincubation of the WERI-1 extract with the competitor should show greater inhibition than the preincubation of the HeLa extract with the competitor. Indeed this is the case. The HeLa and WERI-1 extracts were each preincubated with either the N70W RNA competitor or the splicing substrate before being mixed. When the HeLa extract was preincubated with an excess of N70W RNA and the WERI-1 extract with the splicing substrate, N1 splicing was not affected (Fig. 2C, lanes 2–4). In contrast, when the WERI-1 extract was preincubated with N70W RNA and the HeLa extract with the substrate, N1 splicing was inhibited to near completion at 0.16 μM of N70W RNA (Fig. 2C, lanes 5–7). Thus, the activation N1 splicing requires at least one neuron-specific factor that binds the DCS RNA.

A WERI-1-specific protein complex binds to the DCS RNA

To identify activities that bind to the DCS, gel mobility shift experiments were performed with a uniformly radiolabeled RNA fragment containing the DCS sequence

(the N70 probe; Fig. 2A). Unlike N70W, N70 contains short non-*src* sequences at the ends. The N70 and N70W probes behaved equivalently in all of the assays used here (data not shown). The gel mobility of the N70 RNA probe differed after incubation in the HeLa or the WERI-1 extracts. The WERI-1 extract exhibited the formation of a specific high molecular weight complex, whereas both extracts showed a prominent constitutive complex (Fig. 3A, lanes 3–5). The HeLa/WERI-1 extract mixture gave the same pattern of complexes as the WERI-1 extract alone (data not shown). There was also a lower molecular weight complex that appeared to be HeLa specific. This complex, however, became apparent in WERI-1 extracts when competition experiments were performed (see Fig. 3B). Extracts from LA-N-5 neuroblastoma cells, which splice the N1 exon, also contain the high molecular weight complex seen in WERI-1 extracts. Alternatively, extracts from three other non-neuronal cell lines did not show this complex (data not shown). Thus, among the cell lines tested the neuronal complex was specific to cells that splice the N1 exon.

Previous mutagenesis analyses of *src* splicing *in vivo* showed that a 6-nucleotide mutation within the 38–70 sequence abolishes the activation of N1 splicing by this regulatory element (Black 1992). To test whether this mutation affects the neural complex formation, these same base changes were introduced into the N70 construct and this mutant RNA fragment (N70M; Fig. 2A) was subjected to the gel mobility shift reaction. The neuronal complex failed to form on N70M RNA (Fig. 3A, lanes 6,7), whereas the constitutive complex formation was unaffected by the mutation. Thus, nucleotides that are critical for the *in vivo* activity of the DCS are also required for the neuronal complex assembly.

To compare the binding specificity of the two complexes, competition experiments were performed. The labeled N70 RNA probe was incubated in WERI-1 extract in the presence of increasing amounts (0.05, 0.10, 0.20, or 0.40 μM) of unlabeled N70 RNA, N70M RNA, or a nonspecific RNA fragment of similar length. The WERI-1-specific complex was not affected by 0.40 μM of the nonspecific competitor (Fig. 3B, lanes 4–7), whereas it was competed away nearly completely by 0.10 μM of N70 RNA (Fig. 3B, lanes 8–11). The N70M competitor did not compete as strongly as N70, eliminating the WERI-1-specific complex at 0.20 μM of N70M (Fig. 3B, lanes 12–15). This twofold difference in competitor strength is not surprising given that multiple proteins are binding to the RNA and making up the WERI-1-specific complex (see below). A mutation that eliminates one protein-binding site could leave the others intact allowing the competitor to continue to sequester components of the complex.

The constitutive complex was competed away strongly with all three competitors. At lower concentrations, however, the N70 and N70M RNAs competed for the constitutive complex better than the nonspecific RNA (data not shown). These results indicate that the RNA binding of the neuronal complex is quite sequence specific, whereas the constitutive complex is not.

Figure 3. (A) Gel mobility shift assays in HeLa and WERI-1 extracts. (Lanes 1,2) N70 (wt) or N70M (m) probes alone without extract. (Lanes 3–5) Samples containing the N70 probe incubated in either WERI-1 extract (lane 4) or two different preparations of HeLa extract (H1, lane 3; H2, lane 5). (Lanes 6,7) Similar reactions with an N70M probe. (B) Competition experiments with N70, N70M, or nonspecific RNA (N. sp.) probes. Lanes 1,2, and 3 are the same as lanes 1, 3, and 4, respectively in A. The competition reactions in WERI-1 extract contained 0.05, 0.10, 0.20, or 0.40 μ M of the nonspecific competitor RNA (lanes 4–7), N70 RNA (lanes 8–11), or N70M RNA (lanes 12–15). (H) HeLa extract; (W) WERI-1 extract.

probe (Fig. 4A, lane 1–3). In the WERI-1 extract, an additional protein of 75 kD was detected along with additional light bands in the 55-kD region of the gel (Fig. 4A, lanes 2,3). Note that the 43-kD protein shows a more variable signal than the others but is consistently seen in both extracts. Significantly, with the N70M mutant RNA as the probe, the WERI-1-specific 75-kD protein was not detected, and the intensity of the 90- and 43-kD bands was decreased substantially. The mutation in

N70M had no effect on the cross-linking of the 55-kD protein (Fig. 4A, cf. lanes 4–6 to lanes 1–3). The fact that the WERI-1-specific complex failed to form on the N70M probe in the gel mobility shift assay (Fig. 3A) implies that the loss of signal seen here in the UV cross-linking (Fig. 4A) results from differences in binding and not solely to differences in cross-linking efficiency (also see below). Cross-linking in the presence of the N70, N70M, and nonspecific RNA competitors produced similar results

Figure 2. (A) The RNAs used for the in vitro splicing, gel mobility shift, and cross-linking assays. BS-7 is an RNA splicing substrate that contains exons N1 and 4. It also contains the N1 splice acceptor site and a shortened intron between N1 and 4. The approximate position of DCS is shown. N70W contains the wild-type sequence from 29 to 70 nucleotides downstream of mouse N1 exon and a GGG at the 5' end derived from the T7 promoter. N70 contains the mouse sequence 38–70 nucleotides downstream of N1 (uppercase letters) and extra sequences at the two ends corresponding to *Clal* and *Bam*HI sites (lowercase letters). N70M is identical to N70 except for the 6-nucleotide mutation in the DCS indicated by the underlined lowercase letters. The non-specific (N. Sp.) RNA is a part of the *src* exon 2 sequence. (B) Competition of neural-specific splicing activity with a DCS RNA in vitro. (Lane 1) Splicing reaction in a HeLa extract; (lane 2) splicing reaction in a WERI-1 extract; (lane 3) splicing reaction in a HeLa/WERI-1 extract mixture. Splicing reactions in the HeLa/WERI-1 extract mixture were competed with 0.08, 0.16, 0.24, or 0.32 μ M of unlabeled nonspecific RNA (N. Sp.) (lanes 4–7), or N70W RNA (lanes 8–11). The splicing substrate (BS-7) and products are indicated at left of the gel by schematic drawings. (C) Competition in individual extracts. Lane 1 is equivalent to lane 3 of Fig. 2B, above. (Lanes 2–4) 8 μ l of WERI-1 extract (W) was incubated with 2.8 fmoles of labeled substrate at 30°C, and 5 μ l of HeLa extract was incubated with 0.04 μ M (lane 2), 0.08 μ M (lane 3), or 0.16 μ M (lane 4) of unlabeled N70W RNA. After 10-min preincubation, the WERI-1 and HeLa mixtures were combined. The final mixture had the same composition as the splicing reactions in (Fig. 2B) above (see Materials and methods). (Lanes 5–7) Reactions were performed as in lanes 2–4, respectively, except that the WERI-1 extracts were preincubated with unlabeled N70W RNA and the HeLa extract with the labeled splicing substrate RNA. The splicing substrate (BS-7) and products are indicated as in Fig. 2B above.

Figure 4. (A) UV cross-linking to the N70 and N70M probes. (Lanes 1–3) Cross-linking to the N70 probe in HeLa (H, lane 1) or two different preparations of WERI-1 extracts (W1, lane 2; W2, lane 3). (Lanes 4–6) Cross-linking to the N70M probe in the same extracts as in lanes 1–3. (B) Two-dimensional gel electrophoretic analysis of proteins in the constitutive and WERI-1-specific complexes. (Lanes 1,2) Different exposures of the control cross-linking reaction in HeLa nuclear extract. (Lanes 4,5) Different exposures of the control cross-linking reaction but performed in WERI-1 nuclear extract. (Lanes 3,6) Cross-linked proteins contained in the constitutive complex (c) formed in HeLa and WERI-1 extracts, respectively. Lane 7 shows the cross-linked proteins in the WERI-1-specific complex (w).

to the gel shift experiments. The p55 protein was easily competed with any RNA, whereas the other proteins were strongly reduced by the N70 competitor, less strongly by the N70M RNA and not at all by the non-specific RNA (data not shown).

To determine which proteins seen by the UV cross-linking were in the complexes detected by the gel mobility shift assay, the complexes were separated by a native gel after UV cross-linking (Siebel et al. 1992). The cross-linked complexes showed the same mobilities as uncross-linked reactions and were eluted from the gel and analyzed by SDS-PAGE after RNase treatment. The constitutive complex in both the HeLa and WERI-1 extracts contained the 43- and 55-kD proteins (Fig. 4B, lanes 3,6). The WERI-1-specific complex contained the 90- and 75-kD proteins and a 58-kD protein (Fig. 4B, lane 7). This complex also contained proteins of 43, 32, and 28 kD and other proteins in the 50- to 60-kD region, whose intensities were weaker than the 90-, 75-, and 58-kD bands. Thus, the neuronal complex contains several proteins that are not tissue specific, and at least one, p75, that is found only in the WERI-1 extract. The strongly cross-linking p55 protein is not found in the neuronal complex. Hence, the constitutive complex is not likely to be a precursor to the neuronal complex.

Separation of the nonspecific complex from the WERI-1-specific complex

The p55 protein in the nonspecific complex obscures observation of some of the cross-linked proteins in the neu-

ronal complex (e.g., p58, Fig. 4B). To characterize further the proteins involved in the neuron-specific complex, partial fractionation of the WERI-1 extract was carried out to separate the neuronal binding activity from that of the nonspecific complex. WERI-1 extract was subjected to ultracentrifugation at 360,000g for 30 min to remove large particles and aggregates. This cleared extract (S360) was fractionated by precipitation in a range of ammonium sulfate concentrations from 10% to 70%. Precipitate and supernatant fractions were dialyzed and subjected to the gel mobility shift assay with labeled N70 RNA. The ultracentrifugation step increased the specific activity of the neural-specific complex about twofold (Fig. 5A, cf. lanes 1 and 2). In 40% ammonium sulfate, the WERI-1-specific binding activity was present in the pellet fraction, whereas the nonspecific binding activity stayed in the supernatant fraction (Fig. 5A, lanes 3,4). The specific activity of the WERI-1 complex was increased substantially by ammonium sulfate fractionation, whereas the binding activity of the nonspecific complex went down.

UV cross-linking experiments were also performed with these extract fractions. The S360 fraction showed a similar cross-linking pattern to the original extract except that the intensity of the p75 increased (Fig. 5B, cf. lanes 1 and 2). The 40% ammonium sulfate supernatant showed decreased cross-linking overall, with the most prominent band being p55 (Fig. 5B, lane 3). The p55 protein was greatly reduced in the 40% ammonium sulfate pellet fraction which contained the p90, p75, and p43 proteins. In addition, the cross-linking of 58-, 53-, and

A **B**

Figure 5. (A) Gel mobility shift experiment performed using the WERI-1 nuclear extract (lane 1), S360 extract (lane 2), the 40% ammonium sulfate supernatant (s, lane 3) or pellet fractions (p, lane 4). The gel mobility shift pattern of this preparation of extract shows a novel complex just below the WERI-1-specific complex (indicated by an X). The level of X-binding activity differed from extract to extract (including HeLa extract) and stayed in the supernatant at 40% ammonium sulfate (lane 3). This complex has not been characterized further. (B) UV cross-linking experiment performed with the WERI-1 nuclear extract (lane 1), S360 extract (lane 2), the 40% ammonium sulfate supernatant (lane 3), or the pellet fraction (lane 4)(A.S.) Ammonium sulfate fraction.

28-kD proteins was greatly enhanced in this fraction (Fig. 5B, lane 4). The p75 band was now seen as a doublet. The upper band of the doublet may be a degradation product of p90 because the intensity of p90 decreases in this fraction. The relative band intensity does not likely reflect the stoichiometry of binding in the neuronal complex, because not all of the protein seen cross-linking in the fraction is necessarily in a complete complex or is cross-linking with equal efficiency. Nevertheless, the sizes of the proteins cross-linked in the 40% pellet fraction are equivalent to those seen in the neuron-specific complex (Fig. 4B). Thus, the removal of p55 allows better visualization of proteins that we know from other assays to be in the neuronal complex such as p58 (Fig. 4B) and p53 (see below).

hnRNP F is a component of the WERI-1-specific complex

There are many ubiquitous RNA-binding proteins in nuclear extracts, some of which are similar in size to proteins seen in the neuronal complex. To identify proteins contained in this complex, several assays were performed with antibodies to known RNA-binding proteins. After incubation of the extract with anti-Sm, anti-hnRNP HF, or anti-hnRNP KJ antibodies, the neuronal complex was examined by the gel mobility shift assay (Fig. 6A). The anti-Sm and anti-hnRNP KJ antibodies did not affect the mobility of the protein-RNA complexes in the WERI-1 nuclear extract (Fig. 6A, lanes 2,4). Interestingly, the anti-hnRNP HF antibody caused a large shift in the size of the WERI-1-specific complex (lane 3, *), without affecting the nonspecific complex. Similar su-

pershift assays, performed with anti-hnRNP C, anti-SR protein, and anti-SF2 antibodies, failed to detect changes in the mobility of either complex (data not shown). The anti-hnRNP HF antibody specifically recognizes the hnRNP H (56 kD), hnRNP F (53 kD), and hnRNP E (36 and 40 kD) proteins (Matunis et al. 1994). This indicates that at least one of these nontissue-specific proteins, or a protein with a common epitope, is a component of the neuronal complex.

To determine which of the hnRNP H, hnRNP F, or hnRNP E proteins were bound to the N70 RNA, immunoblots of extract fractions, developed with the anti-hnRNP HF serum, were aligned with the cross-linked proteins from the 40% ammonium sulfate pellet fraction (Fig. 6B). The immunoblot of HeLa and WERI-1 extracts shows the hnRNP H (56 kD) and hnRNP F (53 kD) proteins, and a doublet of hnRNP E proteins (40 kD; Fig. 6B, lanes 1,2). The S360 fraction and the 40% ammonium sulfate pellet fraction contained only the 53-kD hnRNP F protein (Fig. 6B, lanes 3,4). This hnRNP F band comigrated with the p53 cross-linked band in the 40% ammonium sulfate pellet (Fig. 6B, cf. lane 5 with lanes 1-4).

To show that the cross-linked p53 protein is hnRNP F, immunoprecipitation experiments were performed. The N70 probe was cross-linked in the 40% ammonium sulfate pellet fraction, and the reaction was then subjected to immunoprecipitation with the anti-hnRNP HF antibody. The immunoprecipitate contained the p53 protein (Fig. 6C, lane 2). The anti-hnRNP KJ and anti-Sm antibodies failed to bring down any cross-linked proteins (Fig. 6C, lanes 3,4). Thus, hnRNP F is a component of the neuron-specific complex of proteins that binds to the splicing regulatory sequence.

Figure 6. (A) Gel mobility shift experiment performed with WERI-1 nuclear extract alone (lane 1) or in the presence of anti-Sm (lane 2), anti-hnRNP HF (lane 3), or anti-hnRNP KJ (lane 4) antibodies. (B) Western blot analysis with anti-hnRNP HF antibody. (Lane 1) HeLa nuclear extract; (lane 2) WERI-1 nuclear extract; (lane 3) WERI-1, S360 extract; (lane 4) 40% ammonium sulfate pellet fraction; (lane 5) autoradiograph of a UV cross-linking reaction in the 40% ammonium sulfate pellet fraction. The locations of hnRNP H, F, and E and p53 are indicated by arrows. (C) Immunoprecipitation of the cross-linked proteins. The 40% ammonium sulfate pellet fraction was subjected to cross-linking with labeled N70 RNA (lane 1). Immunoprecipitations of this reaction were carried out with anti-hnRNP HF (lane 2), anti-hnRNP KJ (lane 3), or anti-Sm (lane 4) antibodies. The locations of cross-linked proteins are indicated by arrows.

The hnRNP HF antibody specifically inhibits N1 splicing in vitro

To test whether the hnRNP F protein is directly involved in N1 splicing, the BS-7 transcript was subjected to splicing reactions in the presence of the hnRNP HF antibody. The anti-hnRNP HF antibody effectively inhibited BS-7 splicing (Fig. 7A, lanes 2,3) whereas similar concentrations of anti-SF2 antibody did not affect BS-7 splicing (Fig. 7A, lanes 6,7). As expected, the anti-Sm antibody (Y12) also strongly inhibited BS-7 splicing (Fig. 7A, lanes 4,5). This antibody is known to inhibit splicing by binding a common determinant on the spliceosomal small nuclear ribonucleoproteins (snRNPs) (Padgett et al. 1983). The inhibition by the anti-hnRNP HF antibody is transcript specific. When a constitutively spliced adenovirus substrate was subjected to the same conditions as the BS-7 substrate, only the anti-Sm antibody inhibited splicing (Fig. 7B, lanes 4,5). Neither the anti-hnRNP HF nor the anti-SF2 antibody had an effect on the splicing of the adenovirus substrate (Fig. 7B, lanes 2,3 and 6,7). The lack of inhibition by the anti-SF2 antibody may indicate that this particular SR protein is not essential for these introns or that this antibody does not block SF2 function. These antibody inhibition results indicate that the

hnRNP F protein is required for *src* N1 exon splicing in vitro but not for the general splicing reaction.

Discussion

A neural-specific assembly of RNA-binding proteins

In this report we show that splicing of the *c-src* N1 exon specifically requires neural factor(s) that bind to an intronic RNA sequence, the DCS. This sequence is thus a neural-specific splicing regulatory element. We also identify a set of proteins in neural cell extracts that bind to the DCS in a sequence-specific manner. RNA competition and antibody inhibition experiments indicate that this RNA-protein complex is an essential regulator of the neural-specific splicing of the *src* N1 exon.

The two-dimensional gel analyses (Fig. 4B) and the cross-linking pattern of partially fractionated WERI-1 extract (Fig. 5B) indicate that this complex contains 90-, 75-, 58-, 53-, 43-, and 28-kD proteins. Other proteins may be present that do not cross-link to the RNA. Of the six cross-linked proteins, at least one, p75, is specific to the WERI-1 extract. The p90 protein is seen binding in the HeLa extract by UV cross-linking, but in the gel mobility shift assay p90 is seen only in the neural complex and

Figure 7. (A) BS-7 splicing reactions in the presence of various antisera. Splicing reactions were carried out in HeLa-WERI-1 extract mixture and contained no antibody (lane 1), anti-hnRNP HF (lanes 2–3), anti-Sm (lanes 4–5), or anti-SF2 (lanes 6–7). (B) Adenovirus splicing reactions in the presence of antisera. Conditions and lanes are the same as in A.

not in a constitutive complex. Similarly, the hnRNP F protein (p53) is present in both extracts but is seen binding by the gel mobility shift assay only in the WERI-1 extract. These nontissue-specific proteins may bind weakly to the RNA on their own but are stripped off in a gel mobility shift assay. The neural-specific protein, p75, could thus induce the assembly of regulatory proteins that do not otherwise bind well to the DCS. In this way, generally expressed proteins could control splice-site choice through their interactions with more specific factors.

Through the use of anti-hnRNP HF antibodies, we show that the p53 protein in the neuronal complex is the hnRNP F protein. The antibody inhibition experiments indicate that the F protein is required for the splicing of the N1 exon. The hnRNP family of RNA-binding proteins has been presumed to be involved in various aspects of mRNA metabolism (Dreyfuss et al. 1993). HnRNP F is a fairly typical member of this family, containing three RNP-CS type RNA-binding domains. The F protein is reported to have a very high affinity for poly(G) (Matunis et al. 1994). Although no specific function has been assigned previously to hnRNP F, other members of this family have also been implicated in aspects of the splicing reaction (Choi et al. 1986; Swanson and Dreyfuss 1988; Garcia-Blanco et al. 1989; Patton et al. 1991; Horowitz and Krainer 1994). In one example, the hnRNP I protein is thought to repress specific splicing patterns in the α - and β -tropomyosin transcripts (Guo et al. 1991; Singh et al. 1995). In contrast, in the *src* system hnRNP

F is involved in the positive control of splice-site choice. These results are interesting in relation to experiments on the kinetics of spliceosome assembly. In *in vitro* splicing reactions, different RNA substrates bind different constellations of hnRNP proteins, forming what is called the initial H complex (Michaud and Reed 1991; Bennett et al. 1992). This H complex is then converted into a series of spliceosome complexes containing the snRNPs. Thus, in both regulated and unregulated transcripts splice-site choices seem to be determined in the context of specific sets of bound hnRNP proteins.

The DCS is apparently comprised of several different binding elements, and the nucleotides that make it neural specific are not clear. A substitution mutation in the DCS that abolished neural specific splicing *in vivo* inhibited both the formation of the WERI-1-specific complex, and cross-linking to the WERI-1-specific 75-kD protein. This mutation partially disrupts the hexanucleotide sequence UGCAUG (nucleotides 57–62). This hexanucleotide is present in multiple copies downstream from the fibronectin EIIIB exon and is required for EIIIB splicing (Huh and Hynes 1994). Interestingly, the UGCAUG hexanucleotide in the fibronectin system is not neural specific and its activity depends on its adjacent sequence. Immediately upstream of the hexanucleotide is the sequence CUCUCU in the mouse or CUGUCU in the human, which is also altered by the DCS mutation. This element is also found in the N1 exon splice acceptor site, where it also has regulatory effects (Chan and Black 1995). Upstream of the CU-

CUCU sequence is a conserved GGGGGCUG sequence, which is possibly the hnRNP F-binding site, given its affinity for poly(G) (Matunis et al. 1994). It thus appears that the DCS is made up of multiple RNA elements, each binding to different proteins.

Neuronal regulation of splicing

The mechanisms that normally prevent N1 exon splicing are complex. One effect is the nonoptimal short length of the exon (Black 1991; Dominski and Kole 1991, 1992; Sterner and Berget 1993). There are also specific proteins that can repress N1 exon splicing (Chan and Black 1995). In overcoming this repression of N1 splicing, the neuronal activation of splicing is apparently directed to the downstream intron B (Fig. 1A), as it is spliced before the upstream intron A in vitro. Thus, the proteins bound to the DCS may act to facilitate spliceosome assembly at the N1 5' splice site. This model is similar to the *Drosophila dsx* system where the specific factors Tra and Tra-2, and the SR general splicing factors are components of a complex bound to a series of regulatory elements within the regulated *dsx* exon. This complex apparently stabilizes spliceosome assembly at the weak acceptor splice site upstream (Tian and Maniatis 1993; Wu and Maniatis 1993; Staknis and Reed 1994; Lynch and Maniatis 1995). In the *src* case, the DCS again binds both general and neural-specific factors to positively regulate N1 splicing. Here, however, it is an intronic element that likely alters spliceosome assembly at a splice donor site. Moreover, N1 splicing apparently also involves factors binding to the N1 splice acceptor site (Black 1991; Chan and Black 1995).

The proteins that regulate *src* splicing are likely to be involved in the neural-specific splicing of other transcripts. Although many examples of neuronal regulation of splicing have been described, in no case are the regulatory factors identified. Neuron-specific exons showing a similar developmental expression pattern to *src* N1 are found in both the *N-CAM* and the *agrin* mRNAs (Tacke and Goridis 1991; Hoch et al. 1993). Interestingly, the neuron specific exon in the *N-CAM* gene also seems to be regulated at its 5' splice site (Tacke and Goridis 1991). The purification of the *src* DCS-binding proteins will answer many questions regarding their function and role in neuronal gene regulation.

Materials and methods

Oligonucleotides used for PCR

The oligonucleotides used for PCR are as follows: (1) 5'-CGCG-TAATACGACTCACTATAGGGATCGATGAGGCTGGGG-GCTG-3'; (2) 5'-GATGGATCCAGGAAGCACATGCAGAG-3'; (3) 5'-GACGGATCCAGGAAGCACATGGGAATTCCAG-CCCCAGCC-3'; (4) 5'-CGCGTAATACGACTCACTATAG-GGTGCTTCGCTGAGGCTGGGGGCTG-3'; (5) 5'-AGGAAG-CACATGCAGAGAGCAGCCCCAGCCTCAGCGAA-3'.

Template construction and T7 transcription

Templates for T7 transcription were prepared either by PCR or

by linearization of plasmids with appropriate restriction enzymes. The BS-7 construct was derived from the BS-3 construct reported previously (Black 1992) by deletion from the *Pst*I site in exon 3 to the *Apal* site in intron A (Chan and Black 1995). Templates for N70, N70M, and N70W were produced by PCR amplification. The PCR reactions contained 0.5 μ M of each primer, 50 ng of DNA template, 200 μ M of each dNTP, 1.5 mM $MgCl_2$, 10 mM Tris-HCl (pH 8.4 at room temperature), 50 mM KCl, and 2.5 units of AmpliTaq DNA polymerase (Perkin-Elmer). The total reaction mixture was 100 μ l, and each reaction was subjected to 30 cycles of 1 min at 94°C, 1 min at 55°C, and 1 min 45 sec at 72°C by use of a GeneAmp PCR system 9600 (Perkin-Elmer). The N70 RNA template used oligonucleotides 1 and 2 as primers and pSS10 DNA (Black 1992) as the PCR template. N70M used oligonucleotides 1 and 3 as primers and pSS60 DNA (Black 1992) as the PCR template. For N70W, complementary oligonucleotides 4 and 5 were used without plasmid DNA template. Each PCR product was gel purified.

The splicing substrate and the probes for the UV cross-linking and gel mobility shift reactions were transcribed with T7 RNA polymerase from the linearized BS-7 plasmid and the N70 or N70M PCR products as templates, respectively. The adenovirus splicing substrate contains the first two exons of the adenovirus major late transcription unit. It was transcribed with SP6 RNA polymerase from the linearized SPAd plasmid (Solnick 1985). Transcription reactions (50 μ l for 2 hr at 37°C) contained 100 μ M each of ATP, GTP, CTP, 10 μ M of UTP, 20 mM dithiothreitol, 100 μ Ci of [α - 32 P] UTP (800 Ci/mmol; NEN DuPont), 40 mM Tris-HCl (pH 7.5), 6 mM $MgCl_2$, 2 mM spermidine, 10 mM NaCl, 40 units of T7 RNA polymerase (New England Biolabs), and 1.5 μ g of DNA template. The resulting transcripts were gel purified.

Competitor RNA fragments were transcribed in larger (150 μ l) reactions under the same conditions as above except they contained 400 μ M of each NTP, no [α - 32 P] UTP, and 2.5–3 μ g of each PCR template. The nonspecific RNA competitor was transcribed from a plasmid (pBnan, 10 μ g) containing cDNA sequence of *src* exon 2 cut with *Alu*I resulting in a 58-nucleotide transcript. The cold competitor RNA fragments were gel purified and quantified by UV absorbance.

In vitro splicing assays

The splicing reactions were performed as described previously (Black 1992). Five microliters of HeLa nuclear extract (40–50 μ g total protein) was used for the splicing reaction in lane 1, and 8 μ l of WERI-1 nuclear extract (40–50 μ g total protein) was used in lane 2. Each complementation reaction contained 5 μ l of HeLa extract and 8 μ l of WERI-1 extract (lanes 3–11). Each splicing reaction also contained 2.8 fmoles of the substrate RNA (50,000 cpm), 2% polyethylene glycol instead of 2.5% polyvinyl alcohol as described previously, 0.4 mM ATP, 2.2 mM magnesium chloride, 20 mM creatine phosphate, 15.6 units of RNase inhibitor (Pharmacia), and 0.1 μ g/ml of heparin as a nonspecific competitor. For competition experiments, each reaction mix was preincubated with competitor RNA for 10 min at 30°C before addition of the probe. For antibody inhibition reactions, 5–10 μ g (as judged by a Coomassie-stained protein gel) of anti-hnRNP HF (8A6), anti-Sm (Y12), or anti-SF2 antibody was preincubated with each reaction mix for 10 min at 30°C before addition of the probe. Splicing reactions were incubated for 90 min (for the adenovirus substrate) or 4 hr (for the BS-7 substrate) at 30°C. The deproteinized samples were then separated on denaturing 8% polyacrylamide gels (Ausubel et al. 1987) and autoradiographed.

Gel mobility shift assays

HeLa and WERI-1 extracts used for the gel mobility shift reactions were prepared as reported previously (Black 1992). Each gel mobility shift reaction (25 μ l) contained 3–10 μ l of nuclear extract or ultracentrifuged nuclear extract (40–50 μ g of total protein) or 2.5–3 μ l of 40% ammonium sulfate precipitation fractions (20 μ g of protein), 0.4 mM ATP, 20 mM creatine phosphate, 3.2 mM magnesium acetate, and 50 fmoles of N70 or N70M RNA fragment uniformly labeled with [α - 32 P] UTP (2×10^5 cpm). tRNA (400 μ g/ml) or heparin (12 μ g/ml) was also added as a nonspecific competitor. All reaction components except the probe were mixed and incubated for 8 min at 30°C, after which probes were added for another 15-min incubation at 30°C. For competition reactions, the RNA competitors were added after the 8-min preincubation, and the reactions were incubated for 5 min more before the addition of probe. For supershift reactions, ~5 μ g of anti-hnRNP KJ (3C2), anti-hnRNP HF (8A6), or anti-Sm (Y12) antibodies from ascites were added to the reaction after the 15-min incubation with probe. The reactions were put on ice, and 5 μ l of each reaction was separated on a 6% native polyacrylamide gel (29:1, acrylamide/bis) in Tris-borate EDTA buffer (Ausubel et al. 1987). The gels were run at 240 V for 2.5 hr at 4°C and were dried down and autoradiographed on film or PhosphorImager screen.

UV cross-linking assays

Binding reactions were the same as in the gel mobility shift reaction described above. After a 15-min incubation, samples were put on ice and irradiated with UV light at a distance of 4.5 cm from the UV source (254 nm at 11.5 mW/cm², UVG-54 UVP, Inc.) for 10–15 min. Each sample was then incubated with RNase A (200 units) for 30 min at 30°C. An equal volume (25 μ l) of 2 $\times$ protein sample buffer (0.125 M Tris-HCl at pH 6.8, 4% SDS, 20% glycerol, 10% 2-mercaptoethanol) was then added to each reaction, which was boiled (3 min) and loaded (18 μ l) onto an SDS-gel (4% stacking gel, 10% separating gel) (Ausubel et al. 1987). The gels were fixed and dried for autoradiography on X-OMAT film (Kodak) or a PhosphorImager screen (Molecular Dynamics). Both the constitutive (43, 55, and 90 kD) and WERI-1-specific (75 kD) proteins were observed to bind under a number of different conditions, including in the presence of 400 μ g/ml of tRNA, 40 μ g/ml of poly[d(A–C)], or 12 μ g/ml of heparin as nonspecific competitors (data not shown). The binding of these proteins was increased in the presence of Mg²⁺ ion or the absence of ATP (data not shown).

Partial fractionation of the WERI-1-specific complex

The WERI-1 nuclear extract was ultracentrifuged in the Beckman 100.2 rotor for 30 min at 90,000 rpm (360,000g). The supernatant of ultracentrifuged fraction was subjected to ammonium sulfate fractionation (10%–70%), and each supernatant and pellet fraction was dialyzed against buffer DG (Black 1992) for 5 hr. At 40% ammonium sulfate (226 mg/ml) most of the constitutive complex stayed in the supernatant fraction, whereas the WERI-1-specific complex was present in the pellet fraction.

Two-dimensional gel electrophoresis

UV cross-linking reactions were performed as described above except with 17 min of irradiation, and the RNase digestion was omitted. The entire reaction was then loaded onto a 6% native gel as described above. The constitutive and WERI-1 specific

complexes were cut out of the native gel, and these gel slices were soaked in 50 μ l of RNase A (10 mg/ml) for 30 min in 37°C with shaking at 250 rpm. After RNase A digestion, the excess liquid was removed. The gel slices were soaked in 2 $\times$ protein sample buffer for 1 hr at 37°C, boiled for 3 min, and loaded onto a 10% SDS gel (1.5 mm thickness).

Immunoblotting

The immunoblotting experiments were performed as described previously (Ausubel et al. 1989). The SDS gels were run as in the UV cross-linking experiments. The proteins were transferred to a nitrocellulose membrane. A 1:1000 dilution of anti-hnRNP F antibody (8A6; Matunis et al. 1994) from ascites was used as the primary antibody. A 1:2500 dilution of ImmunoPure horseradish peroxidase-labeled goat anti-mouse IgG was used as the secondary antibody (Pierce). The bands were detected by use of the ECL Western Blotting Detection Kit (Amersham).

Immunoprecipitation of UV cross-linked reaction

UV cross-linking reactions were performed as described above and subjected to immunoprecipitation as described previously (Firestone and Winguth 1990) except that GammaBind Plus Sepharose beads (Pharmacia) were used in place of fixed *Staphylococcus aureus* as an immunoadsorbent. One microliter of each antibody ascites fluid was used to precipitate two UV cross-linking reactions.

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The generally expressed hnRNP F is involved in a neural-specific pre-mRNA splicing event.

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