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Conserved Intron Elements Repress Splicing of a Neuron-Specific c-*src* Exon In Vitro

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The neuron-specific N1 exon of the mouse c-*src* transcript is normally skipped in nonneuronal cells. In this study, we examined the sequence requirements for the exclusion of this exon in nonneuronal HeLa cell nuclear extracts. We found that the repression of the N1 exon is mediated by specific intron sequences that flank the N1 exon. Mutagenesis experiments identified conserved CUCUCU elements within these intron regions that are required for the repression of N1 splicing. The addition of an RNA competitor containing the upstream regulatory sequence to the HeLa extract induced splicing of the intron downstream of N1, indicating that the competitor sequence binds to splicing repressor proteins. The similarities between this mechanism for *src* splicing repression and the repression of other regulated exons point to a common role of exon-spanning interactions in splicing repression.

A common mechanism for the regulation of gene expression entails the use of alternative splice sites to produce multiple protein-coding sequences from the same mRNA precursor (pre-mRNA). The regulation of splice site selection is often specific to tissue type or developmental state, so that an alteration in the splice site choice can produce a functional change in the encoded protein. Although examples of regulated pre-mRNA splicing are common, in most cases the mechanisms that govern splice site choice remain unclear (30).

Two of the best-understood systems of alternative splicing are found in the *Drosophila melanogaster* sexual differentiation pathway (27, 33). The female-specific protein Sex-lethal (Sxl) acts as a repressor of the default or male splicing patterns of several transcripts in this genetic pathway. By binding to specific sequence elements in its target transcripts, Sxl blocks the binding of general splicing factors to certain splice sites (15, 19, 36, 37, 40). In contrast to Sxl, the transformer (tra) and transformer-2 (tra-2) proteins are positive regulators of splicing in the sex determination pathway. These proteins activate the splicing of a female-specific exon in the doublesex (*dsx*) transcript. *dsx* exon 4 has a poor splice acceptor site, which contributes to the default pattern of exon 4 skipping in male flies (7). In females, the tra and tra-2 proteins bind to a specific sequence element in *dsx* exon 4, recruit splicing factors of the SR family, and apparently enhance spliceosome assembly at the splice acceptor site upstream (13, 18, 35, 41, 43, 44, 48). These *Drosophila* systems thus provide examples of both positive and negative control of splicing and of how general and transcript-specific splicing factors may interact to affect the use of a particular splice site.

Although mechanisms of alternative splicing in mammalian cells are less well characterized, they are likely to be similar to the *Drosophila* systems. For example, RNA sequence elements that positively or negatively regulate splice site selection in certain mammalian transcripts have been identified (8, 11, 12, 17, 23, 25, 47, 49). SR proteins are also found in mammalian cells and are known to have effects on splice site choice (16). However, highly cell type-specific or transcript-specific splicing factors, equivalent to Sxl or tra, have not been identified in mammalian cells.

Alternative splicing in the mammalian nervous system controls the production of many proteins important for neuronal development and function (6). We are studying the splicing of the mouse c-*src* transcript as a model for understanding neuron-specific splicing regulation. The *src* primary transcript contains an 18-nucleotide exon (N1) that is inserted between exons 3 and 4 in neurons and skipped in other cells (24, 26). Another neuron-specific exon (N2) is also occasionally inserted between exons N1 and 4 (32). The inclusion of the N1 and N2 exons gives rise to the neural *src* (n-*src*) mRNAs. Previously, we showed that the N1 exon is skipped when expressed from a mouse *src* gene transfected into nonneuronal HeLa cells or in vitro when the *src* transcript is incubated in HeLa cell nuclear extracts (3, 4). In contrast, the N1 exon is spliced into the *src* mRNA in transfected LA-N-5 neuroblastoma cells in vivo and in a WERI-1 retinoblastoma cell nuclear extract in vitro (3, 4). Analyses of *src* splicing both in vivo and in vitro indicate that the neuronal inclusion of the N1 exon requires regulatory sequences within nucleotides 38 to 142 downstream of exon N1 (3). The 5′ portion of this region (nucleotides 38 to 70) binds to regulatory factors that allow N1 splicing in the neuronal cells (29). This more defined regulatory sequence is called the downstream control sequence (DCS). In addition, there seem to be other regulatory sequences downstream of the DCS in the 70 to 142 region (3). Thus, the regulated splicing pattern of the N1 exon is under positive control in neurons, while the default splicing pattern appears to be the exon skipping.

The causes of exon N1 skipping in nonneuronal cells are complex. The short length of the N1 exon is one critical feature in preventing its constitutive use. Thus, lengthening the N1 exon from 18 nucleotides to 109 nucleotides allows its efficient inclusion in nonneuronal cells (4). We postulated previously that this short-exon effect may stem from steric hindrance occurring between two spliceosome complexes assembling in close proximity. Subsequent data from other systems indicate that the skipping of a short internal exon is more likely due to

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the lack of an enhancing interaction between the splice sites on each side of the exon (9, 10, 14, 34, 41, 42). The short-exon effect in the N1 exon could be accounted for by the need for this exon-bridging interaction to allow spliceosome assembly in the absence of positive regulatory factors.

In this study, we look at the splicing in vitro of single-intron, two-exon *src* transcripts. In these transcripts, the N1 splice sites are regulated by a different repression mechanism from that mediating the short-exon effect. The inhibition of splicing of these single-intron *src* transcripts did not depend on the presence of adjacent splice sites but instead on the binding of a regulatory factor(s) to conserved intron elements flanking the N1 exon.

MATERIALS AND METHODS

DNA constructions. All DNA constructs were made by standard cloning techniques (1, 38). BS3, BS9, and BS27 were previously described as B3, B9, and B27, respectively (3). BS7 is similar to BS3 except that the sequence from the *Pst*I site (exon 3) to the *Apa*I site (intron A) is deleted, removing 19 nucleotides at the 3′ end of exon 3 and leaving just the last 55 nucleotides of intron A, containing the N1 acceptor site. The following clones are similar to BS7 except for alterations at the N1 acceptor site. BS49, BS50, and BS74 have the N1 acceptor replaced with the adenovirus acceptor from clone S16 (4), the *src* exon 4 acceptor from clone S17 (4), and the β-globin exon 2 acceptor from pSP64HBΔ6 (22), respectively. BS51 has the same intron A branch point mutation as clone S9 (4), which has the branch point AG changed to CC. BS73 was generated by primer-directed PCR mutagenesis of the BS7 polypyrimidine tract. It has the mutation shown in Fig. 3.

BS41 was made by an *Asp*718 site (T7 start site)-to-*Apa*I site (intron A) deletion of BS3, which is equivalent to BS7 minus the exon 3 sequences. BS82 has all but the last 4 nucleotides of intron A removed from BS7. BS103 has the CUCUCU(G/C) tracts at nucleotides 52 to 58 and nucleotides 94 to 100, downstream of exon N1, mutated by primer-directed PCR mutagenesis to GAGAG AC and GAGAGAG, respectively. BS14 has a 13-nucleotide *Cla*I-to-*Rsa*I deletion in BS3, removing the N1 donor site. BS13 contains the *Cla*I-*Bam*HI fragment from clone SS15 (3) subcloned in place of the equivalent fragment in BS3, deleting intron B nucleotides 38 to 142 from BS3. BS54 is similar to BS13 except that it was constructed in the BS7 background.

PCR amplification. The PCR amplification for the primer-directed mutagenesis was performed as described before (3). For BS103, the annealing temperature was 55°C for 25 cycles. For BS73, the first five cycles were carried out at a 45°C annealing temperature, followed by 20 cycles at a 55°C annealing temperature.

Cell extract preparation. Nuclear extracts were prepared as described before (3) except that the high-salt extraction buffer (buffer C) contained 0.42 M NaCl instead of 0.4 M KCl (1). The protein concentrations were determined by the Coomassie protein assay (Pierce) with bovine gamma globulin (Sigma) as a standard.

Transcription of substrates and competitors. All substrates and competitors were transcribed from linearized plasmids with T7 RNA polymerase as described before (3). The two-exon intron B templates were made by linearizing the plasmid at the *Not*I site within exon 4. The two-exon intron A templates were made by linearizing the plasmid at the *Bam*HI site within intron B. The RNA competitors containing acceptor sequences were transcribed from plasmids linearized either at the *Cla*I site in the middle of exon N1 or at the *Bsu*36I site terminating one nucleotide upstream of exon N1.

Splicing reactions. The conditions of the in vitro splicing reactions were as previously described (3) except that 2% polyethylene glycol (molecular weight, 3350) was used in place of 2.5% polyvinyl alcohol. The splicing reaction mixes were incubated at 30°C for 4 h except for the intron A transcripts in Fig. 4, which were incubated for 3.5 h. In each reaction, the nuclear extracts were brought up to a 15-μl volume with buffer DG (3). For the HeLa reactions, 12 μl (216 μg) of nuclear extract protein was used. For Fig. 1, the WERI-1 splicing reaction mixes contained 15 μl (57 μg) of nuclear extract protein. The HeLa/WERI-1 mixture consisted of 10 μl (180 μg) of HeLa nuclear extract protein and 5 μl (22.5 μg) of WERI-1 nuclear extract protein. Each reaction mix contained 50,000 cpm (≈0.25 to 0.5 ng) of labeled RNA substrate. The gel separation of the splicing products was done as described before (3). The amount of spliced product in each lane was quantified with a PhosphorImager (Molecular Dynamics).

For the competition assays, 10 μl (180 μg) of HeLa nuclear extract was incubated with 2 μl of RNA competitor (0.1 to 15 pmol) in the presence of ATP and $MgCl_2$ at 30°C for 10 min prior to the addition of the labeled BS7 transcript and polyethylene glycol. The HeLa/WERI-1 positive controls contained 5 μl (90 μg) of HeLa extracts and 8 μl (36 μg) of WERI-1 extracts. The mixtures were then incubated at 30°C for an additional 4 h, followed by RNA isolation and electrophoresis of the labeled RNA. The final concentrations of ATP, $MgCl_2$, creatine phosphate, and polyethylene glycol were the same as in the splicing reaction mixes described above.

RNA-protein complex analysis. Native gel analysis of prespliceosome complexes was done by a method similar to that of Konarska (20). Reaction mixtures (25 μl) containing HeLa or WERI-1 nuclear extract were incubated for 40 min under splicing conditions as described above to allow spliceosome complexes to form. In this case, 100,000 cpm of labeled transcript and a 2.5% final concentration of polyvinyl alcohol were used. After the incubation, 5 μl of heparin stop solution (50% glycerol, 50 mM Tris base, 50 mM boric acid, 1 mM EDTA, 1.5 mg of heparin sulfate per ml, 0.25% xylene cyanol) was added, and each reaction mix was incubated at 30°C for 3 min. Of this mixture, 5 μl was loaded onto a 0.5% agarose–3% acrylamide gel (60:1, acrylamide-bisacrylamide) in modified TBE buffer (50 mM Tris base, 50 mM boric acid, 1 mM EDTA). The gel was run at 200 V until the xylene cyanol reached the bottom of the gel, approximately 14 cm. The gel was then dried and subjected to autoradiography.

RESULTS

A single-intron *src* substrate is regulated in vitro. We previously reported that the neuron-specific splicing of c-*src* can be recapitulated in vitro by using a synthetic *src* RNA substrate and an extract of WERI-1 retinoblastoma cells (3). The BS3 transcript contains exons 3, N1, and 4 and shortened introns between them (Fig. 1A). In a HeLa cell extract, the BS3 transcript is spliced from exon 3 to exon 4, skipping exon N1, as nonneuronal cells do in vivo (3). In contrast, both exons 3 and N1 are spliced to exon 4 in the neuronal WERI-1 extract, removing introns C and B, respectively. Once intron B has been removed, the upstream intron A can be spliced in either extract (3). Thus, neuronal cells seem to control N1 splicing by regulating the splicing of intron B.

Unlike the BS3 transcript, in a synthetic substrate in which exon 3 is fused to exon N1, intron B is spliced in either extract (BS27 [Fig. 1A]) (3). To investigate what prevented intron B splicing in the longer three-exon transcript, we replaced portions of intron A into the BS27 substrate. We found that replacing the upstream N1 acceptor sequence into BS27 was sufficient to repress intron B splicing in the HeLa extract (BS7 [Fig. 1A]). The BS7 RNA was only weakly spliced when incubated in the HeLa extract (Fig. 1B, lane 1). In contrast, the BS27 transcript, which lacks the N1 splice acceptor sequence, was readily spliced in this extract (Fig. 1B, lane 3).

In addition to exhibiting intron B repression in the HeLa extract, the BS7 substrate showed regulation of intron B splicing in the neuronal extract. In Fig. 1B, the level of BS7 splicing in the WERI-1 extract is significantly enhanced over that in the nonneuronal extract (lanes 1 and 2). Typically, this splicing enhancement is about sixfold. As with the three-exon BS3 substrate, the splicing of BS7 in the WERI-1 nuclear extract requires neuronal factors bound to regulatory sequences in intron B (3, 29). Also as seen before, intron B was spliced in a mixture of HeLa and WERI-1 extracts, indicating that the neuronal activation of N1 splicing is dominant over the default pattern of N1 exclusion (Fig. 2C, lane 2) (3, 29). The WERI-1-complemented HeLa extract behaved similarly to the WERI-1 extract alone in all assays used here; therefore, both extracts will hereafter be referred to as WERI-1 extract.

Specific elements within the N1 acceptor sequence repress intron B splicing. The difference between BS7 and BS27 splicing could stem from the N1 splice acceptor's repressing intron B splicing in BS7. Alternatively, the removal of intron A in BS27 could place an enhancing element closer to intron B. Moreover, if the N1 splice acceptor represses splicing, it is not clear whether any splice acceptor sequence could have this effect. Thus, we next examined whether the block to BS7 splicing by the N1 splice acceptor was sequence specific or if it was a distance effect. When the N1 splice acceptor in BS7 was replaced with acceptor sequences from the adenovirus major late exon 2, *src* exon 4, or human β-globin exon 2, the splicing

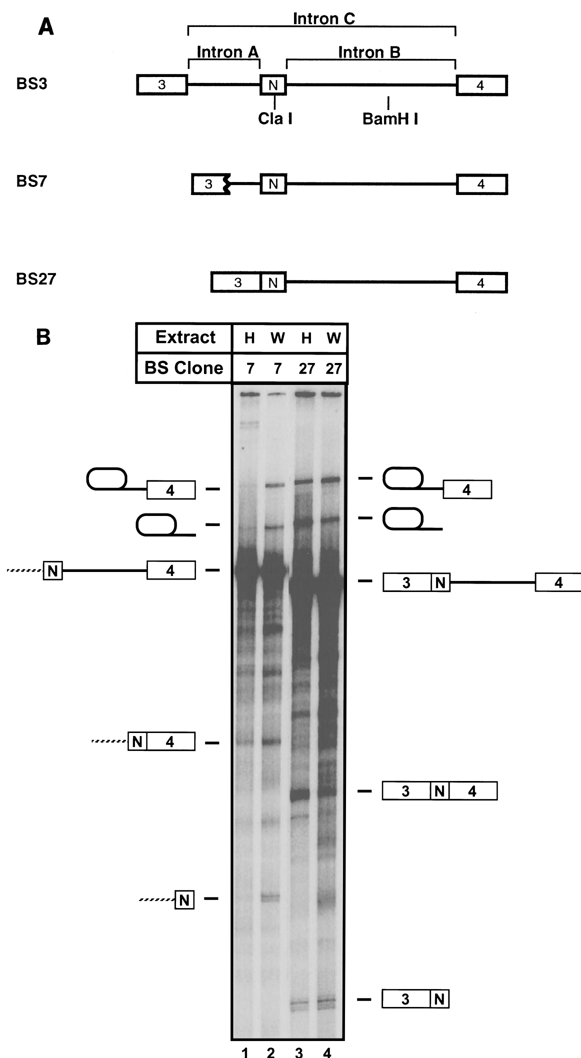

FIG. 1. Two-exon BS7 transcript is repressed in the HeLa extract and spliced in the WERI-1 extract. (A) Maps of the two-exon *src* intron B constructs. The three-exon BS3 construct is shown as a reference. The lines represent introns, and the boxes represent exons. The exon and intron designations are indicated. The BS7 and BS27 constructs contain deletions of BS3. BS27 has intron A removed. BS7 has a deletion from 19 nucleotides 5′ of the exon 3 donor site to 55 nucleotides upstream of exon N1, where the jagged line of exon 3 denotes the lack of a 5′ splice site. (B) In vitro splicing reactions with the two-exon *src* transcripts. The uniformly labeled BS7 and BS27 transcripts were incubated with HeLa and WERI-1 extracts under splicing conditions. The RNA from each reaction mix was fractionated by gel electrophoresis followed by autoradiography. The lariat intermediate, intron lariat, unspliced transcript, spliced product, and 5′ exon intermediate are diagrammed from top to bottom beside the gel. These products were identified previously (3). The bands near the top of the gel in the BS7 lanes may be lariats from a cryptic donor site. The transcript and extract used in each reaction mix are indicated at the top. Transcripts were spliced in HeLa extract (lanes 1 and 3) or in WERI-1 extract (lanes 2 and 4).

of intron B increased significantly in the HeLa extract (Fig. 2B, compare lane 2 with lanes 3 to 5). These substitutions improved splicing by 6- to 10-fold over that in BS7, similar to the deletion of the entire acceptor site (BS27 [Fig. 2B, lane 1]). This indicates that a specific feature of the N1 splice acceptor represses the splicing of intron B in the HeLa extract.

In the WERI-1 extract, these splice acceptor changes showed less dramatic effects on intron B splicing (Fig. 2C, compare lane 2 with lanes 3 to 5). All of the RNAs were spliced to similar levels, within 30% of that of the BS7 control.

We next made specific mutations in the N1 splice acceptor to determine the features required to repress splicing. Mutations in the branch point and the terminal AG dinucleotide of the N1 splice acceptor did not enhance splicing in either extract (data not shown). A comparison of the mouse, human, and chicken N1 splice acceptor sequences identified a conserved nine-nucleotide sequence within the polypyrimidine tract (Fig. 3). This sequence contains a CUCUCU element, which is repeated two to three times in the N1 splice acceptor of all three transcripts and was noted previously (4). Both of these CUC UCU motifs in BS7 were mutated (BS73 [Fig. 2A and 3]). This mutation led to an approximately 10-fold splicing enhancement in the HeLa extract (Fig. 2B, lane 6), while splicing in the WERI-1 extract increased slightly (about 30% [Fig. 2C, lane 6]). Thus, the CUCUCU motifs are required for the repression of intron B. Interestingly, the globin splice acceptor contains a single CUCUCUCU motif, but this sequence alone was apparently not sufficient to repress intron B splicing (Fig. 2B, lane 5) (see below).

Sequences within intron B itself are required for splicing repression in the HeLa extract. Two additional CUCUCU elements are found within intron B at nucleotides 52 to 57 and nucleotides 94 to 99 downstream of exon N1 (Fig. 3). These downstream elements are within the region necessary for the activation of n-*src* splicing in neuronal cells and are also present in the human *src* pre-mRNA (3, 29). To test the involvement of these downstream elements in intron B splicing, they were mutated to GAGAGA (BS103). This mutation caused a sevenfold increase in intron B splicing in the HeLa extract (BS103 [Fig. 2B, lane 7]). A four- to sixfold increase in splicing was observed when each intron B CUCUCU element was changed individually (data not shown). Thus, in addition to the N1 acceptor, these downstream sequences are required for the repression of intron B. Combining the upstream (BS73) and the downstream (BS103) CUCUCU mutations did not further enhance intron B splicing over that by BS73 or BS103 (data not shown). This lack of an additive effect suggests that all four elements are required together to repress intron B splicing.

In previous experiments, the intron B nucleotides 38 to 142 were shown to be important for N1 inclusion in neuronal cells (3, 29). Mutation of the CUCUCU elements within this region did not affect intron B splicing in the WERI-1 extract (Fig. 2C, compare lanes 2 and 7). The activation of intron B splicing in WERI-1 extract must require sequences other than these CU CUCU elements (29).

Repression of the upstream intron A requires sequences downstream of N1. Since sequences in intron A repressed the splicing of intron B, we wanted to test the converse, whether sequences in intron B repressed the splicing of intron A. To examine intron A splicing, we created a second series of two-exon substrates. The BS3-Bam substrate contains exon 3, a shortened intron A, exon N1, and 153 nucleotides of intron B, including the N1 donor site and the downstream control region but terminating before the exon 4 acceptor (Fig. 4A). The BS9 RNA has the same exon 3 and intron A sequences as BS3-Bam, but exon N1 is fused to exon 4, removing all of intron B. As shown previously, intron A is efficiently removed from BS9 in either extract (Fig. 4B, lanes 1 and 5) (3). In contrast, splicing of the BS3-Bam transcript was repressed in the HeLa extract (Fig. 4B, lane 2). Unlike intron B splicing, the BS3-Bam intron A transcript also failed to splice in the WERI-1 extract (Fig. 4B, lane 6).

To localize the sequences downstream of N1 that inhibit intron A splicing, two deletion mutants were tested. The BS14 transcript is similar to BS3-Bam except that 13 nucleotides

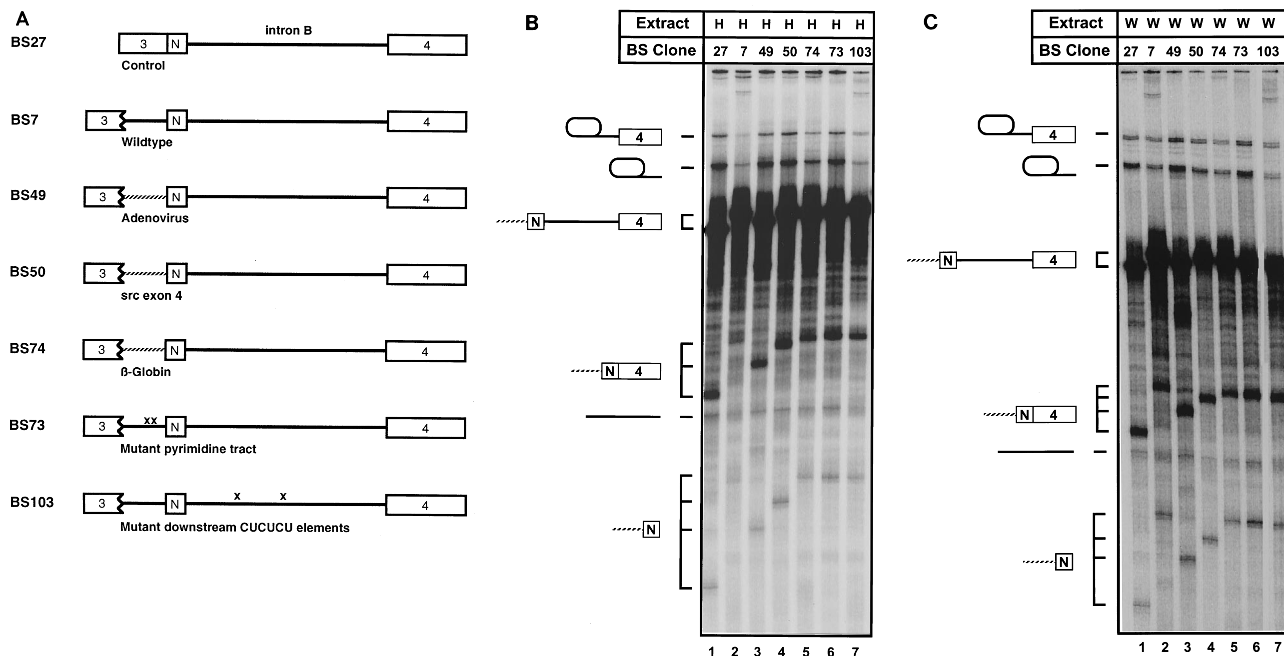

FIG. 2. Sequences in the N1 splice acceptor and the downstream control region are required for splicing repression of BS7 in the HeLa extract. (A) Maps of intron B *src* constructs containing different upstream splice acceptors and a mutant downstream control region. The constitutively spliced BS27 transcript is used as a positive control for splicing. Substituted acceptor sequences are indicated by stippled lines. BS49, BS50, and BS74 have acceptor sequences upstream of N1 from the adenovirus major late exon 2, *src* exon 4, and human β-globin exon 2, respectively. Mutations at the CUCUCU elements in BS73 and BS103 are indicated by X's. (B and C) In vitro splicing reactions in HeLa (H) extract and HeLa/WERI-1 (W) extract mixture, respectively. The extract and splicing substrate are indicated at the top. Differences in the relative mobilities of the lariat bands in panels B and C are caused by slight variations in polyacrylamide solutions.

encompassing the N1 donor site are deleted. The removal of the N1 donor site did not improve intron A splicing in either extract (Fig. 4B, lanes 4 and 8). In contrast, removing intron B nucleotides 38 to 142 in BS13 allowed intron A splicing in both extracts (Fig. 4B, lanes 3 and 7). Thus, sequences within intron B repress the splicing of the upstream intron A, similar to the N1 acceptor sequence's repressing the splicing of the downstream intron B.

Inhibition of intron B splicing does not correlate with prespliceosome assembly upstream at the N1 acceptor site. In an earlier model for the repression of N1 splicing, the splicing complex formed at the N1 splice acceptor was thought to block spliceosome assembly at the N1 splice donor (4). In this steric hindrance model, a spliceosome complex should not assemble at the intron A splice acceptor under conditions in which intron B is spliced. All of the substrates containing a new splice acceptor upstream from N1 showed splicing of the downstream intron B (Fig. 2). Therefore, we examined prespliceosome complex formation at the new acceptor sites in these RNAs to test whether splicing was occurring in the presence of an upstream splicing complex.

Labeled transcripts from the various clones were incubated in nuclear extract to assemble splicing complexes and resolved by native gel electrophoresis (20). The transcripts were terminated within intron B to prevent complex formation at the exon 4 acceptor while allowing assembly on the N1 splice acceptor (Fig. 5A). RNAs containing just a splice acceptor site will usually form two complexes identifiable by native gel electrophoresis (30). Complex H is a nonspecific complex consisting of heterogeneous nuclear ribonucleoprotein particle (hnRNP) proteins and the RNA (2, 28). In the presence of ATP and the splicing factor U2AF, the U1 and U2 small nuclear RNPs

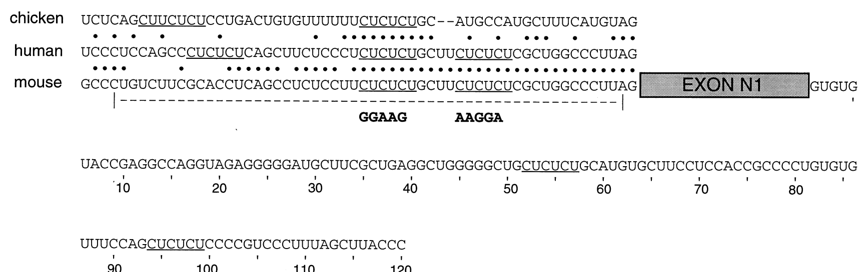

FIG. 3. Map of the intron sequences flanking the *src* N1 exon. The sequences immediately upstream of the N1 exon from the mouse, human, and chicken *src* genes and the mouse intron sequences downstream of the N1 exon are shown. Each dot beneath the chicken and human sequences indicates a nucleotide match with the mouse acceptor sequence. The CUCUCU repeat elements are underlined. The BS73 mutation within the mouse N1 polypyrimidine tract is indicated below. The dashed line denotes the RNA competitor sequence that derepresses BS7 splicing (see Fig. 7). The nucleotides downstream of exon N1 are numbered from the first nucleotide of intron B. The downstream control region defined previously (3) includes nucleotides 38 to 142.

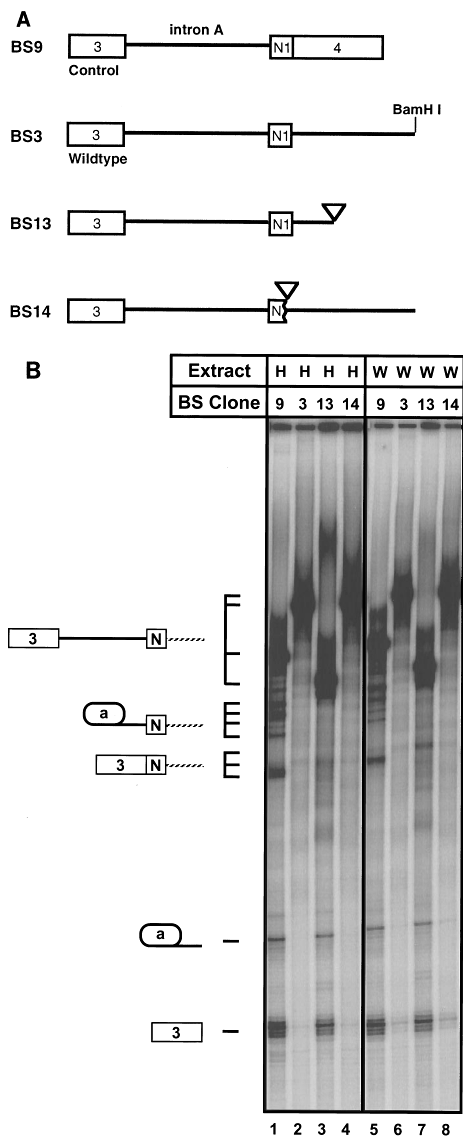

FIG. 4. Intron A splicing is repressed by the downstream control region. (A) Maps of the intron A *src* constructs. The BS9 transcript contains exon N1 already spliced to exon 4. The BS3-Bam substrate is transcribed from the BS3 plasmid (3) linearized at the *Bam*HI site before the *src* exon 4 splice acceptor. BS13 has the downstream control region deleted from nucleotide 38 to the *Bam*HI site. BS14 has the N1 donor site (13 nucleotides, AUGUCAG/GUGUGU) deleted; the jagged line denotes the lack of a 5′ splice junction. (B) In vitro splicing reactions with the intron A transcripts. The unspliced transcripts, lariat intermediate, spliced product, intron lariat, and 5′ exon intermediate are diagrammed to the left of the gel. These products were identified previously (3). The spliced product of BS13 is relatively unstable in the extracts. Splicing is best measured by the level of intron lariat or 5′ exon intermediate. Lanes 1 to 4, splicing reactions in HeLa extract; lanes 5 to 8, splicing reactions in HeLa/WERI-1 extract mixture. Constructs: BS9 (lanes 1 and 5), BS3 (lanes 2 and 6), BS13 (lanes 3 and 7), and BS14 (lanes 4 and 8).

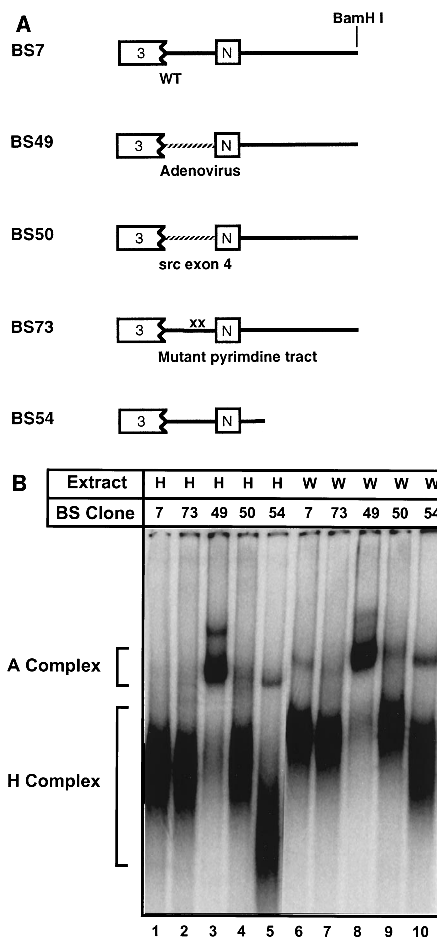

FIG. 5. Prespliceosome complex formation at the N1 acceptor does not correlate with intron B splicing. (A) Maps of the RNA probes. All probes are truncated at the *Bam*HI site within intron B upstream of the exon 4 splice acceptor. BS7 contains the wild-type N1 splice acceptor and downstream control region. The various substitution mutations in the N1 splice acceptor and the downstream control region are as shown in Fig. 2A and 4A. BS54 has intron B nucleotides 38 to 142 deleted. (B) RNA-protein complex gel. The uniformly labeled transcripts were incubated with nuclear extract under splicing conditions for 40 min to allow complexes to form. The complexes were then resolved on a native agarose-polyacrylamide composite gel and detected by autoradiography. The H and A complexes formed on these probes are indicated to the left of the gel. The identity of complex A was confirmed by its dependence on ATP and its sensitivity to U2 snRNA degradation (data not shown). Lanes 1 to 5, reactions in HeLa extract; lanes 6 to 10, reactions in WERI-1 extract. Constructs: BS7 (lanes 1 and 6), BS73 (lanes 2 and 7), BS49 (lanes 3 and 8), BS50 (lanes 4 and 9), and BS54 (lanes 5 and 10).

(snRNPs) assemble onto a 3′ splice site to form prespliceosome complex A (21, 28, 50). This complex is blocked from further assembly by the absence of an upstream 5′ splice site. All six transcripts tested showed complex H formation (Fig. 5B). The BS7 transcript showed no complex A formation in the HeLa extract (Fig. 5B, lane 1) and a slight amount of complex A formation in the WERI-1 extract (Fig. 5B, lane 6). As expected, introducing purines into the polypyrimidine tract decreased the amount of complex A formed in the WERI-1 extract (BS73 [Fig. 5B, lane 7]). When the strong adenovirus 3′ splice site was substituted for the N1 acceptor, substantial complex A formation was observed in both HeLa and WERI-1 extracts (BS49 [Fig. 5B, lanes 3 and 8]). This RNA also gave a larger complex in the HeLa extract; this may be complex B, arising from a cryptic donor site in the RNA. In contrast to the adenovirus acceptor, the *src* exon 4 acceptor gave only weak complex A formation (BS50 [Fig. 5B, lanes 4 and 9]). Recall that all three mutant substrates (BS49, BS50, and BS73) were spliced readily in the HeLa extract (Fig. 2B). Under these conditions, prespliceosome complex A formed efficiently on the BS49 upstream splice site, whereas the BS73 transcript does not show complex assembly. Thus, there is no apparent correlation, positive or negative, between complex A forma-

tion upstream of exon N1 and the splicing of the downstream intron. Apparently, steric hindrance from an upstream spliceosome does not repress intron B splicing in these two-exon transcripts. Conversely, the presence of complex A at the N1 acceptor site does not seem to enhance splicing of the downstream intron, consistent with findings that positive exon-bridging interactions require a minimum exon length (9, 10, 34, 42).

The native gel analysis also indicates that intron sequences downstream of exon N1 inhibit complex A formation at the N1 splice acceptor. In both extracts, the deletion of intron B nucleotides 38 to 142 led to an enhancement of complex A formation at the upstream N1 acceptor site (BS54 [Fig. 5B, lanes 5 and 10]). The downstream control region apparently represses upstream intron splicing at a step prior to prespliceosome complex A formation.

The N1 acceptor site binds to splicing repressor molecules. The N1 splice acceptor assembled a spliceosome complex very poorly. This could result from binding of other molecules to this sequence, preventing spliceosome assembly and also mediating the repression of the downstream intron. To test whether specific repressor molecules were binding to the N1 acceptor site and inhibiting intron B splicing, we used an RNA competition assay (Fig. 6A). Unlabeled RNAs containing the N1 acceptor sequence were incubated in the HeLa extract prior to the addition of the labeled BS7 transcript. The BS7-Cla competitor contains sequences from the transcription start site to the *Cla*I site within exon N1 of BS7. Normally, the BS7 substrate is spliced poorly in the HeLa extract (Fig. 6B, lane 1). At low levels of competitor (20, 40, and 60 nM), BS7 splicing was enhanced to the level observed in the WERI-1 extract (Fig. 6B, compare lanes 1 and 2 with lanes 3 to 5). Higher levels of competitor inhibited splicing (80 and 100 nM [Fig. 6B, lanes 6 and 7]), presumably because the excess RNA bound to general splicing factors. This competition assay was repeated with a broader concentration range of competitor; splicing was enhanced at 12 to 72 nM competitor, corresponding to a 200- to 1,200-fold excess over the substrate (data not shown). The BS7-Cla RNA competitor had no enhancing effect on the splicing of BS27 or other non-*src* splicing substrates (data not shown). Thus, this enhancement is not likely due to a nonspecific splicing activation but rather to the sequestering of molecules that specifically repress BS7 splicing.

To test whether other splice acceptor sequences had the same effect as BS7-Cla, we tested RNA competitors bearing the adenovirus (BS49) and β-globin (BS74) acceptor sequences (Fig. 6A). Recall that transcripts from these clones were readily spliced in the HeLa extract (Fig. 2B). As expected, neither BS49-Cla nor BS74-Cla was an effective competitor in derepressing BS7 splicing (Fig. 6B, compare lanes 3 to 7 with lanes 8 to 12 and lanes 18 to 22). These competitors inhibited the already low level of splicing. The adenovirus and β-globin acceptor signals presumably lack sequences necessary to bind the repressor molecules, although both competitors contain functional acceptor sequences. Significantly, a competitor from the BS73 clone, with mutations in the CUCUCU motifs of the N1 polypyrimidine tract, also failed to derepress splicing (Fig. 6B, lanes 13 to 17). Thus, the N1 splice acceptor specifically binds repressor molecules that inhibit intron B splicing, and the CUCUCU elements are necessary for this binding.

Deletions of the BS7-Cla competitor defined more precisely the sequences responsible for the derepression of BS7 splicing (Fig. 7A). As shown in Fig. 7B, the BS7-Cla competitor again derepressed BS7 splicing in the 16 to 72 nM range (lanes 3 to 6). The BS51-Cla competitor, which has the branch point AG dinucleotide changed to CC, derepressed BS7 splicing at similar concentrations (Fig. 7B, compare lanes 3 and 4 with lanes

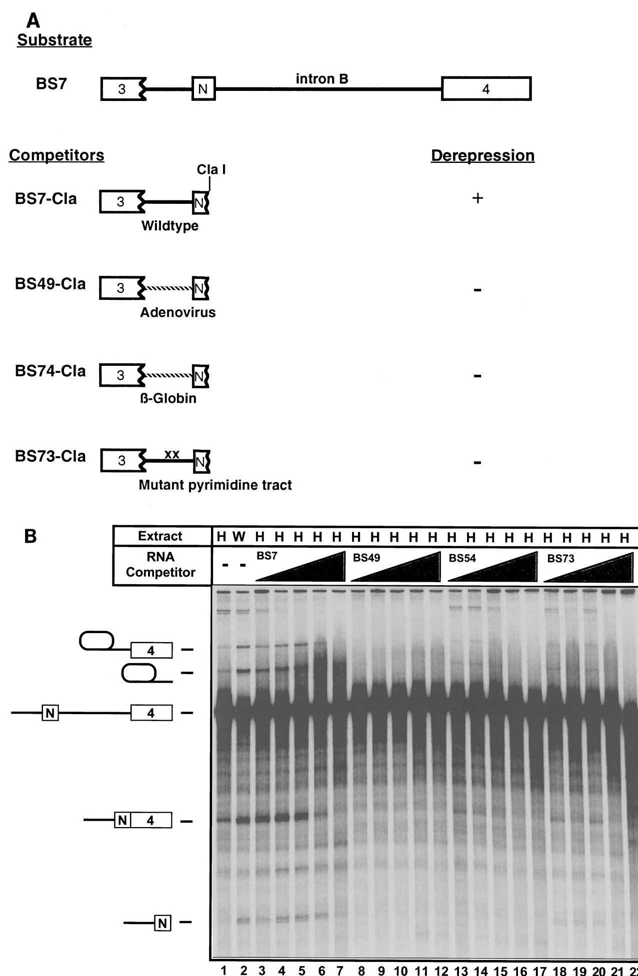

FIG. 6. N1 splice acceptor RNA specifically binds repressor molecules in the HeLa extract. (A) Maps of the labeled BS7 substrate and the various unlabeled RNA competitors. The competitors are truncated in the middle of exon N1 at the *Cla*I site. The mutations in the splice acceptors are shown in Fig. 2A. The ability of the competitor to derepress BS7 splicing in the HeLa extract is indicated by a plus or minus sign to the right of the competitor diagram. (B) Splicing competition assay. The HeLa nuclear extract was incubated with 20, 40, 60, 80, or 100 nM competitor RNA before the addition of the labeled BS7 splicing substrate. The increasing competitor concentration is indicated by the increasing height of the triangle. A negative splicing control with the BS7 transcript in HeLa extract without competitor is shown in lane 1. A positive splicing control with the BS7 transcript in a HeLa/WERI-1 extract mixture is shown in lane 2. Lanes 3 to 22 contain BS7 transcript in HeLa extract containing BS7-Cla competitor (lanes 3 to 7), BS49-Cla competitor (lanes 8 to 12), BS73-Cla competitor (lanes 13 to 17), or BS74-Cla competitor (lanes 18 to 22).

11 and 12). Deleting the exon N1 sequences (BS51-Bsu [Fig. 7B, lanes 19 and 20]) or the remaining exon 3 sequences (BS41-Cla [lanes 7 and 8]) still allowed the RNA to compete for repressor binding. Thus, the branch point, exon 3, and exon N1 sequences are all unnecessary for the derepression effect. Consistent with the CUCUCU element mutation (BS73 [Fig. 6B]), the deletion of the polypyrimidine tract and other intron sequences (BS82-Cla) abolished the derepression effect (Fig. 7B, lanes 15 to 18). In competition experiments with polyribonucleotides, the addition of poly(CU) RNA again derepressed BS7 splicing in HeLa extracts, whereas poly(C) and poly(U) RNA had no effect (data not shown). Therefore, the sequences important for repressor binding include the CUCUCU elements and perhaps more of the polypyrimidine tract.

FIG. 7. Derepression effect of the RNA competitors is dependent on the N1 polypyrimidine tract. (A) Maps of the mutant competitors. The various RNA competitors have either deletions or mutations in the BS7-Cla sequences. BS41-Cla has the exon 3 sequences deleted. BS82-Cla has an internal deletion of BS7-Cla, removing the N1 splice acceptor sequences; the deleted sequence is denoted by the dashed line in Fig. 3. The BS51 competitor contains an AG-to-CC change at the N1 branch point; the truncation at the *Bsu*36 I site removes the last nucleotide of intron A and the exon N1 sequences. (B) Splicing competition assay. The competitors were titrated by mass rather than by molar amounts, as in Fig. 6. The HeLa nuclear extract was incubated with 0.02, 0.1, 0.2, or 0.4 μg of competitor before the addition of the labeled BS7 transcript. A negative splicing control with the BS7 transcript in HeLa extract without competitor is shown in lane 1. A positive splicing control with the BS7 transcript in a HeLa/WERI-1 extract mixture is shown in lane 2. Lanes 3 to 22 contain the BS7 transcript in HeLa extract containing BS7-Cla competitor (lanes 3 to 6), BS41-Cla competitor (lanes 7 to 10), BS51-Cla competitor (lanes 11 to 14), BS82-Cla competitor (lanes 15 to 18), or BS51-Bsu competitor (lanes 19 to 22).

DISCUSSION

Nonneuronal repression of the *src* N1 exon is mediated by flanking intronic elements. This paper describes a repression mechanism regulating the splicing of the *src* N1 exon. Normally, the short internal N1 exon is subject to repression because of its suboptimal length (4). It was proposed earlier that this short-exon effect was due to steric hindrance between spliceosome complexes assembling on both sides of the short N1 exon. However, we show here that prespliceosome complex A can form upstream of N1 without hindering the splicing of the downstream intron B. This argues against the steric hindrance model. An alternative explanation is that the length of a very short exon may prohibit positive interactions between the two splice sites flanking the exon (14, 34, 42). These positive exon-bridging interactions have been observed in other exons, in which the need for this positive effect can be overcome by strengthening the splice sites, either by improving their match to the consensus sequence or by specific *trans*-acting factors (9, 10, 14, 34, 46). In fact, *trans*-acting factors are needed to activate the N1 splice site(s) in neurons (3, 29); without these neuronal factors, the N1 exon is skipped.

In the experiments presented here, the N1 exon is present as either a 5′- or 3′-terminal exon rather than an internal exon. In these truncated *src* transcripts, the repression of intron B is maintained by the interaction of specific repressor molecules with CUCUCU elements and perhaps other sequences flanking the N1 exon. Mutations that disrupt these negative elements activate N1 splicing in a two-exon transcript. However, deletion of these elements in a three-exon transcript had little effect in vivo, presumably because in this context the exon is still repressed by its short length (3, 4, 10). Thus, the role of this CUCUCU element-mediated repression is not clear when N1 is in its normal context as an internal exon. Nevertheless, the specificity of the CUCUCU element repression and its similarity to other repression systems argue for an important role in N1 regulation (see below).

The repression of N1 splicing apparently does not occur through a simple blockage mechanism, where regulatory proteins binding at or near the repressed splice site block its utilization by general splicing factors. Otherwise, one set of the CUCUCU elements on either side of the exon should still confer some degree of repression. Instead, the results suggest a coupling between the upstream and downstream regulatory regions. Copies of the element CUCUCU are found both within the N1 splice acceptor sequence and downstream of the N1 donor site. Mutating the upstream and downstream regulatory sequences individually or in combination derepressed intron B splicing to comparable levels. Moreover, the presence of the downstream regulatory region inhibited spliceosome complex assembly across the N1 exon at the upstream splice acceptor. Together, these results imply that factors must be bound to both the upstream and downstream sequences to see repression, perhaps forming a bridge that loops out the exon and sequesters the splice sites.

Repeated sequence elements are also involved in repressing the splicing of the *Drosophila sxl* and rat α-tropomyosin exons (11, 15, 36). Results obtained with these systems again indicate that regulatory proteins must bind both sides of the regulated exon to interfere with splicing (11, 15, 36). Multiple U_8 elements in the *Sxl* transcript facilitate the cooperative binding of its splicing regulator, Sxl (45). For *src*, removing one of the two downstream CUCUCU elements in *src* gave nearly full derepression of the N1 exon (data not shown), again suggesting that the repressor molecules bind cooperatively to the CUC UCU elements. Exon-spanning interactions between repeated sequence elements may be a common means of repressing splicing of regulated exons.

Multiple levels of regulation. The regulation of the c-*src* N1 exon appears to involve at least three distinct mechanisms. These include the short-exon effect that represses N1 splicing when it is present as an internal exon (4), the activation of splicing in neuronal cells that involves neuron-specific complex assembly at the downstream control sequence (3, 29), and finally, this second repression mechanism involving factors binding on either side of the N1 exon. In most other analyzed cases of alternative splicing, two principal factors govern splice site selection. First, the strength of the regulated splice site(s)

relative to that of surrounding splice sites establishes a default splicing pathway. A second process involving regulatory factors then alters the balance of splice site use by either strengthening or weakening specific splice sites. *src* regulation apparently uses two specific sets of regulatory factors to both repress and activate splicing.

The need for the seemingly redundant repression mechanisms is obscure. The short-exon effect may not repress N1 splicing sufficiently, so that an additional level of control is needed. The repression factors binding to the *src* RNA may also regulate the splicing of other *src* exons. A second neuron-specific *src* exon, N2, is occasionally inserted between exons N1 and 4 but is never included in the absence of N1 (32). A coupling mechanism may be needed to prevent the insertion of N2 in cells in which N1 is also skipped. Another possibility is that the mechanisms of activation and repression are functionally related. Other studies in our laboratory have identified a specific protein complex that assembles onto the downstream control sequence in the WERI-1 splicing extract (29). This complex contains non-tissue-specific proteins found in both HeLa and WERI-1 cells as well as at least one neuron-specific protein. The non-tissue-specific proteins may repress splicing, while the addition of a neuron-specific factor could alter the complex and activate splicing. In transcription initiation, regulatory factors may repress transcription in one context but activate transcription in another context (5, 31, 39). The purification and the identification of the factors that bind at the N1 acceptor site and at the downstream regulatory sequences should prove illuminating.

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