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A Complex Intronic Splicing Enhancer from the c-*src* Pre-mRNA Activates Inclusion of a Heterologous Exon

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The mouse c-*src* gene contains a short neuron-specific exon, N1. To characterize the sequences that regulate N1 splicing, we used a heterologous gene, derived from the human β-globin gene, containing a short internal exon that is usually skipped by the splicing machinery. Various fragments from the *src* gene were inserted into the globin substrate to measure their effects on the splicing of the test exon. These clones were transiently expressed in neuronal and nonneuronal cell lines, and the level of exon inclusion was measured by primer extension. Several sequences from the N1 exon region induced the splicing of the heterologous exon. The most powerful effect was seen with a sequence from the intron downstream of the N1 exon. This sequence acted as a strong splicing enhancer, activating splicing of the test exon when placed in the intron downstream. The enhancer was strongest in neuronal LA-N-5 cells but also activated splicing in nonneuronal HEK293 cells. Deletion and linker scanning mutagenesis indicate that the enhancer is made up of multiple smaller elements that must act in combination. One of these elements was identified as the sequence UGCAUG. Three copies of this element can strongly activate splicing of the test exon in LA-N-5 neuroblastoma cells. These component elements of the *src* splicing enhancer are also apparently involved in the splicing of other short cassette exons.

In eukaryotic cells, alternative RNA splicing patterns are used to generate mRNAs encoding multiple protein products from a single gene. The choice of alternative splicing pattern is often tissue specific or developmentally regulated and can profoundly affect the activity of the encoded protein (1, 10, 25). In general, the mechanisms that regulate splice site choice, particularly in mammalian cells, are poorly understood (6, 19, 28, 29). Splice sites and branchpoints adhere to specific consensus sequences needed for proper recognition and assembly by the spliceosome. In addition, a variety of other regulatory sequences within the precursor RNA can have both positive and negative effects on spliceosome assembly at certain splice sites.

Positively acting splicing regulatory sequences can be classified by their location in either exons or introns. Purine-rich exonic sequences are found in a number of genes and can induce the splicing of an exon that is normally excluded from the mRNA (8, 14, 36, 43). These exonic splicing enhancers bind to specific members of the SR protein family, a set of splicing regulatory factors that have strong effects on splice site choice and spliceosome assembly (16, 23, 39). The *Drosophila* doublesex gene (*dsx*) contains a highly regulated exonic enhancer, whose activity depends on the regulatory factors Tra and Tra-2, in addition to specific SR proteins (reference 22 and references therein). Other exonic enhancers seem to act constitutively, although they may also require non-SR protein factors (35, 44). It is thought that SR proteins bound at an exonic enhancer interact with the required splicing factor U2AF to stabilize its binding to the 3′ splice site upstream (33, 40–42). This leads to the activation of a splice site that is not normally recognized by the general splicing machinery.

In addition to splicing enhancers found in exons, intronic splicing enhancer sequences have also been identified. These sequences are generally found in the introns downstream of very short or highly tissue specific exons (3, 5, 9, 13, 17, 18, 30, 32). On the whole, intronic splicing enhancers are not well characterized. With the partial exception of an intronic splicing enhancer found in the c-*src* gene, the proteins that activate splicing through these elements and how they function are unknown (26, 27).

We are using the mouse c-*src* gene as a model system to study the splicing of a neuron-specific exon. The c-*src* gene encodes a 60-kDa protein tyrosine kinase and is expressed in a wide variety of cells (7). In most neurons, an 18-nucleotide exon (N1) is included in the *src* mRNA between the constitutive exons 3 and 4, generating a neuron-specific *src* mRNA and protein (20, 24). Mutational analysis has identified certain features of the N1 exon region as important in controlling its tissue-specific splicing. The short length of the exon is essential to its repression in nonneural cells (4). In addition, sequences in the 3′ splice site upstream of the exon are needed for the repression of N1 splicing in nonneuronal cell extracts (12). The splicing of the exon in neuronal cells requires an intronic region downstream from exon N1 (5). This sequence (nucleotides 37 to 70 downstream of N1) is conserved phylogenetically and has been called the downstream control sequence (DCS) (27). The reconstitution of N1 splicing in vitro allowed the identification of some of the proteins responsible for the effects of these regulatory sequences. The repression of splicing in nonneuronal cell extracts is mediated, at least in part, by the binding of the polypyrimidine tract binding protein at the 3′ splice site (11). The DCS assembles a multiprotein complex, specific to neural extracts, that is required to allow splicing of the exon. This DCS complex contains at least six polypeptides, including the proteins hnRNP F and KH-type splicing regulatory protein (KSRP) (26, 27). The proteins in the complex are required for N1 splicing, but they are not specific to the neuronal extract. The source of the neural specificity in the assembly of the DCS complex and in N1 splicing is not yet clear.

Mutagenesis analyses performed on the *src* gene itself are subject to limitations on their interpretation. Although specific

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sequences were shown to be required for the regulated splicing of exon N1, it was not clear whether all of the regulatory sequences had been identified or whether the known elements were sufficient for the regulation. Moreover, since multiple sequences were required for the splicing of the exon, it was not known which individual elements produced the neuron specificity of splicing. To look at these issues of sufficiency and tissue specificity, we analyzed the effects of individual *src* sequence elements on the splicing of a heterologous pre-mRNA. We find that the *src* intronic activating sequence is indeed sufficient to induce the splicing of a heterologous exon and thus has the properties of a splicing enhancer. However, this enhancer is significantly larger and more complex than could be determined previously. In addition, this experimental system has allowed us to more fully dissect this enhancer, to identify some of its component sequence elements, and to assess their neuron specificity.

MATERIALS AND METHODS

Plasmid construction. All DNA constructs were made by using standard cloning procedures and confirmed by sequencing (2, 31). The parent plasmid from which all further clones were made was CMV (cytomegalovirus) DUP33, a gift from Z. Dominski and R. Kole (15). To facilitate the cloning of *src* fragments, restriction sites were introduced for *Apa*I, 47 nucleotides upstream from the middle exon, and for *Bgl*II, 46 nucleotides downstream from the middle exon. This clone was assembled from multiple PCR products by using the following primers, described by their sequence relationship to the transcribed DUP RNA. DUP1 (GCAGCTCACTCAGTGTGGCA) is complementary to CMV DUP33 exon 3 (globin exon 2). DUP2 (CCAATAGATCTGGGCATGTG) is homologous to CMV DUP33 intron 2 but with an inserted *Bgl*II site. DUP3 (CACAT GCCCAGATCTATTGG) is complementary to intron 2 and to primer DUP2 and also contains the *Bgl*II site. DUP4 (GCTGCTGGTGGTGCCATGGC) is homologous to CMV DUP33 exon 2. DUP5 (CTGCCCAGGGCCTGCCAT GG) is complementary to CMV DUP33 exon 2 and to primer DUP4. DUP6 (TTCTGATAGGGCCCACTGACTCT) is homologous to CMV DUP33 intron 1 but contains an inserted *Apa*I site. DUP7 (AGAGTCAGTGGGCCCTATCA GAA) also contains the *Apa*I site and is complementary to intron 1 and primer DUP6. DUP8 (GACACCATGCATGGTGCACC) is homologous to DUP exon 1. A series of PCRs was performed with the following primers and templates. Fragments of CMV DUP33 DNA were amplified by using primer pairs 1-2, 3-4, 5-6, and 7-8. The second set of PCRs used the following: primers 1 and 4 with the 1-2 and 3-4 PCR products as templates, and primers 5 and 8 with the 5-6 and 7-8 PCR products as templates. A final PCR assembled a complete fragment by using primers 1 and 8 with the 1-4 and 5-8 PCR products as templates. This final PCR product was cleaved with *Nsi*I and *Dra*III and inserted into the CMV DUP33 vector also cleaved with *Nsi*I and *Dra*III. This clone was designated DUP4-1 (Fig. 1B).

DUP4-5 (Fig. 2A) was made by digesting a *src* minigene construct (Fig. 1A) with *Apa*I and *Bam*HI and inserting the fragment containing the N1 exon into DUP4-1 at the *Apa*I and *Bgl*II sites. DUP4-4 and DUP4-66 were made by inserting the appropriate *Apa*I/*Cla*I or *Cla*I/*Bam*HI fragment of pBS1 (12) into DUP4-1 cut with *Apa*I/*Nco*I or *Nco*I/*Bgl*II. Before ligation, the *Cla*I and *Nco*I ends were made flush with T4 DNA polymerase. Clones 4-21 and 4-74 were made by two-step PCR mutagenesis using oligonucleotide DUP9 (5′-CGCTGG CCCTTAGGCTGCTGGTGGTGCCATGGCAGG) or DUP10 (5′-CCTGCCA TGGCACCACCAGCAGCCTAAGGGCCAGCG) as the upstream primer and DUP122 (5′-CCACCCTTAGGAGGAAGGTGCCATCGGCA) or DUP123 (5′-TGCCATGCGATGGCACCTTCCTAAGGGTGG) as the downstream primer with the DUP4-4 plasmid as a template. DUP4-69 was made by annealing synthetic oligonucleotides DUP116 (5′-CATGGCGATGT CAGGTGTGTACC GAGGCCA) and DUP117 (5′-GATCTGGCCTCGGTACACACCTCGCC) and ligating this fragment into DUP4-1 cut with *Nco*I and *Bgl*II.

Clones depicted in Fig. 3A and 4A were all made by PCR using primers specific to the sequences in the *src* gene. Specific regions were amplified in standard 100-μl PCRs with oligonucleotides carrying restriction sites for *Bgl*II and *Bam*HI or *Apa*I (DUP4-72 and DUP4-73) at the ends. Amplified fragments were cut with the appropriate enzymes, gel purified, and ligated to DUP4-1 linearized with either *Apa*I or *Bgl*II. Multiple copies of these sequences were inserted by ligating a new copy of the PCR fragment into a plasmid containing a single insert. Clones 4-107, 4-108, and 4-109 were made by annealing complementary oligonucleotides and ligating the resulting fragments into the DUP4-1 plasmid cut at the *Bgl*II site.

The scanning mutations across the duplicated 17–70 region (Fig. 5) were made by oligonucleotide-directed PCR mutagenesis. PCR was performed with DUP4-30 as template DNA and with the Bam70 primer paired with the DUP100, -101, -102, -103, and -104 primers to make clones DUP4-80 through DUP4-88, respectively, or with the Bgl17 primer paired with the DUP105, -106, -107, -108, and -109 primers to make clones DUP4-90 through DUP4-98, respectively. These primers had the following structures: Bam70, CCAGGATCCCACCGCC CTGTGTG; DUP100, CCAGATCT**TTCGAA**GGGGGATGC; DUP101, CCA GATCTGGTAGA**TTCGAA**ATGCTTCGC; DUP102, CCAGATCTGGTAGA GGGGG**TTCGAA**CGCTGAGGC; DUP103, CCAGATCTGGTAGAGGGGG ATGCT**TTCGAA**AGGCTGGGGG; DUP104, CCAGATCTGGTAGAGGGG GATGCTCGCTG**TTCGAA**GGGGGCTGC; Bgl17, CCAGATCTGGTAGAG GGGGATGCTT; DUP105, GATGGATCCAGGAGCACATGCAGAGAGCA G**TTCGAA**GCCTCAG; DUP106, GATGGATCCAGGAAGCACATGCAGA**T TCGAA**CCCCCAGCC; DUP107, GATGGATCCAGGAAGCACATG**TTCGA A**AGCAGCCCCC; DUP108, GATGGATCCAGGAAG**TTCGAA**CAGAGAG CAG; and DUP109, GATGGATCC**TTCGAA**CACATGCAGAGAGCAG (boldface indicates nucleotides altered from the wild-type *src* sequence). The products from the various PCRs were digested with *Bgl*II and *Bam*HI and ligated into the DUP4-1 plasmid, linearized at the *Bgl*II site. After screening for clones with a single insert in the proper orientation, the resulting plasmids were linearized at the *Bgl*II site again, and another copy of a specific mutant fragment was inserted to yield the double mutants tested.

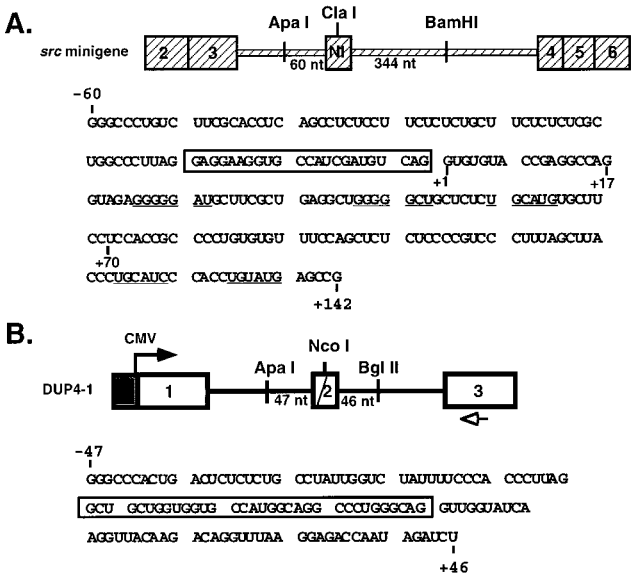

FIG. 1. Maps of the *src* and globin minigenes. (A) The *src* minigene with exon N1 (23 nucleotides [nt] with an inserted *Cla*I site) and flanking exons. Exons are shown as boxes, and the introns are shown as lines. The sequence from 60 nucleotides upstream to 142 nucleotides downstream of N1 is shown below the diagram. The exon sequence is boxed, and repeated elements are underlined. (B) The chimeric β-globin minigene DUP4-1. The globin sequences are fused to the CMV immediate-early promoter (shaded box); the transcription start site is indicated by the filled arrow. DUP exon 1 is β-globin exon 1, and DUP exon 3 is β-globin exon 2. There is an additional exon downstream that is not shown (globin exon 3 or DUP exon 4). The diagonal line in the second DUP exon indicates that it is a fusion of the second and first β-globin exons to make a 33-nucleotide hybrid exon. The arrow below the third exon indicates the position of the oligonucleotide used for primer extension analysis. The sequence from the *Apa*I site to the *Bgl*II site is shown below.

Transfection and cell culture. All plasmid DNAs for transfection were purified by banding twice on CsCl gradients. Nonneuronal HEK293 cells were grown in Dulbecco modified Eagle medium (DMEM; Gibco-BRL) supplemented with 10% newborn calf serum and split 1:5 every 3 days in 10-cm-diameter plates. The human neuroblastoma cell line LA-N-5 was grown in DMEM–F-12 (Gibco-BRL) supplemented with 15% fetal calf serum (FCS) and split 1:2 every 3 days in 15-cm-diameter plates. All cells were grown in an incubator containing 5% CO_2 at 37°C.

For transfection of the HEK293 cells, confluent plates were split 1:4, and 15 μg of plasmid DNA was transfected per plate by using a standard $CaCl_2$-HEPES-buffered saline (HBS) procedure (2).

Confluent 15-cm-diameter plates of LA-N-5 cells were split into four 10-cm-diameter plates on the day before transfection. Two hours before transfection, the DMEM–F-12 was removed and 10 ml of fresh DMEM containing 15% FCS was added. The cells were transfected with 15 to 20 μg of plasmid DNA by a modified $CaCl_2$ method. The plasmid and 32 μl of 2 M $CaCl_2$ were diluted to a final volume of 250 μl in ultrapure H_2O. Then 250 μl of modified 2× HBS (50 mM HEPES-NaOH, 280 mM NaCl, 2.8 mM Na_2HPO_4 [pH 7.05 at room temperature]) was added slowly to the DNA-$CaCl_2$ solution and incubated for 20

min at room temperature. The mixture was then added directly to the medium on the plates and incubated at 37°C. After 4 h, the medium was aspirated and 2.5 ml of prewarmed 15% (vol/vol) glycerol in 1× HBS was added directly to the cells for 90 s. After this solution was aspirated, the cells were washed once with prewarmed DMEM–F-12, and 15 ml of DMEM–F-12 containing 15% FCS was added.

RNA preparation and analysis. Cytoplasmic RNA was prepared from cells 40 to 48 h after transfection by Nonidet P-40 lysis as described previously (4).

The oligonucleotide used for the primer extension analysis was DUPRT#3 (5′-AACAGCATCAGGAGTGGACAGATCCC), complementary to nucleotides 48 to 73 in the third exon of the DUP mRNA. The oligonucleotide was labeled with [γ-^{32}P]ATP (6,000 Ci/mmol; NEN DuPont) and T4 polynucleotide kinase and purified on a G-25 spin column (5′-3′ Inc.).

For each primer extension reaction, 30 μg of HEK293 RNA or 50 μg of LA-N-5 RNA was ethanol precipitated. The RNA pellet was dried and resuspended in 10 μl containing 50 mM Tris-HCl (pH 8.3), 75 mM KCl, 3 mM $MgCl_2$, and 100,000 cpm of ^{32}P-labeled DUPRT#3 primer (approximately 25 fmol). The mixture was heated to 85°C for 5 min, and the oligonucleotide was annealed to the RNA at 50°C for 1 h. The reaction mixture was then placed in a 42°C water bath, and 10 μl of prewarmed reverse transcription mix (50 mM Tris-HCl [pH 8.3], 75 mM KCl, 3 mM $MgCl_2$, 40 μM dithiothreitol, 1 mM each deoxynucleoside triphosphate, 100 U of Superscript II reverse transcriptase [Gibco-BRL]) was added. After a 1-h incubation, LA-N-5 samples were treated with 30 μl of RNase A in Tris-EDTA (30 μg/ml), and the reaction mixture was incubated for 30 min at 42°C. Finally, 30 μl of Tris-EDTA was added to the HEK samples, 50 μl of 2× PCA buffer (0.6 M sodium acetate, 100 mM Tris-HCl [pH 8.0], 10 mM EDTA, 0.4% sodium dodecyl sulfate) was added, and the mixture was extracted once with phenol-chloroform (50:50) and precipitated with 2.5 volumes of ethanol. The pellet was resuspended in 6 μl of gel loading buffer (95% deionized formamide, 5 mM EDTA, 0.025% each bromophenol blue and xylene cyanol, 0.2 M NaOH) and heated for 10 min at 85°C. After the tubes were placed on ice, 3 μl was loaded onto an 8% polyacrylamide (19:1 acrylamide:bis)–7.5 M urea–1× Tris-borate-EDTA gel and electrophoresed at 60 W until the xylene cyanol reached the bottom of the 39-cm gel. The gel was dried on Whatman 3MM paper and exposed to a PhosphorImager screen for 24 h with HEK293 samples or 72 h with LA-N-5 samples. The amount of product was measured on a Molecular Dynamics PhosphorImager.

RESULTS

Multiple sequences contribute to activating N1 exon splicing. Mutagenesis studies using a transfectable *src* minigene indicated that multiple regulatory sequences were involved in the neuron-specific splicing of the N1 exon (4, 5). These regulatory elements included a downstream intronic sequence and the 3′ splice site upstream from the exon (12, 27). An additional feature required for the repression of splicing in nonneural cells was the short length of the exon (4). To characterize the role of each individual sequence, we tested them in a heterologous context in the absence of other *src* sequences. The heterologous gene was the CMV DUP33 clone made by Dominski and Kole (15) (Fig. 1B). DUP33 is derived from the human β-globin gene but contains four exons and three introns (15). Between the standard β-globin exons 1 and 2 there is an additional 33-nucleotide hybrid exon containing the duplicated 3′ splice site of globin exon 2 and the duplicated 5′ splice site of globin exon 1. Thus, the first three exons of this DUP transcript have two identical 5′ splice sites and two identical 3′ splice sites. In this study, we will be considering the splicing of these first three exons (Fig. 1A). Most importantly for our purposes, the hybrid central exon 2 is normally skipped due to its short length (15). By placing *src* sequences within this gene, we could test the ability of each sequence to induce the splicing of the central DUP exon 2.

The parent clone CMV DUP33 contained the CMV promoter/enhancer and the β-globin polyadenylation site for expression in mammalian cells. This clone was modified by the addition of unique *Apa*I and *Bgl*II restriction sites into the two introns for insertion of the *src* fragments. The new clone, DUP4-1, was transfected into either nonneuronal HEK293 cells or LA-N-5 neuroblastoma cells. After transient expression, RNA was isolated from the cells and the splicing pattern was determined by primer extension analysis using an oligonucleotide primer complementary to the third DUP exon. As expected, both HEK and LA-N-5 cells showed the product generated by the splicing of exon 1 to exon 3, skipping the central exon (Fig. 2B, lanes 1 and 10). Neither cell line showed significant inclusion of the central exon, which yields a 33-nucleotide-longer extension product.

To confirm the ability of the N1 exon to maintain its neural specificity in the context of the DUP4-1 globin sequences and the CMV promoter, a fragment of the *src* gene containing the N1 exon and flanking intron sequence was cloned in place of the central DUP exon (clone 4-5 [Fig. 2A]). This clone was transfected into LA-N-5 cells, where ~29% of the expressed transcripts showed inclusion of the N1 exon. This was in contrast to the HEK cells, where splicing of the N1 exon was barely detectable (Fig. 2B, lanes 2 and 11). Thus, the N1 exon maintained its neural specificity in the globin gene context. A similar result was obtained previously, using adenovirus exons flanking a larger N1 exon genomic fragment (4). In this case, the splicing of the N1 exon to the adenovirus exons was also neuron specific, but the exon was included to a lower extent (~10%).

To test the contributions of the different regions surrounding the N1 exon to its regulation, various sequences were individually inserted into the DUP4-1 clone (Fig. 2A). The 3′ half of the N1 exon and the downstream *src* intron sequence induced significant splicing of the hybrid exon (5′ half DUP/3′ half N1; clone 4-66). This clone showed 31% exon inclusion in LA-N-5 cells and also some splicing in HEK293 cells (6% [Fig. 2B, lanes 3 and 12]). This region was further divided into two fragments. One fragment contained the 3′ portion of the N1 exon and its 5′ splice site (clone 4-69), and the second fragment contained the intron sequence from 17 to 142 nucleotides downstream of the exon, including the previously defined DCS (Fig. 2A, clone 4-28). The N1 exon fragment with the 5′ splice site induced a small amount of splicing that was higher in neuronal cells (6% inclusion in LA-N-5 [Fig. 2B, lanes 4 and 13]). In contrast, the downstream fragment had a large effect, inducing 62% inclusion of the exon in LA-N-5 cells (Fig. 2B, lanes 5 and 14). This sequence also had a significant effect in HEK cells, producing approximately 18% inclusion of the exon. Thus, the 17–142 fragment has the properties of a splicing enhancer, activating a normally excluded exon. This sequence has some neuronal specificity, as its effect is threefold stronger in LA-N-5 cells than in HEK cells. However, there is also activation of splicing in the HEK cells that was not seen with the N1 exon as a whole.

The converse hybrid exon was also tested. A fragment containing the N1 3′ splice site and the 5′ portion of the N1 exon was fused to the 3′ half of the DUP exon (clone 4-4). This clone showed a small amount of splicing of the hybrid central exon in LA-N-5 cells (Fig. 2B, lanes 6 and 15). The 5′-half exon fragment was further subdivided. The 3′ splice site sequence itself had no measurable effect on the splicing of the central DUP exon (clone 4-21 [Fig. 2B, lanes 7 and 16]). The 5′ portion of the N1 exon had a small effect, producing 2 to 4% inclusion of the exon in the two cell types (clone 4-74 [Fig. 2B, lanes 8 and 17]). This exon sequence contains a stretch of eight purine residues reminiscent of an exonic splicing enhancer.

Delineation and properties of the intronic splicing enhancer. Several individual *src* sequences were found that activated splicing of the DUP exon, but by far the largest effect was seen with nucleotides 17 to 142 downstream of the N1 exon. This sequence activated splicing strongly in the LA-N-5 cells and moderately in the HEK cells. The region from 37 to 142 nucleotides downstream of N1 was previously identified as containing splicing regulatory sequences including the con-

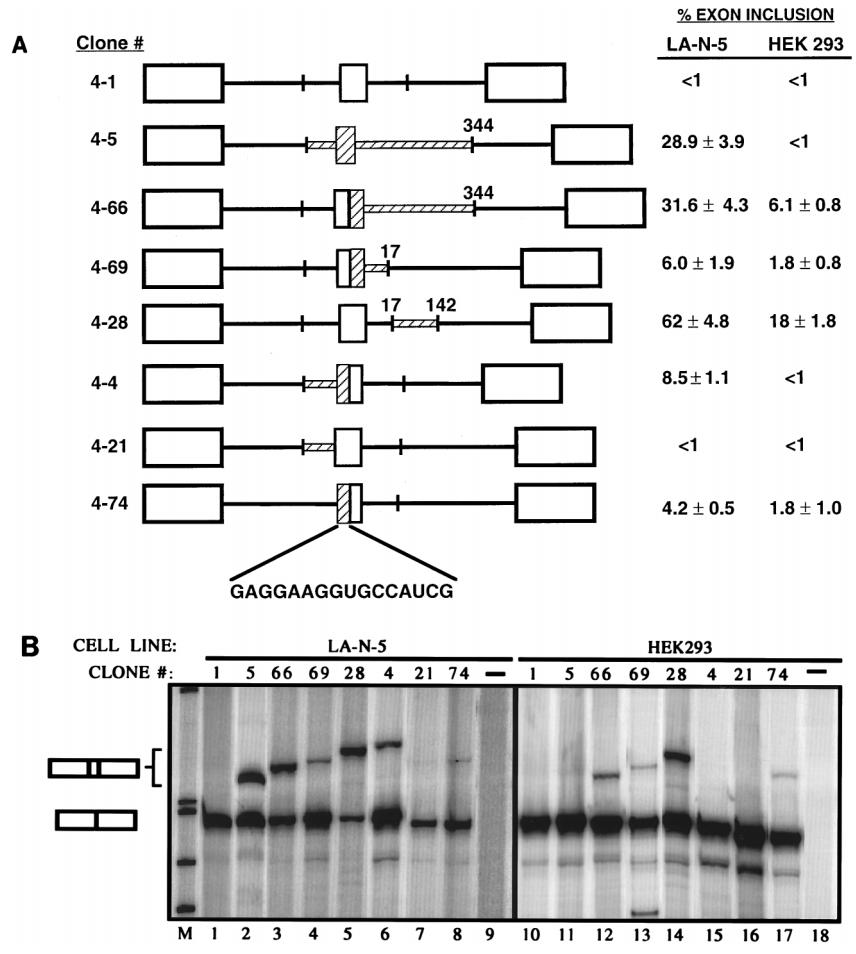

FIG. 2. Multiple sequences contribute to activating N1 splicing. (A) Maps of DUP minigenes containing portions of exon N1 and its flanking sequences. Sequences from *src* are shown with diagonal cross-hatching; and globin sequences are shown with solid black lines. Clone numbers are indicated on the left. DUP4-5 has *src* sequences from 60 nucleotides upstream to 344 nucleotides downstream of exon N1. DUP4-66 contains a *src* fragment from the *Cla* site in the N1 exon to 344 nucleotides downstream of N1, while DUP4-69 has *src* sequence from the *Cla* site to 17 nucleotides downstream of N1. The intronic sequence from 17 to 142 nucleotides downstream of N1 was inserted to make DUP4-28. *src* sequence from 60 nucleotides upstream of N1 to the *Cla* site within the exon was inserted in DUP4-4. DUP4-21 contains 60 nucleotides of *src* sequence upstream of N1 with no exon sequence, while DUP4-74 has the 5′ half of exon N1 (exact sequence shown below) with no *src* intron sequence. Plasmid DNA was transiently expressed in LA-N-5 and HEK293 cells. The mRNA splicing phenotype was assayed by primer extension using a ^{32}P-labeled oligonucleotide complementary to DUP exon 3. Percent central exon inclusion was quantified on a PhosphorImager and defined as [total counts in the exon included product/(counts in the exon included product + counts in the exon excluded product)] × 100. The averages from at least three independent transfections and standard deviations are shown. (B) Primer extension analysis of the clones diagrammed in panel A. Clone numbers are indicated along the top. Lane M contains DNA size markers (from top to bottom, 307, 242, 238, 217, and 201 nucleotides in size). Lanes 1 to 9, RNA from LA-N-5 cells; lanes 10 to 18, RNA from HEK293 cells. Lanes 10 and 18 are primer extension reactions with RNA from mock-transfected cells. The identity of each band is indicated at the left as either the exon skipped (lower) or the exon included (upper) product. Due to differences in clone construction, the size of exon 2 varies between 23 nucleotides (DUP4-5), 28 nucleotides (DUP4-66), 29 nucleotides (DUP4-69), 33 nucleotides (DUP4-1, -28, -21, and -74), and 34 nucleotides (DUP4-4).

served DCS (5, 27). In the DUP context, this fragment activates splicing but not as strongly as the 17–142 fragment, indicating that the extent of the regulatory region is larger than could be delineated earlier (compare clones 4-3 [Fig. 3B, lanes 3 and 11] and 4-28 [lanes 2 and 10]). The splicing activation from this region is sequence specific, as the 37–142 fragment inserted in the reverse orientation has no effect on splicing (clone 4-19 [lanes 1 and 9]). This result indicates that simply placing any insertion into this position of the DUP4-1 clone and increasing the size of the second intron does not activate splicing of the central exon.

The activating region extends through most of the 17–142 fragment. Many possible subportions of this sequence were inserted into DUP4-1 and found to have no effect (Fig. 3A and data not shown). As seen in Fig. 3B, inserting the 37–70, 70–110, or 110–142 fragment alone did not activate splicing in either cell line (lanes 4 to 6 and 12 to 14). Similarly, nucleotides 17 to 70 and 37 to 110 had no effect when inserted as a single copy into DUP4-1 (Fig. 4B, lanes 5 and 11, and data not shown). From these results, the enhancer could be comprised of a single long sequence element or structure that cannot be reduced in size, or it could contain multiple smaller elements that must be present in combination for splicing activation to occur.

An intronic splicing enhancer from the chicken cardiac troponin T gene was reported to continue to function when moved from downstream of the activated exon to a position upstream of the exon (9). This was not true of the *src* N1 enhancer. When either the 17–142 or the 37–142 fragment was placed upstream of the central DUP exon, no effect on splicing was observed (clones 4-70 and 4-71 [Fig. 3B, lanes 7, 8, 15, and 16]). Thus, in our assay, the N1 splicing enhancer was position

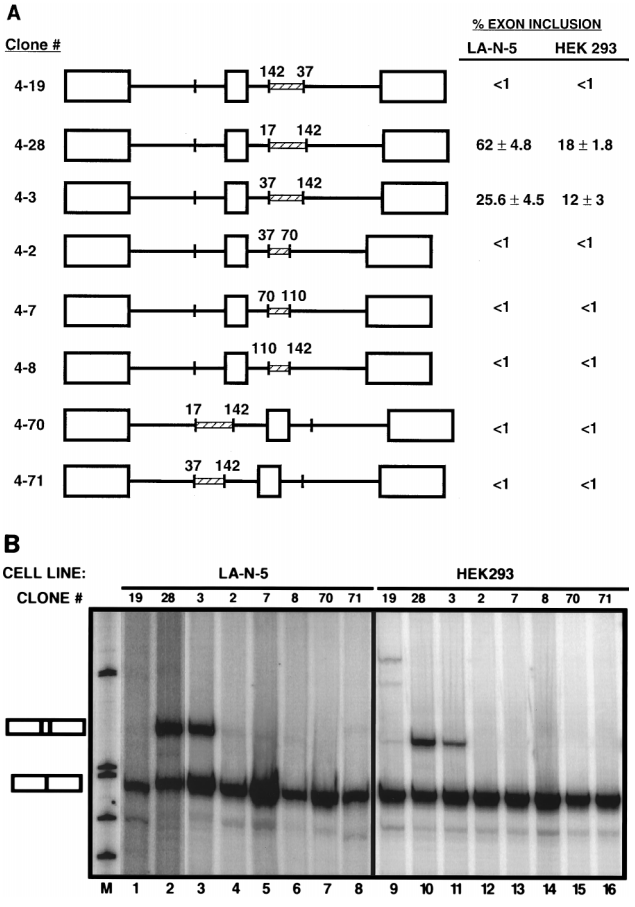

FIG. 3. Delineation of the intronic splicing enhancer. (A) Maps of DUP minigenes containing various sequences from the intron downstream of N1. Solid black lines indicate globin sequences, and *src* inserts are shown with diagonal cross-hatching. The numbers over each insert indicate the nucleotides in the *src* intron downstream from exon N1 where the sequence begins and ends. DUP4-19 has an inverted copy of nucleotides 37 to 142. DUP4-70 and -71 have nucleotides 17 to 142 and 37 to 142 from the intron downstream of N1 inserted 47 nucleotides upstream from the central DUP exon. The level of exon inclusion was measured as for Fig. 2. (B) Primer extension analysis of the clones depicted in panel A. The positions of the primer extension products are indicated to the left. Clone numbers are indicated along the top. Lane M is a DNA size marker as in Fig. 2. Lanes 1 to 8 clones expressed in LA-N-5 cells; lanes 9 to 16 clones expressed in HEK293 cells.

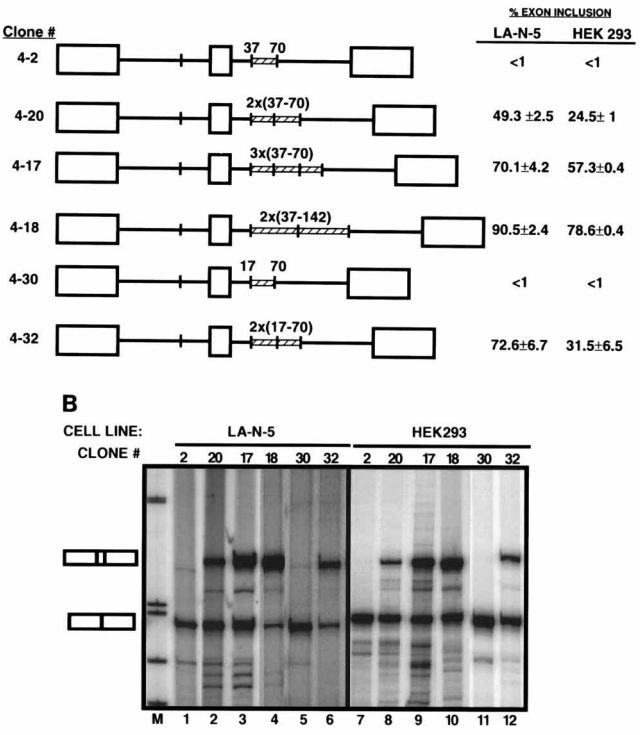

FIG. 4. Enhancer sequences are stronger in combination. (A) Constructs containing repeated inserts from the enhancer region. Globin and *src* sequences are indicated as in Fig. 3A. DUP4-2 contains one copy of the *src* intron sequence from 37 to 70 nucleotides downstream of N1. DUP4-20 and DUP4-17 contain two and three copies, respectively, of this sequence. DUP4-18 contains two copies of nucleotides 37 to 142. DUP4-30 and DUP4-32 contain one and two copies of nucleotides 17 to 70. The levels of exon inclusion were determined as described for Fig. 2. (B) Primer extension analysis of clones depicted in panel A. Clone numbers are indicated along the top. The positions of the primer extension products are indicated on the left. The band seen between the two major products is apparently a premature reverse transcription stop. Lane M is a DNA size marker as in Fig. 2. Lanes 1 to 6 and lanes 7 to 12 are from LA-N-5 and HEK293 transfections, respectively.

dependent, needing to function from downstream of the activated exon.

Multiple copies of the enhancer sequence strengthen its effect. Different portions of the enhancer region gave different levels of splicing of the central DUP exon. Therefore, we tested whether the level of splicing was dependent on the number of positive elements present downstream. Indeed, this proved to be true. The 37–142 sequence induced 25% inclusion of the exon in LA-N-5 cells and 12% inclusion in HEK cells (Fig. 3B, lanes 3 and 11). Two copies of this sequence gave 90% inclusion of the DUP exon in LA-N-5 cells and 78% inclusion in HEK cells (clone 4-18 [Fig. 4B, lanes 4 and 10]). In the *src* gene itself, a similar duplication increased N1 splicing in LA-N-5 cells but not in the nonneuronal HeLa cells (5). Here, in the context of the DUP exon, the activation of splicing was seen in both cell types.

The augmented splicing by specific sequence duplications was particularly striking with smaller portions of the enhancer region. The 37–70 sequence gave no splicing of the exon when present in single copy (clone 4-2 [Fig. 4B, lanes 1 and 7]). Simply duplicating this sequence made it into a strong activator, yielding ~49% inclusion of the exon in LA-N-5 cells and 24% in HEK cells (clone 4-20 [Fig. 4B, lanes 2 and 8]). Three copies of the sequence gave still more splicing of the exon (clone 4-17 [lanes 3 and 9]). The single and double copies of the 17–70 sequence showed an even greater difference in activity. This sequence has no effect in single copy (clone 4-30 [lanes 5 and 11]) but in duplicate yields 72% exon inclusion in LA-N-5 cells and 31% exon inclusion in HEK293 cells (clone 4-32 [lanes 6 and 12]). These fragments of the enhancer region are apparently made up of smaller sequence elements that must synergize or cooperate with other elements to produce an activating effect.

Individual elements within the core enhancer. To refine our picture of the individual elements that make up the splicing enhancer, we constructed a series of linker scanning mutations across the 17–70 region (Fig. 5A). The 17–70 sequence has little effect on splicing by itself but has a strong effect when present as a tandem duplication (Fig. 4; compare lanes 5 and 6 or lanes 11 and 12). To see a maximal effect from the mutations, they were introduced into both copies of the duplicated 17–70 fragment. Mutations in the 5′ portion of the 17–70 sequence had modest effects, although some showed reduced splicing compared to the parent 4-32 clone (Fig. 5). Mutations

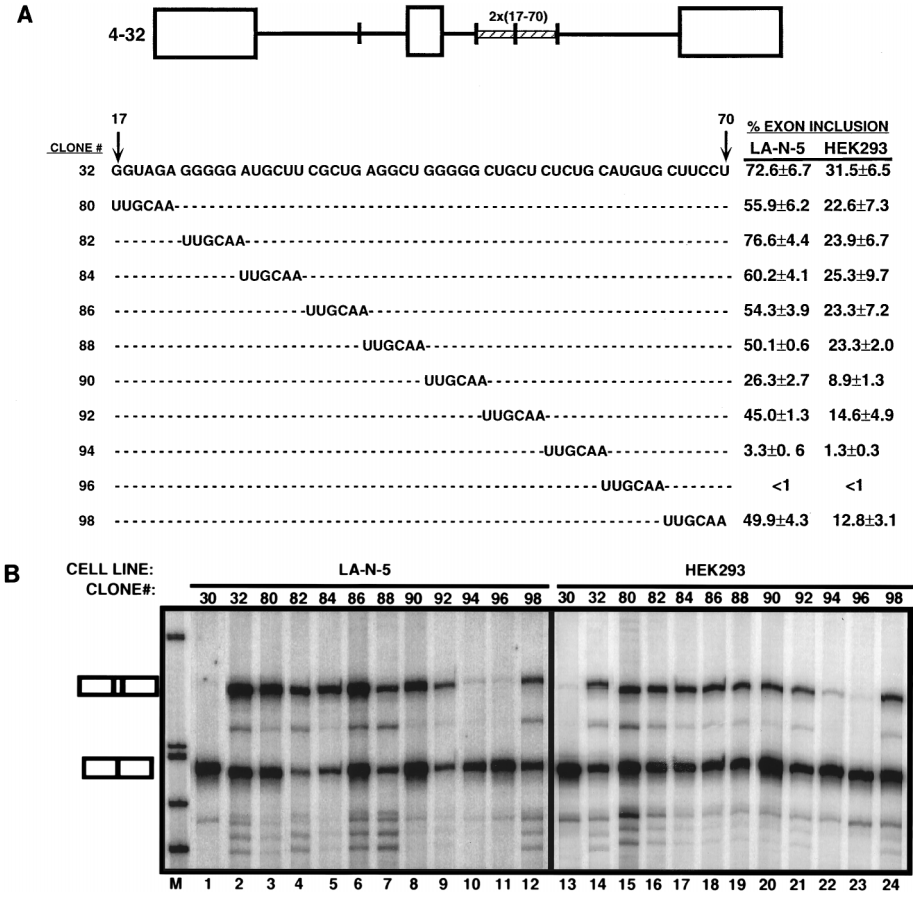

FIG. 5. Individual elements within the core enhancer. (A) Scanning mutagenesis of the *src* intronic sequence from 17 to 70 nucleotides downstream of exon N1. As indicated in the map of DUP4-32 (Fig. 4A), each clone contains two copies of the 17–70 region. The site of each mutation is indicated below the wild-type *src* 17–70 sequence. The amount of exon inclusion for each clone was determined as described for Fig. 2. (B) Primer extension analysis of the clones depicted in panel A. Clone numbers are shown above the lanes. DUP4-30 has a single copy of nucleotides 17 to 70. Other clones have either two copies of the wild-type 17–70 sequence or two copies of the mutated 17–70 sequence. Lane M is a DNA size marker. Lanes 1 to 12 are from LA-N-5 transfections, and lanes 13 to 24 are from HEK293 transfections.

in the 3′ portion of the sequence had much stronger effects. Altering the poly(G) tract, starting at intron nucleotide 44, reduced exon inclusion almost threefold, to 26%, in LA-N-5 cells (clone 4-90 [Fig. 5B, lanes 8 and 20]). By far the largest effect was seen from two adjacent linker mutations in clones 4-94 and 4-96. In clone 4-94, central exon splicing was reduced 22-fold, to 3.3 and 1.3% in LA-N-5 and HEK cells, respectively (lanes 10 and 22). The mutation in clone 4-96 virtually eliminated splicing of the DUP exon in both cell types (lanes 11 and 23). These mutations interrupt a UGCAUG element within the most conserved portion of the enhancer.

Cell-type-specific activation of splicing by a simple repeated element. The scanning mutations identified several possible elements involved in activating splicing by the intronic enhancer. Mutation of the GGGGG tract had a moderate effect on the splicing of the central DUP exon. In contrast, the UGCAUG element appeared to be essential. Mutation of a third sequence element between nucleotides 17 and 23 (GGU AGAG) had only mild effects, reducing splicing by 23%. To test whether these sequences could function without other surrounding elements and to quantify their effects, we inserted them individually into the DUP4-1 clone. A sequence element GGGGCUG was previously implicated in the splicing of a troponin T exon (9). There is an adjacent CUG in the *src* sequence; therefore, we included this in our repeated G-rich element. Each sequence was made in triplicate, with each copy separated by 9 to 10 nucleotides of spacer sequence derived from the globin intron (Fig. 6A). Neither the triple GGGGG CUG sequence nor the triple GGUAGAG sequence had an effect on splicing in this context (Fig. 6B, lanes 2, 4, 6, and 8). In contrast, the triple UGCAUG sequence had a strong effect in LA-N-5 cells, activating splicing to 48% (lane 3). Interestingly, only minimal activation was seen in HEK cells (2.5% exon inclusion [lane 7]). This is much lower than the activation seen in HEK with the entire enhancer sequence and may indicate that the difference in activation in the two cell types results from the use of different elements within the larger enhancer. A single copy of this UGCAUG element had no effect on the splicing of the DUP exon (data not shown). As with the larger enhancer fragments, the cooperative interactions between multiple elements are evidently needed for splicing to be affected.

DISCUSSION

A splicing enhancer and its components. We have dissected the sequence components that regulate the splicing of the c-*src* N1 exon. The sequence with the largest effect on the splicing of this neuron-specific exon is a complex regulatory region located in the downstream intron. We showed previously that

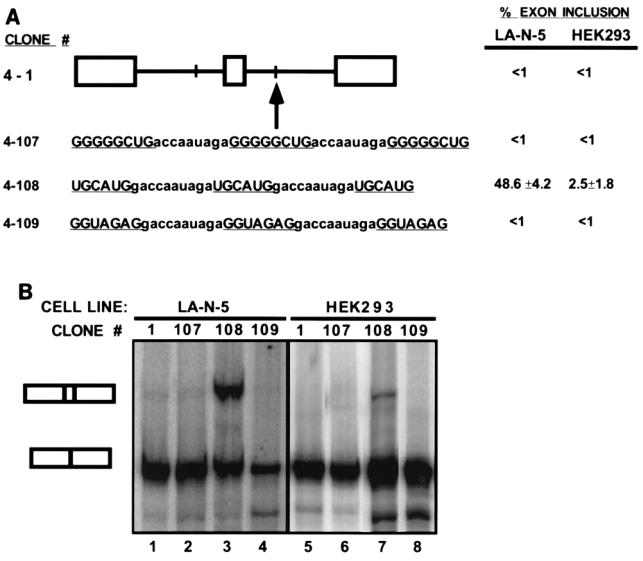

FIG. 6. Activation of splicing by a simple repeated element. (A) DUP4-1 containing only globin sequences, with the arrow indicating the site where the repeated *src* elements were inserted. The inserts contained three copies of GGGGGCUG (clone 4-107), UGCAUG (clone 4-108), or GGUAGAG (clone 4-109) separated by spacer sequences from the globin intron indicated in lowercase letters. The level of exon inclusion was determined as for Fig. 2. (B) Primer extension of clones depicted in panel A. Products are indicated at the left; clone numbers are shown over above the lanes. Lanes 1 to 4 are from LA-N-5 transfections, and lanes 5 to 8 are from HEK293 transfections.

this sequence is critical to the splicing of the N1 exon both in vivo and in vitro (5, 27). We now show that this region alone is sufficient to activate the splicing of a heterologous exon that is normally excluded from the mRNA. This *src* intron sequence is thus a splicing enhancer, activating splice sites that are usually ignored by the general splicing machinery.

The N1 splicing enhancer, extending over at least 120 nucleotides, is quite complex in structure. We find that subportions of the enhancer that do not function alone give strong activation when they are duplicated. Thus, the enhancer is made up multiple copies of smaller elements that can function in various combinations. This explains earlier results where substitution mutations within the *src* gene itself were found to have only small effects, presumably due to the presence of redundant elements within the enhancer (27a). To observe larger effects from the substitution mutations, we tested them as double mutants in a duplicated portion of the enhancer. Some of these mutations had strong effects, identifying two individual enhancer elements.

Mutation of a GGGGG tract reduced but did not eliminate central exon splicing. This element was previously shown to be important for the assembly of splicing regulatory proteins onto the N1 enhancer (27). Similar elements have been implicated in the splicing of exons from the chicken β-tropomyosin and cardiac troponin T genes (9, 32). In the troponin T study, a GGGGCUG sequence could activate the splicing of a test exon when the element was multimerized. This was not the case here; a triple repeat of the GGGGGCUG sequence found in the *src* enhancer had no effect in our assay. Although needed for full enhancer activity, this element in the N1 enhancer apparently requires other enhancer sequences to function.

By far the largest effect on splicing was seen from two adjacent linker mutations in clones 4-94 and 4-96 (Fig. 5). Both mutations altered nucleotides in the sequence, UGCAUG, and nearly eliminated central exon splicing. A mutation in this sequence was previously shown to reduce N1 splicing in a *src* gene containing just this part of the enhancer and also to block the binding of splicing regulatory proteins to the core enhancer in vitro (5, 27). The *src* enhancer contains one exact copy of the UGCAUG sequence in the 17–142 region. There are additional partial matches to the element starting at intron nucleotides 121 and 132 (Fig. 1A). Moreover, a fragment containing these nucleotides (110–142 fragment) activated splicing weakly when added to a clone containing the 37–70 fragment (data not shown). This may indicate that the 37–70 and 110–142 fragments can cooperate to activate splicing. However, this effect was weak, and a single synthetic UGCAUG element did not show this cooperative effect with the downstream portion of the enhancer (data not shown).

Most interestingly, the UGCAUG element by itself had strong effects on splicing when multimerized downstream from the DUP exon. Activation from the multimerized element was strong in the LA-N-5 cells but very weak in HEK293 cells. We also tested this multimerized element in HeLa, 3T3, and N1E 115 neuroblastoma cells. HeLa showed little activity (5% inclusion), whereas 3T3 and N1E cells showed moderate levels of inclusion (15 and 25% respectively [data not shown]). This apparent neural specificity was somewhat surprising because mutation of the UGCAUG element in the 17–70 enhancer fragment affected splicing in both HEK and LA-N-5 cells.

A UGCAUG element has also been implicated in the splicing of exons from the fibronectin and nonmuscle myosin II heavy-chain-B genes (17, 18). The myosin II exon is neuron specific, but the fibronectin exon is not. Multiple copies of the element are found in the intron downstream of the fibronectin EIIIB exon and are required for its splicing. In this system, the UGCAUG element had stronger effects in F9 cells than in HeLa or COS cells (17). The enhancement activity on the fibronectin exon was strongly influenced by the nucleotides flanking the repeated element (17). Thus, the different levels of splicing enhancement that we see in various cell types may be an effect of the sequences chosen to surround the UGCAUG element. Extensive further analyses will be needed to determine how this element cooperates with the sequences around it and which other elements affect its activity.

Tissue specificity and combinatorial control. Some of the neural specificity of the N1 exon stems from the activity of the enhancer. The 17–142 sequence activated splicing three times more strongly in the neuronal LA-N-5 cells than in nonneuronal HEK 293 cells. However, the N1 enhancer is not entirely neuron specific, as it did show some activity in HEK 293 cells. Experiments in an in vitro splicing system indicate that a complex of regulatory proteins assembles onto the core portion of the enhancer (nucleotides 37 to 70) only in neural extracts. However, many of the proteins in this complex are not neuron specific (26, 27). When the whole enhancer is present, these non-neuron-specific proteins may bind strongly enough to affect splicing. Other sequences in the N1 exon region presumably modulate the enhancer to bring about the high degree of tissue specificity seen with the exon as a whole (Fig. 2).

Purine-rich exonic splicing enhancers appear to be less complex than this intronic N1 enhancer. They are generally shorter and contain less diverse sequences (14, 34–36, 43). However, there are similarities between the N1 enhancer and the regulated exonic enhancer in the *dsx* gene. The *dsx* enhancer contains a purine-rich element and a set of 13-nucleotide repeated elements (21). Both of these two types of elements bind to the Tra and Tra-2 proteins in conjunction with specific SR proteins (22). Like the N1 enhancer, the *dsx* enhancer elements must be present in combination or be duplicated to observe enhancement. The *dsx* enhancer loses its Tra dependence and becomes

constitutive when it is moved from its normal context and placed closer to the 3′ splice site that it is regulating (38). This effect is similar to the non-tissue-specific enhancement that we see when the N1 enhancer is moved into the context of the DUP33 clone. Both the N1 and *dsx* enhancers engage in the cooperative assembly of combinations of RNA binding proteins. This is also likely true of the simpler purine-rich enhancers, in that they also require factors in addition to the SR proteins for their function (35, 44). Moreover, like the repeated UGCAUG element, a multimerized SR protein binding site can function as an exonic enhancer (35). For exonic enhancers, there is evidence that the enhancer-protein complex functions by stimulating spliceosome assembly at the upstream 3′ splice site. How the N1 enhancer may interact with the general splicing machinery is still an open question.

The method presented here can be used for testing many different splicing regulatory sequences for their effects on the central DUP33 exon. The low background level of DUP33 exon inclusion allows for sensitive assays of positive-acting sequences. Using this assay, we have identified N1 splicing regulatory sequences that were not observed previously. A purine-rich element from the N1 exon activates low levels of splicing when placed in the DUP33 exon (clone 4-74 [Fig. 2]). This sequence may function as a purine-rich splicing enhancer, although its effect is small. An additional positive-acting sequence was identified between nucleotides 17 and 37 downstream of N1. This sequence clearly strengthens the effect of the downstream enhancer (clones 4-28 and 4-3 [Fig. 3]). The precise element in this region has not been identified, although there is a GGGGG tract similar to an element downstream. Other elements that can be observed to strengthen the effect of the core enhancer are within nucleotides 70 to 110 and 110 to 142 (data not shown). With further analysis, still more regulatory sequences throughout the N1 exon region are likely to be identified. We have also used this system to identify an intronic splicing enhancer adjacent to a neuron-specific exon in the agrin gene. This agrin enhancer seems to be composed of different sequence elements than the N1 enhancer and is active in different cell lines (41a). A similar system has been used by Tian and Kole to characterize exonic enhancer sequences (37). The assay should thus be applicable to a variety of regulated exons and cell types.

Perhaps the most surprising aspect of these results is the wide range of sequences affecting splicing of the N1 exon. Nearly every fragment from this region had some effect on the splicing of the central DUP exon (Fig. 2). The N1 splicing enhancer itself contains an array of sequence elements. Although most studies of splicing regulation have focused on single regulatory elements, it is likely that other regulated exons will show equal complexity. We presume that proteins assemble onto these many RNA elements in various combinations leading to various levels of activation in different cell types. Such a combinatorial system of control will make the question of tissue specificity more difficult to answer. Although there may be some relatively tissue specific splicing factors, there may be no single regulatory sequence whose tissue specificity matches that of the exon as a whole. Instead, a variety of elements, each showing a unique range of activity in different cells, may combine to generate the precise tissue-specific inclusion of an exon. This picture of combinatorial regulation is reminiscent of the complexity of transcriptional enhancers. Splicing enhancer sequences may prove to be equally intricate in structure.

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REFERENCES

1. **Adams, M. D., D. Z. Rudner, and D. C. Rio.** 1996. Biochemistry and regulation of pre-mRNA splicing. Curr. Opin. Cell Biol. **8:**331–339.
2. **Ausubel, F. M., R. Brent, R. E. Kingston, D. D. Moore, J. G. Seidman, J. A. Smith, and K. Struhl (ed.).** 1987. Current protocols in molecular biology. John Wiley and Sons, New York, N.Y.
3. **Balvay, L., D. Libri, M. Gallego, and M. Y. Fiszman.** 1992. Intronic sequence with both negative and positive effects on the regulation of alternative transcripts of the chicken beta tropomyosin transcripts. Nucleic Acids Res. **20:** 3987–3992.
4. **Black, D. L.** 1991. Does steric interference between splice sites block the splicing of a short c-src neuron-specific exon in non-neuronal cells? Genes Dev. **5:**389–402.
5. **Black, D. L.** 1992. Activation of c-src neuron-specific splicing by an unusual RNA element in vivo and in vitro. Cell **69:**795–807.
6. **Black, D. L.** 1995. Finding splice sites within a wilderness of RNA. RNA **1:**763–771.
7. **Brown, M. T., and J. A. Cooper.** 1996. Regulation, substrates and functions of src. Biochim. Biophys. Acta **1287:**121–149.
8. **Caputi, M., G. Casari, S. Guenzi, R. Tagliabue, A. Sidoli, et al.** 1994. A novel bipartite splicing enhancer modulates the differential processing of the human fibronectin EDA exon. Nucleic Acids Res. **22:**1018–1022.
9. **Carlo, T., D. A. Sterner, and S. M. Berget.** 1996. An intron splicing enhancer containing a G-rich repeat facilitates inclusion of a vertebrate micro-exon. RNA **2:**342–353.
10. **Chabot, B.** 1996. Directing alternative splicing: cast and scenarios. Trends Genet. **12:**472–478.
11. **Chan, R. C., and D. L. Black.** 1997. The polypyrimidine tract binding protein binds upstream of the neural cell-specific c-*src* exon N1 to repress the splicing of the intron downstream. Mol. Cell. Biol. **17:**
12. **Chan, R. C., and D. L. Black.** 1995. Conserved intron elements repress splicing of a neuron-specific c-*src* exon in vitro. Mol. Cell. Biol. **15:**6377–6385.
13. **Del Gatto, F., and R. Breathnach.** 1995. Exon and intron sequences, respectively, repress and activate splicing of a fibroblast growth factor receptor 2 alternative exon. Mol. Cell. Biol. **15:**4825–4834.
14. **Dirksen, W. P., R. K. Hampson, Q. Sun, and F. M. Rottman.** 1994. A purine-rich exon sequence enhances alternative splicing of bovine growth hormone pre-mRNA. J. Biol. Chem. **269:**6431–6436.
15. **Dominski, Z., and R. Kole.** 1991. Selection of splice sites in pre-mRNAs with short internal exons. Mol. Cell. Biol. **11:**6075–6083.
16. **Fu, X. D.** 1995. The superfamily of arginine/serine-rich splicing factors. RNA **1:**663–680.
17. **Huh, G. S., and R. O. Hynes.** 1994. Regulation of alternative pre-mRNA splicing by a novel repeated hexanucleotide element. Genes Dev. **8:**1561–1574.
18. **Kawamoto, S.** 1996. Neuron-specific alternative splicing of nonmuscle myosin II heavy chain-B pre-mRNA requires a cis-acting intron sequence. J. Biol. Chem. **271:**17613–17616.
19. **Kramer, A.** 1996. The structure and function of proteins involved in mammalian pre-mRNA splicing. Annu. Rev. Biochem. **65:**367–409.
20. **Levy, J. B., T. Dorai, L. H. Wang, and J. S. Brugge.** 1987. The structurally distinct form of $pp60^{c\text{-}src}$ detected in neuronal cells is encoded by a unique c-*src* mRNA. Mol. Cell. Biol. **7:**4142–4145.
21. **Lynch, K. W., and T. Maniatis.** 1995. Synergistic interactions between two distinct elements of a regulated splicing enhancer. Genes Dev. **9:**284–293.
22. **Lynch, K. W., and T. Maniatis.** 1996. Assembly of specific SR protein complexes on distinct regulatory elements of the Drosophila doublesex splicing enhancer. Genes Dev. **10:**2089–2101.
23. **Manley, J. L., and R. Tacke.** 1996. SR proteins and splicing control. Genes Dev. **10:**1569–1579.
24. **Martinez, R., B. Mathey-Prevot, A. Bernards, and D. Baltimore.** 1987. Neuronal pp60c-src contains a six-amino acid insertion relative to its non-neuronal counterpart. Science **237:**411–415.
25. **McKeown, M.** 1992. Alternative mRNA splicing. Annu. Rev. Cell Biol. **8:**133–155.
26. **Min, H., C. W. Turck, J. M. Nikolic, and D. L. Black.** 1997. A new regulatory protein, KSRP, mediates exon inclusion through an intronic splicing enhancer. Genes Dev. **11:**1023–1036.
27. **Min, H., R. C. Chan, and D. L. Black.** 1995. The generally expressed hnRNP F is involved in a neural-specific pre-mRNA splicing event. Genes Dev. **9:**2659–2671.

27a. **Modafferi, E.** Unpublished data.
28. **Moore, M. J., C. C. Query, and P. A. Sharp.** 1993. Splicing of precursors to mRNA by the spliceosome, p. 303–357. *In* R. F. Gesteland and J. F. Atkins (ed.), The RNA world. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.
29. **Reed, R.** 1996. Initial splice-site recognition and pairing during pre-mRNA splicing. Curr. Opin. Genet. Dev. **6:**215–220.
30. **Ryan, K. J., and T. A. Cooper.** 1996. Muscle-specific splicing enhancers regulate inclusion of the cardiac troponin T alternative exon in embryonic skeletal muscle. Mol. Cell. Biol. **16:**4014–4023.
31. **Sambrook, J., E. F. Fritsch, and T. Maniatis.** 1989. Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.
32. **Sirand-Pugnet, P., P. Durosay, E. Brody, and J. Marie.** 1995. An intronic (A/U)GGG repeat enhances the splicing of an alternative intron of the chicken beta-tropomyosin pre-mRNA. Nucleic Acids Res. **23:**3501–3507.
33. **Staknis, D., and R. Reed.** 1994. SR proteins promote the first specific recognition of pre-mRNA and are present together with the U1 small nuclear ribonucleoprotein particle in a general splicing enhancer complex. Mol. Cell. Biol. **14:**7670–7682.
34. **Tacke, R., and J. L. Manley.** 1995. The human splicing factors ASF/SF2 and SC35 possess distinct, functionally significant RNA binding specificities. EMBO J. **14:**3540–3551.
35. **Tacke, R., Y. Chen, and J. L. Manley.** 1997. Sequence-specific RNA binding by an SR protein requires RS domain phosporylation: creation of an SRp40-specific splicing enhancer. Proc. Natl. Acad. Sci. USA **94:**1148–1153.
36. **Tanaka, K., A. Watakabe, and Y. Shimura.** 1994. Polypurine sequences within a downstream exon function as a splicing enhancer. Mol. Cell. Biol. **14:**1347–1354.
37. **Tian, H., and R. Kole.** 1995. Selection of novel exon recognition elements from a pool of random sequences. Mol. Cell. Biol. **15:**6291–6298.
38. **Tian, M., and T. Maniatis.** 1994. A splicing enhancer exhibits both constitutive and regulated activities. Genes Dev. **8:**1703–1712.
39. **Valcarcel, J., and M. R. Green.** 1996. The SR protein family: pleiotropic functions in pre-mRNA splicing. Trends Biochem. Sci. **21:**296–301.
40. **Wang, Z., H. M. Hoffmann, and P. J. Grabowski.** 1995. Intrinsic U2AF binding is modulated by exon enhancer signals in parallel with changes in splicing activity. RNA **1:**21–35.
41. **Watakabe, A., K. Tanaka, and Y. Shimura.** 1993. The role of exon sequences in splice site selection. Genes Dev. **7:**407–418.
41a. **Wei, N., C. Q. Lin, E. F. Modafferi, W. A. Gomes, and D. L. Black.** A unique intronic splicing enhancer controls the inclusion of the agrin Y exon. RNA, in press.
42. **Wu, J. Y., and T. Maniatis.** 1993. Specific interactions between proteins implicated in splice site selection and regulated alternative splicing. Cell **75:**1061–1070.
43. **Xu, R., J. Teng, and T. A. Cooper.** 1993. The cardiac troponin T alternative exon contains a novel purine-rich positive splicing element. Mol. Cell. Biol. **13:**3660–3674.
44. **Yeakley, J. M., J. P. Morfin, M. G. Rosenfeld, and X. D. Fu.** 1996. A complex of nuclear proteins mediates SR protein binding to a purine-rich splicing enhancer. Proc. Natl. Acad. Sci. USA **93:**7582–7587.