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Combinatorial control of a neuron-specific exon

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ABSTRACT

The mouse c-*src* gene contains a short neuron-specific exon, N1. N1 exon splicing is partly controlled by an intronic splicing enhancer sequence that activates splicing of a heterologous reporter exon in both neural and nonneural cells. Here we attempt to dissect all of the regulatory elements controlling the N1 exon and examine how these multiple elements work in combination. We show that the 3′ splice site sequence upstream of exon N1 represses the activation of splicing by the downstream intronic enhancer. This repression is stronger in nonneural cells and these two regulatory sequences combine to make a reporter exon highly cell-type specific. Substitution of the 3′ splice site of this test exon with sites from other exons indicates that activation by the enhancer is very dependent on the nature of the upstream 3′ splice site. In addition, we identify a previously uncharacterized purine-rich sequence within exon N1 that cooperates with the downstream intronic enhancer to increase exon inclusion. Finally, different regulatory elements were tested in multiple cell lines of both neuronal and nonneuronal origin. The individual splicing regulatory sequences from the *src* gene vary widely in their activity between different cell lines. These results demonstrate how a simple cassette exon is controlled by a variety of regulatory elements that only in combination will produce the correct tissue specificity of splicing.

Keywords: alternative splicing; neuron; regulatory sequence

INTRODUCTION

Alternative pre-mRNA splicing is a common means of gene regulation in eukaryotes that allows the generation of multiple protein isoforms from a single gene (for reviews see Wang & Manley, 1997; Y. Wang et al., 1997). The control of pre-mRNA splicing pattern can be very precise and there are many examples of exons in mammalian cells whose inclusion is highly tissue specific. A variety of pre-mRNA sequence elements and regulatory proteins are known to affect the splicing patterns of different RNA transcripts. Although these regulatory components must ultimately alter spliceosome assembly at certain splice sites, the interactions between the general splicing apparatus and its regulators are mostly unknown. In particular, the molecular events that determine the tissue-specific use of an exon in mammalian cells have not been described.

One difficulty in understanding the precise nature of an exon's regulation is the diversity of mechanisms that can affect its splicing. Splicing enhancer elements activate the use of splice sites that are otherwise ignored by the general splicing machinery (Hertel et al., 1997; Wang & Manley, 1997), whereas repressor sequences are thought to block recognition of certain splice sites. The tissue-specific expression of a particular splicing pattern often depends on a combination of positive and negative control.

Splicing enhancers can be classified by their location in either exons or introns. These two types of enhancer are thought to be mechanistically different and to bind different types of regulatory proteins. Exonic splicing enhancer sequences are often bound by a group of factors called SR proteins (Fu, 1995; Chabot, 1996; Manley & Tacke, 1996; Valcarcel & Green, 1996; Caceres & Krainer, 1997). Proteins in the SR family contain one or two RNA binding domains of the RRM type and a C-terminal domain rich in serine-arginine dipeptides. SR proteins are essential for constitutive splicing and also play an important role in the selection of alternative splice sites. In particular, SR proteins bound with other factors to exonic splicing enhancers are thought to promote spliceosome assembly to upstream 3′ splice sites, although the mechanism for this effect on splicing is not yet clear (Wang et al., 1995; Zuo & Maniatis, 1996; Rudner et al., 1998). In contrast to ex-

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onic enhancers, intronic splicing enhancers are often found in introns downstream of regulated exons, and have not been shown to bind SR proteins (Balvay et al., 1992; Black, 1992; Huh & Hynes, 1994; Del Gatto & Breathnach, 1995; Sirand-Pugnet et al., 1995; Carlo et al., 1996; Kawamoto, 1996; Ryan & Cooper, 1996; Modafferi & Black, 1997; Wei et al., 1997; Lim & Sharp, 1998). Although both exonic and intronic enhancers can be found associated with highly tissue-specific exons, their activity and the known proteins that bind to them are thus far not highly cell-type specific.

Like enhancer sequences, splicing repressor or silencer elements are diverse in sequence and can be found in both exons and introns. Splicing repression is also mediated by specific proteins, the best understood of which is the female specific Sex-lethal protein (Sxl) in *Drosophila* (Inoue et al., 1990; McKeown, 1992; Horabin & Schedl, 1993; Valcarcel et al., 1993; Samuels et al., 1994; Deshpande et al., 1996; J. Wang et al., 1997). In mammalian cells, the polypyrimidine tract binding protein (PTB) is also an apparent negative regulator of splicing (Valcarcel & Gebauer, 1997; Y. Wang et al., 1997). PTB binds to sequence elements in the polypyrimidine tract of certain 3′ splice sites. In some cases this binding is thought to directly block spliceosome assembly, whereas in other systems, dispersed PTB binding sites are needed to repress the use of an exon. PTB is widely expressed, but is implicated in the repression of certain tissue-specific exons (Mulligan et al., 1992; Norton, 1994; Lin & Patton, 1995; Ashiya & Grabowski, 1997; Chan & Black, 1997; Gooding et al., 1998). It is not clear how the repression of these exons by PTB might be relieved in particular cells.

We are using the mouse c-*src* gene as a model system to study the splicing of a neuron-specific exon. C-*src* encodes a 60-kDa protein tyrosine kinase that is expressed in a wide variety of cells (Cooper, 1990). In neurons, an 18-nt exon (N1) is included in the *src* mRNA between the constitutive exons 3 and 4, generating a neural-specific *src* mRNA and protein (Levy et al., 1987; Martinez et al., 1987). This neural form of the *src* protein contains an insertion in the SH3 domain that alters its binding to other proteins (Brown & Cooper, 1996; Santoro et al., 1997).

Mutagenesis analyses have identified features of the N1 exon region that control its splicing (Black, 1991, 1992; Modafferi & Black, 1997). The short length of the exon is essential to its repression in nonneural cells, and is thought to prevent positive exon bridging or exon definition interactions from forming across the exon. In addition to the negative effect of exon length, there are also negative elements in the 3′ splice site upstream of the exon and in the intron downstream that repress the in vitro splicing of N1 in nonneuronal cell extracts (Chan & Black, 1995). In neuronal cells, an intronic splicing enhancer downstream from exon N1 is essential for activating splicing of the exon (Black, 1992; Min et al., 1995; Modafferi & Black, 1997). The N1 splicing enhancer can activate the splicing of a heterologous 33-nt exon within a transfected reporter gene (Modafferi & Black, 1997), but in this context, the splicing is not as tissue specific as the whole N1 exon.

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 thought to be mediated at least partly by PTB (Chan & Black, 1997). The most phylogenetically conserved portion of the intronic enhancer (intron nt 37–70), called the Downstream Control Sequence (DCS), assembles a multiprotein complex that is apparently required for splicing of the exon in vitro and is more stable in neuronal extracts (Min et al., 1995, 1997). This DCS complex contains at least five polypeptides including the proteins hnRNP F, KSRP, and hnRNP H (Min et al., 1995, 1997; Chou et al., 1999). These identified proteins seem to be required for N1 splicing, but are not specific to the neuronal extract. The factors that stabilize their binding in neuronal extracts and the determinants of N1 exon tissue specificity are still unknown.

In this study, we examine how individual elements from the *src* gene combine to regulate splicing of a test exon and thus determine its tissue specificity. We find that the combination of a nonneuronal repressor sequence in the 3′ splice site and a partially neuron-specific downstream enhancer produces a highly neuron-specific exon. We also find that the N1 exon region contains additional splicing regulatory elements, and that individual elements vary widely in their activity between different cell lines.

RESULTS

The N1 exon 3′ splice site inhibits splicing induced by the downstream enhancer

Because of the redundancy of the *cis*-acting sequences that affect the splicing of the N1 exon, it has proven difficult to analyze the role of individual elements through mutagenesis of the *src* gene itself. To better identify these elements and examine how they interact, we developed a gain-of-function assay for splicing regulatory sequences that can induce the splicing of a heterologous exon (Modafferi & Black, 1997). Portions of the *src* gene were transferred into a splicing reporter gene, DUP4-1, containing a short test exon (33 nt) that is normally skipped by the splicing apparatus (Fig. 1A,B). This exon is a hybrid of β-globin exons 1 and 2 sequences and is lacking the enhancer elements identified in the globin exons (Dominski & Kole 1991; Schaal & Maniatis, 1999). Using this heterologous gene, the *src* intron sequence from 17–142 nt downstream of exon N1 was shown to act as a splicing enhancer,

strongly inducing splicing of the test exon (Modafferi & Black, 1997). The level of enhancer-dependent splicing was highest in LA-N-5 neuroblastoma cells. However, significant splicing of the test exon was also seen in nonneural HEK293 cells. Thus, although the enhancer was essential to N1 exon splicing, it was not apparently the sole determinant of the exon's tissue specificity. This indicated that other sequence elements needed to be combined with the enhancer to give the precise tissue-specific regulation of the N1 exon. One candidate for such a sequence was the 3′ splice site upstream of the N1 exon. This sequence had already been shown to inhibit the in vitro splicing of the N1 exon in HeLa cell nuclear extracts (Chan & Black, 1995).

A series of new plasmids were constructed with the 3′ splice site of the *src* N1 exon replacing the globin 3′ splice site of the test exon (Fig. 1C). These plasmids were transfected into LA-N-5 and HEK293 cells. After transient expression, cytoplasmic RNA was isolated and analyzed for test exon inclusion. The results of this primer extension analysis are shown in Figure 1D. As reported previously, a fragment containing the *src* N1 3′ splice site and the 5′ half of exon N1 inserted into the DUP construct resulted in a small enhancement of test exon splicing in LA-N-5 cells (8.1% exon inclusion; Fig. 1D, lane 1), whereas no splicing was observed in the HEK293 cells. As expected, the *src* downstream intronic enhancer induced high levels of exon inclusion in LA-N-5 cells (68%; Fig. 1D, lane 2) and moderate levels in HEK293 cells (20%; Fig. 1D, lane 6). The combination of these two elements had a strikingly different effect: in RNAs carrying the enhancer, the *src* N1 3′ splice site strongly decreased exon inclusion (DUP4-29). Most interestingly, the level of this splicing repression differed between the two cell types. In the LA-N-5 cells, exon inclusion dropped about one-third when the N1 3′ splice site was present, from 68% to 43%. In contrast, in nonneuronal HEK293 cells, exon inclusion dropped nearly 10-fold in the presence of the 3′ splice site (from 20% to 2.3%; Fig. 1D, compare lanes 6 and 7). Thus, the combination of the N1 3′ splice site and the enhancer makes splicing of the test exon much more cell-type specific.

The DUP4-29 transcript contains a portion of the N1 exon along with the N1 3′ splice site. DUP4-75 contains just the *src* N1 3′ splice site sequence, leaving the test exon sequence unchanged. DUP4-75 transcripts show the same repression of splicing in HEK293 cells as DUP4-29, indicating that the 3′ splice site is sufficient for the effect (Fig. 1D, lanes 4 and 8). In summary, there are *cis*-acting sequences within the N1 exon 3′ splice site that repress the activation of splicing by the downstream intronic enhancer. Moreover, the combination of these upstream and downstream sequences determines the different levels of splicing in the two cell types.

The relative number of enhancer and repressor elements determines the overall level of splicing

We previously defined the N1 splicing enhancer within the intron downstream of the N1 exon. The 17–142 sequence (encompassing intron nt 17–142) was the strongest fragment tested, and including more sequence did not further activate splicing of the test exon. Smaller enhancer fragments induced lower levels of splicing, whereas duplicating the enhancer sequences strongly increased splicing. (Black, 1992; Modafferi & Black, 1997). We next tested whether such an enhancer duplication could overcome the repression by the N1 3′ splice site. Plasmids were constructed with repeated portions of the downstream enhancer paired with either the globin or the N1 3′ splice site. As seen previously, the 37–142 sequence was weaker than the 17–142 sequence, inducing 25% exon inclusion in LA-N-5 cells and 8% inclusion in HEK293 cells (DUP4-3; Fig. 2B, lanes 1 and 11). DUP4-22, which combines the N1 3′ splice site with the 37–142 sequence, exhibited an increase in exon inclusion in the LA-N-5 cells (45%), whereas the HEK293 cells showed repression of splicing (~1%). The increase in splicing in LA-N-5 cells is apparently due to synergy between the N1 exon sequence included in these clones and a portion of the enhancer (see Fig. 7). As seen before, duplication of the 37–142 fragment further activates splicing (DUP4-18; Fig. 2B, lanes 3 and 13). Splicing of the test exon increased to 90% in LA-N-5 cells and 70% in HEK293 cells. Combining the N1 3′ splice site with the duplicated 37–142 enhancer reduced exon inclusion in both cell types (DUP4-23). In LA-N-5 cells, exon inclusion dropped from 90% to 70% (Fig. 2B, compare lanes 3 and 4). In the HEK293 cells, exon inclusion was reduced fivefold, from 70% to 14%. Thus, even when multiple enhancer elements induce high levels of splicing, the repression from the N1 3′ splice site was greater in the HEK293 cells than in the LA-N-5 cells.

In earlier results, intron nucleotides 37–70 of the downstream enhancer did not induce splicing of the test exon (Modafferi & Black, 1997). When this sequence element was combined with the N1 3′ splice site there was a small amount of exon inclusion in the LA-N-5 cells (12%) and no detectable splicing in the HEK293 cells (DUP4-6; Fig. 2B, lanes 6 and 16). Duplicating the 37–70 sequence makes it into a strong enhancer yielding 45% exon inclusion in LA-N-5 cells and 19% in HEK293 cells (DUP4-20; Fig. 2B, lanes 7 and 17). When the globin 3′ splice site is replaced with the N1 3′ splice site (DUP4-26; Fig. 2B, lanes 8 and 18), this splicing was reduced to 43% in LA-N-5 cells, and to 1% in HEK293 cells.

Three copies of the 37–70 sequence resulted in still greater splicing of the exon (clone 4-17; Fig. 2B,

lanes 9 and 19). However, unlike other enhancer fragments, combining the N1 3′ splice site with this triple 37–70 sequence did not repress splicing (DUP4-27; Fig. 2B, lanes 10 and 20). Compared to the triple enhancer alone, this combination of elements gave higher exon inclusion in the LA-N-5 cells (84%), and splicing in HEK293 cells was relatively unchanged (49% to 52%). Thus, the particular combination of downstream enhancer elements affects the ability of the N1 3′ splice site to repress splicing. The overall level of splicing is determined by the relative numbers and types of positive and negative elements.

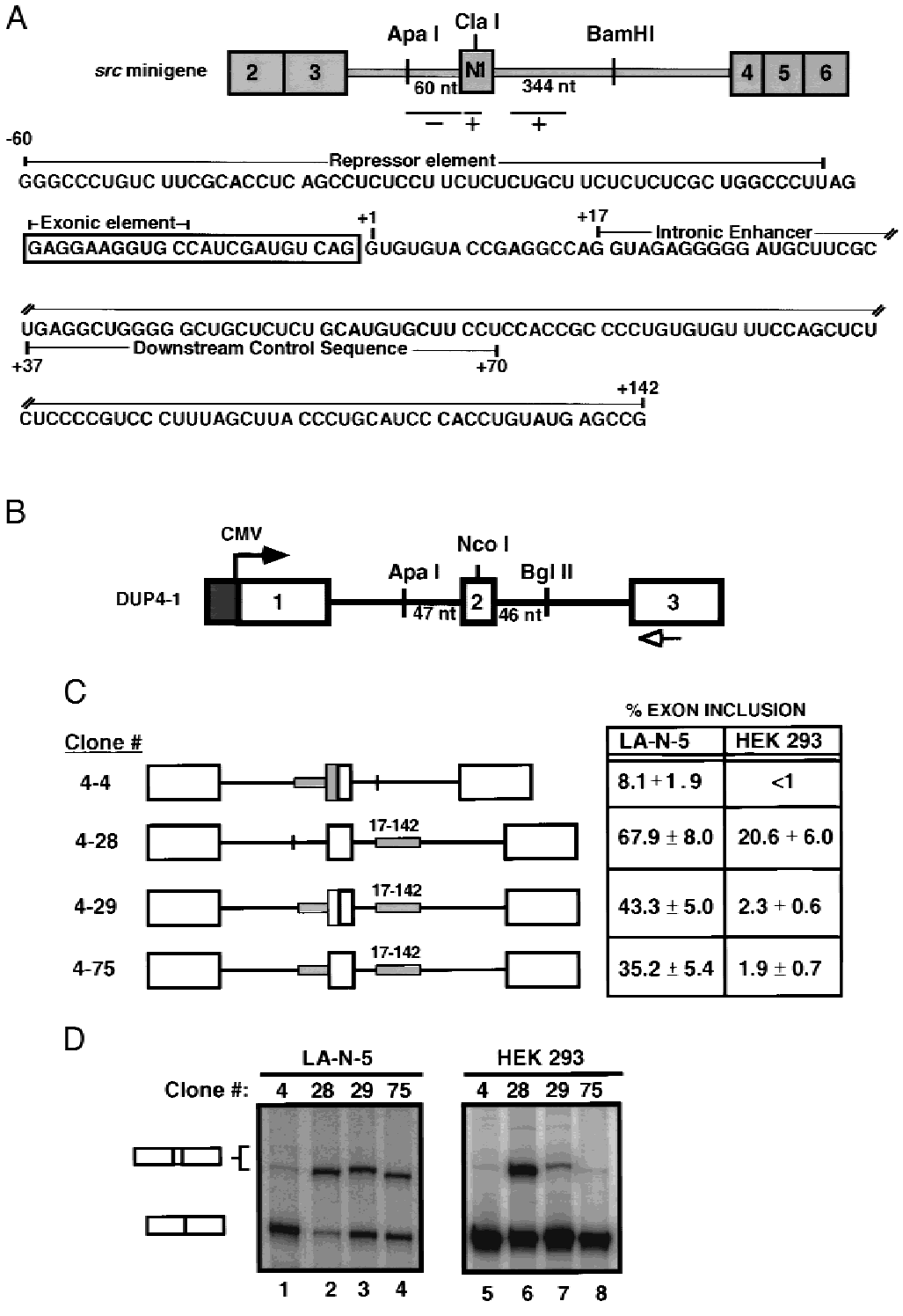


FIGURE 1. (*Legend on facing page.*)

Duplication of the N1 3′ splice site sequence increases splicing repression

Duplication of elements from the downstream enhancer strengthens their activating effect on splicing. We next tested whether multimerizing the repressor elements increases their effect and whether these elements need to be within the functioning 3′ splice site. We constructed plasmids where the polypyrimidine tract region of the N1 3′ splice site was duplicated or placed upstream of the globin 3′ splice site. The AG dinucleotide at the intron 3′ terminus was not duplicated to prevent the formation of a new functional splice site. Inserting an additional copy of the N1 polypyrimidine tract just upstream of the N1 exon branchpoint resulted in complete skipping of the test exon in both cell types (DUP4-104; Fig. 3B, lanes 3 and 10). Activation of splicing from the downstream intron enhancer elements was nearly eliminated with the duplicated polypyrimidine tract (DUP4-137 and DUP4-136; Fig. 3B, lanes 4, 5, 11, and 12).

The N1 polypyrimidine tract also had a potent effect when placed upstream of the globin branchpoint (DUP4-178 and DUP4-180). The DUP4-180 construct contains one copy of the repressor sequence in combination with the 17–142 enhancer. In spite of the presence of the enhancer, this construct exhibited no test exon splicing in the HEK293 cells (Fig. 3B, lane 14), and very little splicing in the LA-N-5 cells (Fig. 3B, lane 7). In control experiments, these sites with extra polypyrimidine tracts were shown to splice properly when tested in the context of a larger constitutive exon (see Fig. 6). Thus, the added upstream repressor sequence did not completely inactivate the 3′ splice sites downstream. Instead, the enhancer appears unable to function when the repressor is moved upstream of its normal position. The ability of cells to overcome the repression is dependent on both the number of negative elements and their position relative to the 3′ splice site.

Repression by the N1 3′ splice site does not require a specific downstream enhancer element

The downstream splicing enhancer contains multiple cooperative sequence elements. The repression of splicing by the N1 3′ splice site could be directed through specific types of enhancer elements or could be a general repression affecting splicing activated by any means. We previously constructed a series of linker scanning mutations in tandem copies of the core of the intronic enhancer (nt 17–70 downstream from the N1 exon; Modafferi & Black, 1997). The duplicated 17–70 sequence (DUP4-32) induced 72% exon inclusion in LA-N-5 cells and 32% in HEK293 cells. Mutations in this enhancer sequence reduce its activity, with the mutations in clones 90, 94, and 96 having the largest effect (Fig. 4 and Table 1; Modafferi & Black, 1997). If the repression required a single specific element of the enhancer, one of these scanning mutations might eliminate repression from the 3′ splice site without affecting activation by the enhancer. Thus, we tested these same linker scanning mutations of the enhancer in combination with the N1 3′ splice site.

These enhancer mutants are diagrammed in Figure 4A and the data on their splicing is presented in Table 1. The results indicate that no single element in this multielement enhancer is essential for repression by the 3′ splice site. This lack of a strong effect from any of the enhancer mutations may stem from the presence of redundant repressible elements in the enhancer. Alternatively, the repressor may have a general effect on any enhancer complex that assembles downstream.

Although the N1 3′ splice site repressed the activity of all the mutant enhancers in HEK293 cells, the repression did vary between the different mutants. The level of repression can be quantified by the ratio of exon inclusion between the pairs of clones containing

FIGURE 1. The N1 exon 3′ splice site inhibits activation of splicing by the downstream splicing enhancer. **A**: Map and sequences of the src minigene. The introns and exons are shown as narrow and wide gray boxes, respectively. The N1 exon is boxed in the sequence and the adjacent intron sequence is numbered upstream and downstream from this exon. The regulatory sequences described in the text are the upstream repressor, the exonic enhancer element, and the intronic enhancer and its core region, the downstream control sequence or DCS. These are noted with bars above or below the sequence and with bars and + or − signs below the diagram. **B**: Map of the parent DUP minigene. DUP exon 1 is human β-globin exon 1. DUP exon 3 is human β-globin exon 2. DUP exon 2 is a 33-nt hybrid of β-globin exon 1 with its 5′ splice site and β-globin exon 2 with its 3′ splice site. This plasmid is as described in Dominski & Kole (1991) with the modifications described in Modafferi & Black (1997). The open arrow indicates the position of the primer extension primer used to assay exon 2 inclusion. The CMV promoter is denoted with a closed arrow. **C**: Diagrams of globin/src hybrid constructs. Globin sequences are indicated as open boxes and solid lines. Src sequences are indicated as gray boxes. Clone numbers are indicated to the left. DUP4-4 contains the N1 exon 3′ splice site and the 5′ half of exon N1. The *src* intron sequence from 17–142 nt downstream of exon N1 was inserted into the downstream intron in DUP4-28. DUP4-29 contains the 3′ splice site and 5′ half of exon N1, and the downstream 17–142 enhancer, whereas DUP4-75 contains only the 3′ splice site of exon N1 with the 17–142 sequence. Average exon inclusion with standard deviations are shown to the right of each construct. **D**: Primer extension analysis of cytoplasmic RNA from LA-N-5 and HEK293 cells transiently expressing the clones diagrammed in **C**. Clone numbers are indicated along the top. Lanes 1–4 contain RNA from LA-N-5 cells. Lanes 5–8 contain RNA from HEK293 cells. The identity of each band is indicated at the left as either the exon skipped (lower) or the exon included (upper) product. The size of exon 2 is either 33 nt (DUP4-28 and DUP4-75) or 34 nt (DUP4-4 and DUP4-29).

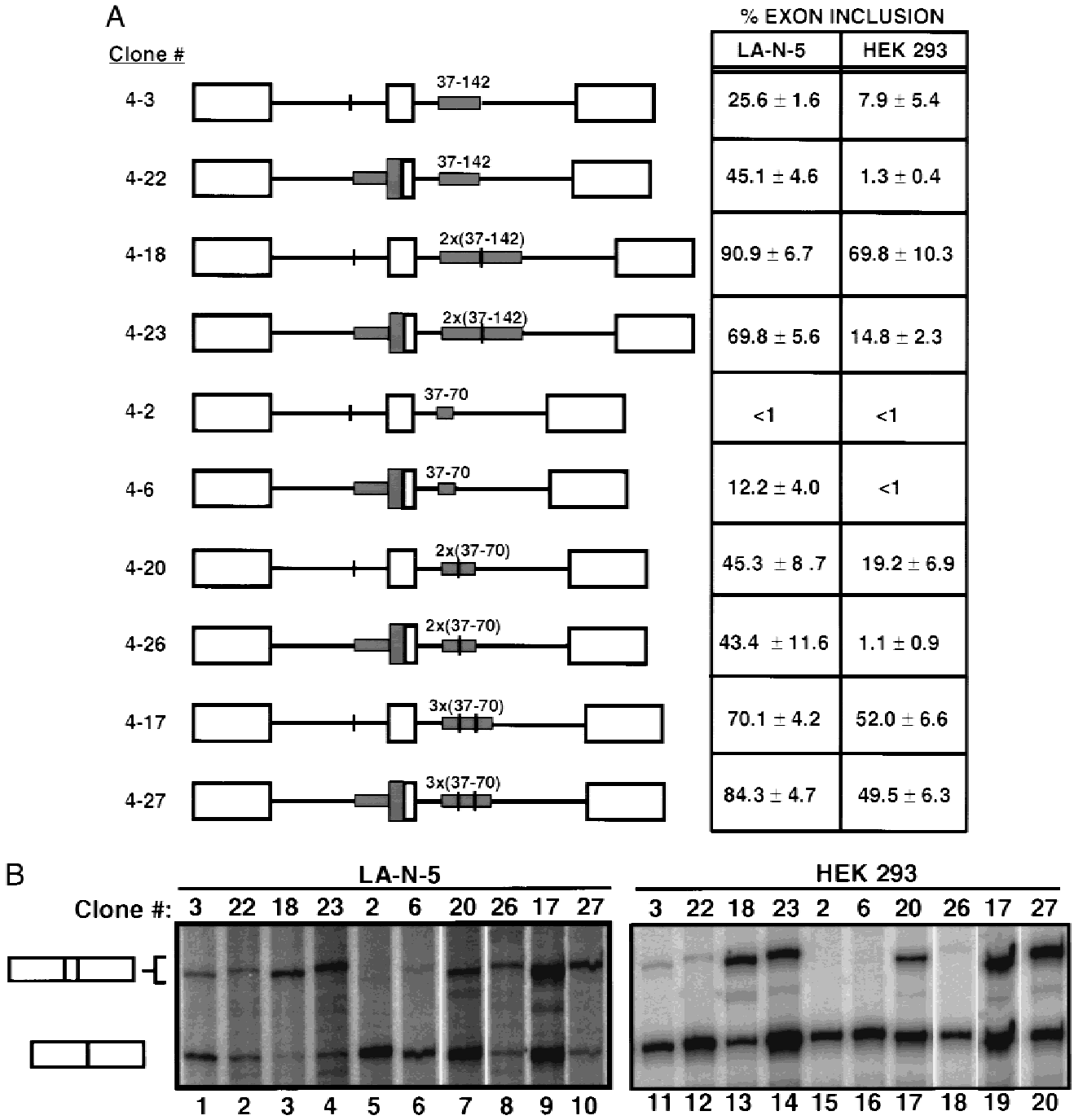


FIGURE 2. Duplication of enhancer elements overcomes repression of splicing by the N1 3′ splice site region. **A**: Constructs containing repeated inserts from the enhancer region. Globin and *src* sequences are indicated as in Figure 1. Enhancer sequence nucleotides are noted above the clone diagrams. Average exon inclusion is indicated to the right of the diagrams. **B**: Primer extension analysis of the clones depicted in **A**. Clone numbers are shown above the lanes. Notations are as in Figure 1.

the globin and src 3′ splice sites (Table 1, columns D and F, and Fig. 4B). For the clones with no enhancer mutation (DUP4-32 and DUP4-33), this ratio is 1.4 in LA-N-5 cells and 6.9 in HEK293 cells. Thus, the N1 3′ splice site represses splicing about sevenfold in HEK293 cells. Some mutations in the enhancer change this ratio. For example, the exon inclusion ratio between clones 84 and 85 is 3.5 in HEK293 cells, indicating that this mutant enhancer is less repressed by the N1 3′ splice site (Fig. 4B). In contrast, the mutation in clones 82 and 83 caused splicing to be more repressed by the N1 3′ splice site than the wild-type. The ratio of splicing for these clones increased to 9.2 in HEK293 cells. In LA-N-5 cells, the N1 3′ splice site always had a smaller

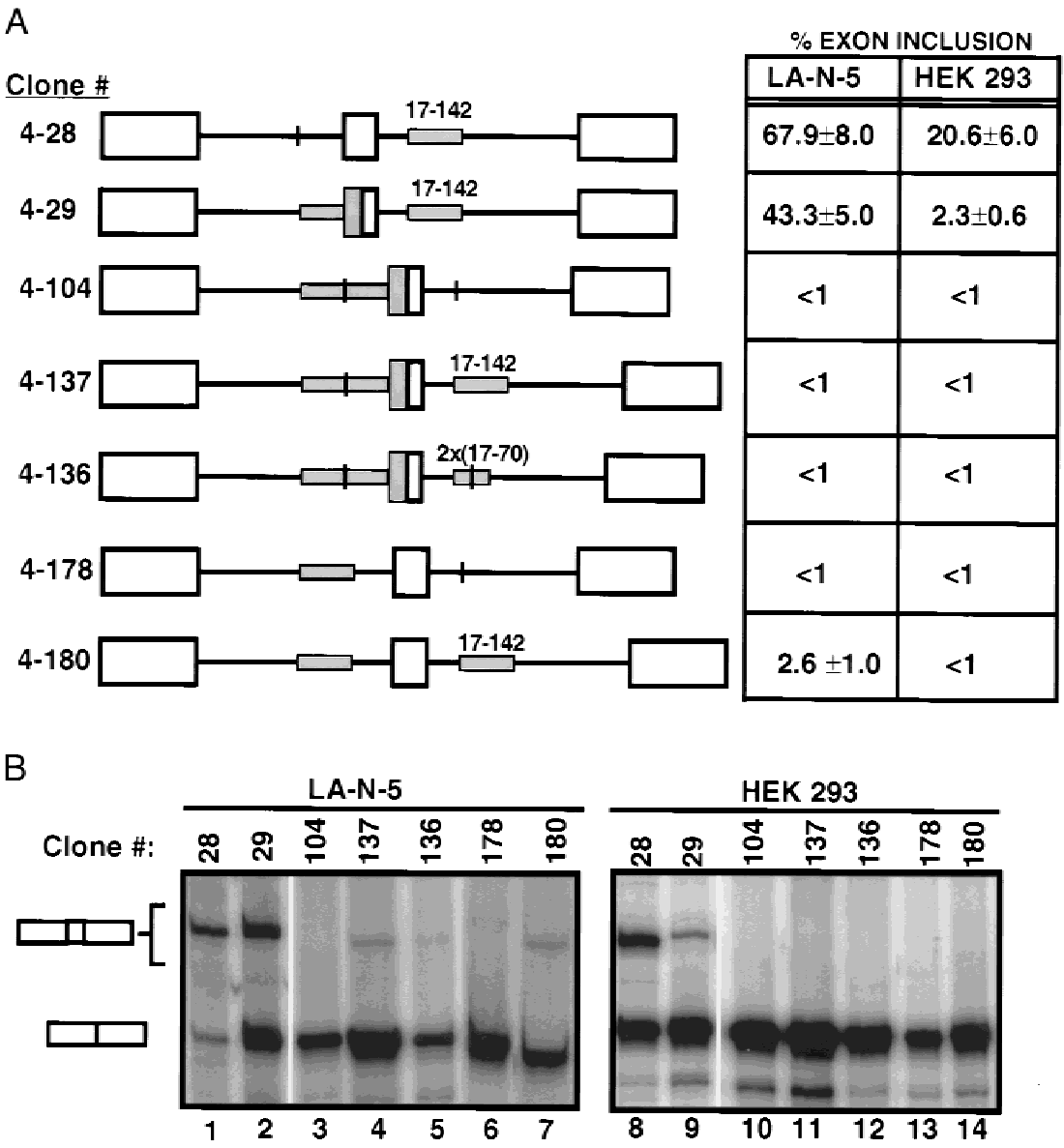


FIGURE 3. Activated splicing can be further repressed by additional N1 3′ splice site sequences. **A**: DUP minigene diagrams. DUP4-104, DUP4-136, and DUP4-137 contain two copies of the *src* 3′ splice site in the upstream intron. DUP4-178 and DUP4-180 each have the *src* exon N1 3′ splice site without the AG dinucleotide inserted upstream of the globin 3′ splice site. The levels of exon inclusion are shown to the right. **B**: Primer extension analysis of the clones shown in **A**. Clone numbers are indicated along the top. Lanes 1–7 are from plasmids expressed in LA-N-5 cells and lanes 8–14 are from plasmids expressed in HEK293 cells.

effect on exon inclusion, and some enhancer mutants were not repressed at all, bringing the ratio to 1 or lower (see clones 84–89). Overall, these changes in splicing are relatively small, and understanding the sequences needed for the N1 3′ splice site to repress the enhancer will require further work.

A more interesting analysis of this data is seen in the ratio of exon inclusion between LA-N-5 and HEK293 cells for the individual clones (Table 1, column G, and Fig. 4C). The presence of the N1 3′ splice site increases this ratio for each of the enhancers. For example, adding the N1 3′ splice site to the wild-type enhancer increases the LA-N-5/HEK293 ratio from 2.3 to 11. Thus, for clone 33 there was 11 times more exon inclusion in LA-N-5 cells than in HEK293 cells. As shown graphically in Figure 4C, this ratio varies between 2 and 4 with the globin 3′ splice site, and between 8 and 16 with the src 3′ splice site. The N1 3′ splice site is a major contributor to the tissue specificity of splicing in that it strongly increases the difference in exon inclusion between LA-N-5 and HEK293 cells.

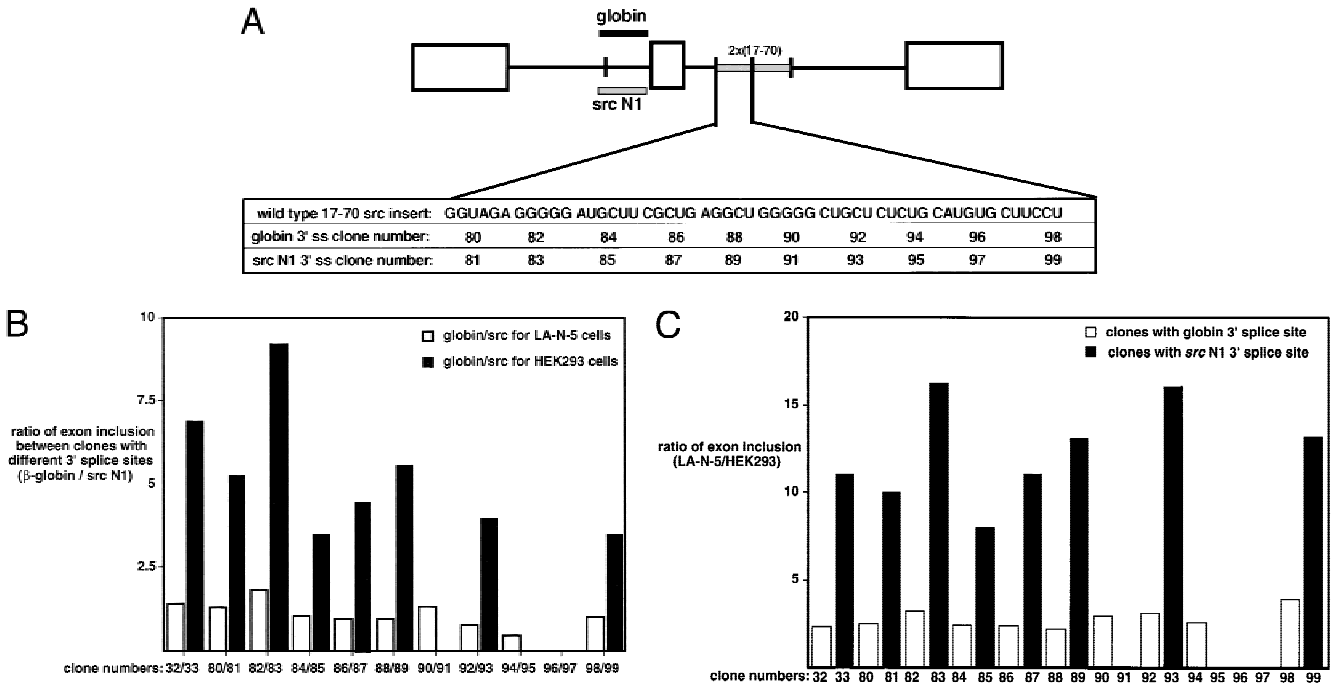


FIGURE 4. **A**: Map of the linker scanning mutations in the splicing enhancer. Each construct contains two copies of the *src* intron sequence from 17–70 nucleotides downstream of exon N1. The wild-type *src* sequence of one repeat is shown. Each block of nucleotides was mutated to UUCGAA in both copies of the repeat. The site of each block mutation is indicated by the position of the plasmid number below the sequence. The even numbered plasmids contain the 3′ splice site from *β*-globin exon 2. The odd numbered plasmids contain the 3′ splice site and 5′ half of the N1 exon. **B**: The *src* 3′ splice site has a larger effect on splicing in HEK293 cells than in LA-N-5 cells. The data in Table 1, column F, is plotted as a bar graph. The exon inclusion was measured for pairs of clones containing the same enhancer sequence combined with either the globin or *src* 3′ splice site. The ratio of exon inclusion with the globin site to exon inclusion with the *src* site was determined for LA-N-5 cells (open bars) and HEK293 cells (filled bars). In LA-N-5 cells, this ratio is always close to 1, indicating a small effect from the src splice site. In HEK293 cells, the ratio ranges from 4–9, indicating significant repression of splicing by the *src* 3′ splice site. **C**: The *src* 3′ splice site increases the tissue specificity of test exon splicing. The data in Table 1, column G, is plotted as a bar graph. The exon inclusion was measured for pairs of clones containing the same enhancer sequence combined with either the globin or *src* 3′ splice site. The ratio of exon inclusion in LA-N-5 cells to that in HEK293 cells was determined for each clone. Clones with the globin 3′ splice site are indicated by open bars and clones with the *src* 3′ splice site by filled bars. Clones with the *src* 3′ splice site show a greater difference in splicing between the two cell lines and hence are more tissue specific.

Activation by the enhancer requires specific elements in the upstream 3′ splice site

The downstream enhancer has been tested in combination with either the 3′ splice site upstream of human *β*-globin exon 2 or the site upstream of mouse *src* exon N1. To refine our understanding of the interactions between the 3′ splice site and the enhancer, we constructed clones where a variety of other 3′ splice sites were placed upstream of the test exon. The 17–142 enhancer in combination with the globin and N1 splice sites behaved as described before (DUP4-28 and DUP4-29; Fig. 5B, lanes 1, 2, 11, and 12). Quite different results were obtained with the strong 3′ splice site from exon 2 of the adenovirus major late transcription unit. This sequence gave 100% exon inclusion in both cell types, whether the enhancer was present or not (DUP4-100 and DUP4-113; Fig. 5B, lanes 3, 4, 13, and 14). This agrees with previous results that a very strong splice site can reverse the inhibition of splicing due to the small size of an exon (Black, 1991; Dominski & Kole, 1992). Interestingly, the 3′ splice site from upstream of the constitutive *src* exon 4 gave the opposite result. With this 3′ splice site, no exon inclusion was observed in either cell type, regardless of the presence of the enhancer (DUP4-101 and DUP4-158, Fig. 5B, lanes 5, 6, 15, and 16). Again, a similar effect was seen in the c-*src* gene itself, when the N1 site was substituted with the exon 4 splice site (Black, 1991).

The N1 3′ splice site contains two conserved CUCUCU elements that are required for the inhibition of N1 splicing in vitro (Chan & Black, 1995). We tested the role of these CUCUCU elements in the splicing of the test exon containing the N1 3′ splice site. To preserve the number of pyrimidines, the CUCUCU repeats were changed to either all cytosine or all uridine residues. In DUP4-105 and DUP4-145, the CUCUCU elements were changed to all C residues. These clones behaved similarly to those containing the *src* exon 4 splice site; no exon inclusion occurred in either cell

TABLE 1. Quantification of splicing with linker-scanner mutations.

A Clone #	B 3′ splice site	C LA-N-5	D globin/src	E HEK293	F globin/src	G LA-N-5/ HEK293
32	globin	72.6 ± 6.5	1.43	31.5 ± 6.5	6.9	2.3
33	src N1	50.6 ± 3.3		4.6 ± 2.8		11.1
80	globin	55.9 ± 6.2	1.31	22.6 ± 7.3	5.3	3.2
81	src N1	42.6 ± 2.3		4.26 ± 1.1		10
82	globin	76.6 ± 4.4	1.81	23.9 ± 6.7	9.2	3.2
83	src N1	42.3 ± 5.1		2.6 ± 0.8		10
84	globin	60.2 ± 4.1	1.03	25.3 ± 9.7	3.5	2.3
85	src N1	58.3 ± 5.6		7.3 ± 4.5		8
86	globin	54.3 ± 3.9	0.95	23.3 ± 7.2	4.5	2.2
87	src N1	57.2 ± 6.6		5.2 ± 1.9		11
88	globin	50.1 ± 0.6	0.94	23.3 ± 2.0	5.6	3
89	src N1	53.5 ± 1.6		4.1 ± 0.9		13
90	globin	26.3 ± 2.7	1.34	8.9 ± 1.3	N.D.	3
91	src N1	19.6 ± 4.4		<1		N.D.
92	globin	45.0 ± 1.3	0.78	14.6 ± 4.9	4.0	3.1
93	src N1	57.6 ± 5.9		3.6 ± 1.0		16
94	globin	3.3 ± 0.6	0.46	1.3 ± 0.3	N.D.	2.5
95	src N1	7.1 ± 6.5		<1		N.D.
96	globin	<1	N.D.	<1	N.D.	N.D.
97	src N1	9.0 ± 0.8		<1		N.D.
98	globin	49.9 ± 4.3	1.02	12.8 ± 3.8	3.5	3.9
99	src N1	49.0 ± 3.9		3.7 ± 2.5		13.4

N.D.: not determined.

type, regardless of the presence of the downstream enhancer (Fig. 5B, lanes 7, 8, 17, and 18). In contrast, changing the CUCUCU elements to all-U residues resulted in exon inclusion that was dependent on the downstream enhancer (DUP4-106 and DUP4-152). When the enhancer is present, splicing of the test exon with the all-U mutation was strong in LA-N-5 cells (79%; Fig. 5B, lane 10) and weaker in HEK293 cells (16%; Fig. 5B, lane 20). These results demonstrate that the enhancer downstream of the regulated exon functions with some upstream polypyrimidine sequences, but not with others. The exact sequence of the polypyrimidine tract in the upstream 3′ splice site is important not only for the repression of splicing in nonneuronal cells, but also for the downstream enhancer to activate splicing in either cell type.

The globin 3′ splice site contains one CUCUCU element instead of two in the N1 3′ splice site. Inserting an additional CUCUCU sequence into the globin polypyrimidine tract, the same distance from the test exon as in the N1 3′ splice site, did not affect the splicing observed with the enhancer (data not shown). Thus, the functional differences between the globin and N1 3′ splice sites are not due solely to the CUCUCU elements.

Some of the 3′ splice site mutations examined here and in Figure 3 gave no splicing of the test exon. To ensure that these splice sites were indeed functional, we placed these sequences upstream of the longer constitutive DUP exon 3 (β-globin exon 2) and removed the test exon and adjacent intron (Fig. 6A). These clones test whether the 3′ splice sites can function in the context of a large constitutive exon instead of the original short exon. A primer complementary to the last DUP exon (DUP exon 4, β-globin exon 3) was used for primer extension analysis of RNA from cells transfected with these clones. This analysis generates products representing the splicing of DUP exons 1–3–4, if exon 3 is included, or exons 1–4 if exon 3 is skipped. As seen in Figure 6B, all of the 3′ splice sites can function as authentic 3′ splice sites when positioned upstream of the larger exon even though they fail to splice in the short test exon. In LA-N-5 cells, exon 3 was completely included in all of the transcripts. In HEK293 cells, exon 3 was spliced with varying efficiencies. Notably, the clone 4-140+ containing two copies of the N1 3′ splice site shows significant exon 3 skipping in HEK293 cells (~50%; Fig. 6B, lane 13). Clone 4-160+ with the CU elements changed to all C residues also showed some exon skipping (Fig. 6B, lane 14). Clones 4-160+ and 4-161+ exhibited an increase in the retention of the upstream intron (Fig. 6B, lanes 14 and 15). Nevertheless, the major spliced product for all of these clones included exon 3, indicating that the various exon 3 splice sites were functional, in spite of their failure to splice in the context of the shorter DUP test exon.

A purine-rich exon sequence cooperates with elements of the intronic splicing enhancer

The N1 exon contains a purine-rich element similar to sequences known to act as splicing enhancers in other exons (Manley & Tacke, 1996; Caceres & Krainer, 1997; Hertel et al., 1997). We next examined whether this *src* exonic element could enhance splicing on its own or in combination with intronic enhancers. DUP4-74 contains the 5′ half of exon N1 fused to the 3′ half of DUP exon 2. This construct showed a slight amount of exon inclusion in LA-N-5 and HEK293 cells (7% and 2% respectively; Fig. 7B, lanes 1 and 10). The exonic element also increased the activity of the 17–142 enhancer (compare clones 4-28 and 4-102, Fig. 7B, lanes 2, 3, 11, and 12). This cooperation between exonic and intronic enhancer elements was most evident when smaller, less active portions of the downstream enhancer were tested. Neither the 37–70 nor the 17–70 *src* intron fragments enhanced splicing by themselves (DUP4-2, Fig. 2, and DUP4-30, Fig. 7B, lanes 4 and 13). However, when combined with the exon sequence, both induced significant test exon splicing. DUP4-125, which combines the exonic element with the 17–70 fragment, showed 17% exon inclusion in LA-N-5 cells (Fig. 7B, lane 5) and 12% inclusion in HEK293 cells (Fig. 7B, lane 14). The smaller 37–70 fragment in DUP4-126 induced lower levels of splicing (13% and 3%; Fig. 7B, lanes 6 and 15).

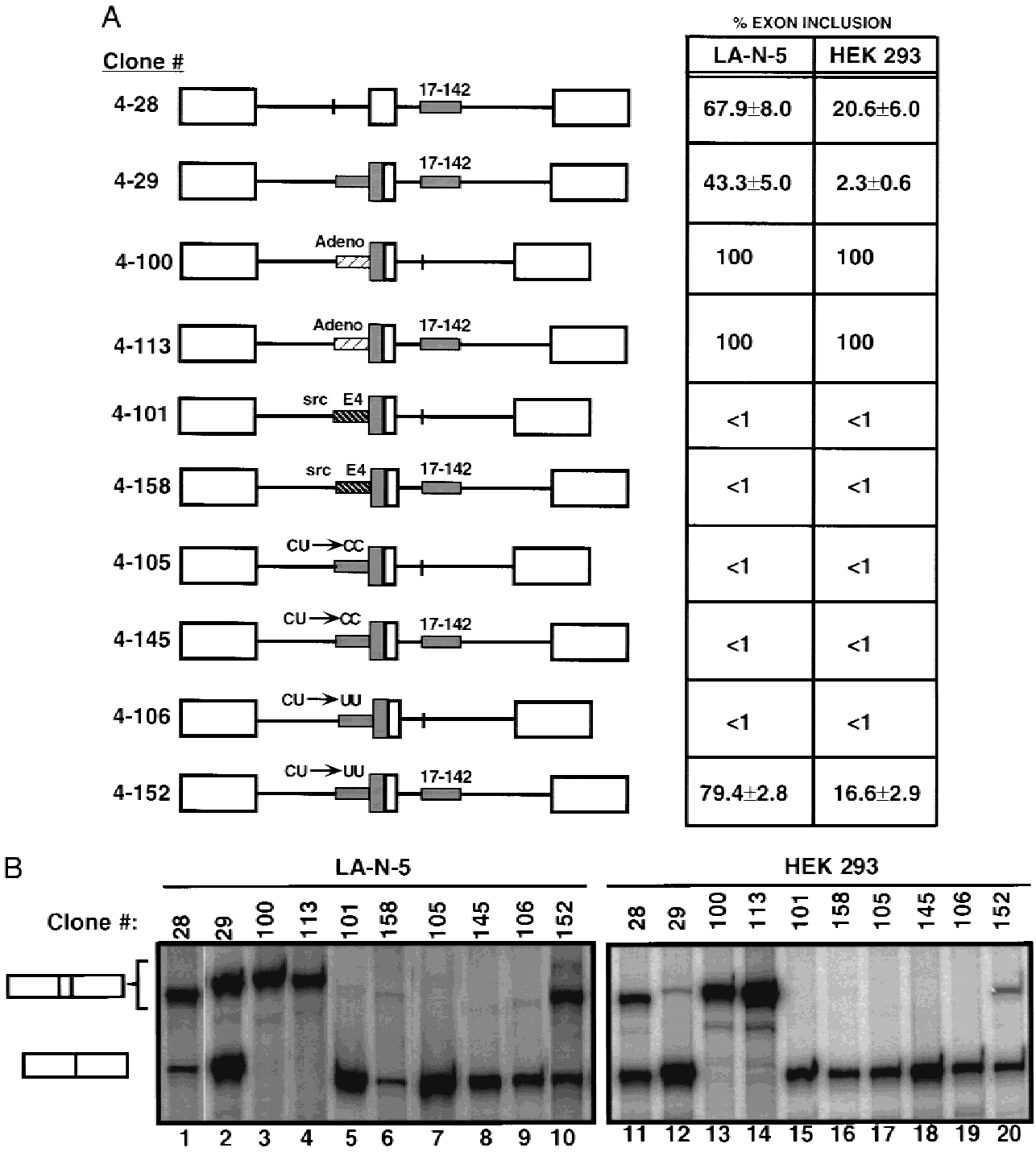


FIGURE 5. The sequence of the 3′ splice site determines the ability of the exon to be activated by the enhancer. **A**: Constructs containing different 3′ splice site sequences. These are diagramed as in Figure 1. Plasmids contain the following 3′ splice sites: globin exon 2 (DUP4-28), *src* exon N1(DUP4-29), Adenovirus major late exon 2 (DUP4-100 and DUP4-113), *src* exon 4 (DUP4-101 and DUP4-158), *src* exon N1 with CUCUCU sequences changed to CCCCCC (DUP4-105 and DUP4-145), and *src* exon N1 with CUCUCU sequences changed to UUUUUU (DUP4-106 and DUP4-152). **B**: Primer extension analysis of the plasmids depicted in **A**. Lanes 1–10 are from LA-N-5 cells and lanes 11–20 from HEK293 cells.

The sequence in the 17–70 fragment missing from the 37–70 fragment, nt 17–36, did not activate splicing when combined with the exonic element (clone 4-186, Fig. 7B, lanes 7 and 16). This small 17–37 fragment contains a G-rich element (GGGGGAUG) similar to a GGGGGCUG element further downstream and previously identified in other splicing enhancers (Carlo et al., 1996; Modafferi & Black, 1997). As found previously,

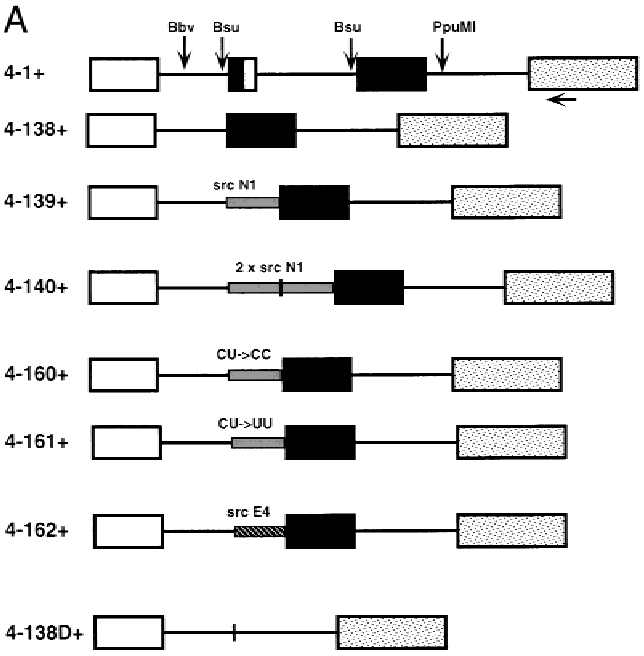
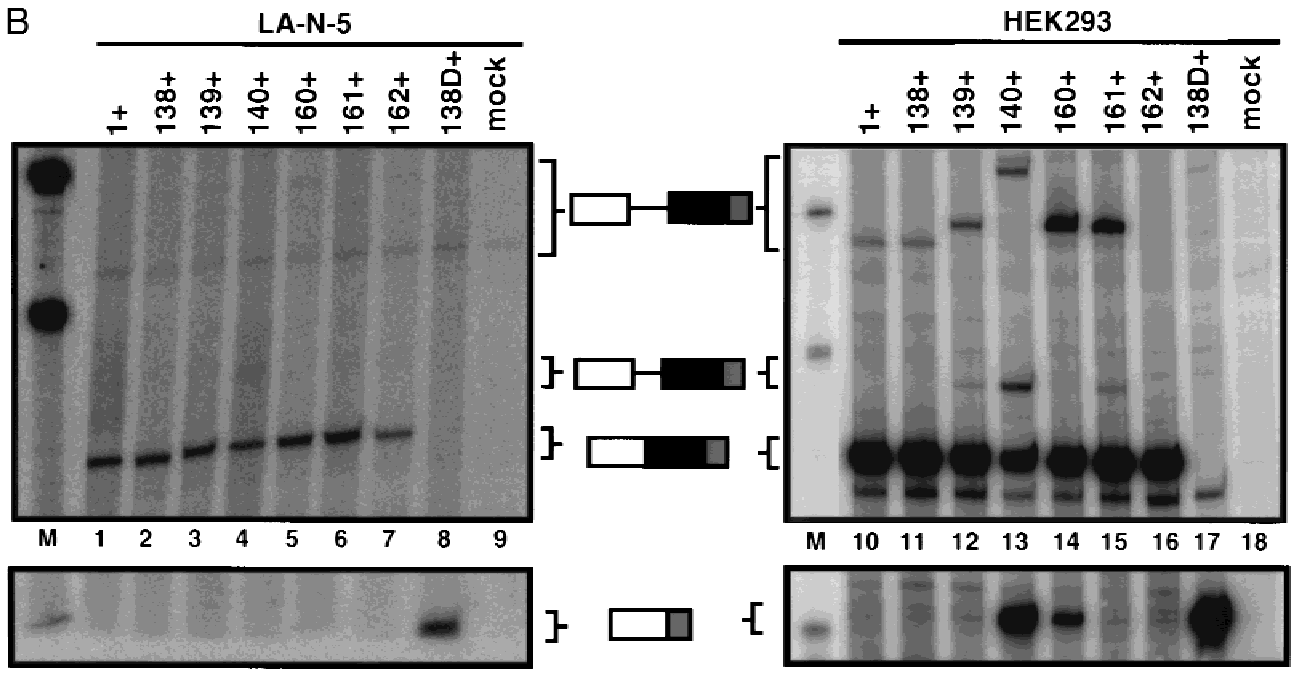


FIGURE 6. Mutant 3′ splice sites function normally within a larger exon. **A**: Clone diagrams. β-globin exon 1 is shown as a white box, β-globin exon 2 (DUP exon 3) as a black box, and globin exon 3 as a stippled box (DUP exon 4). The hybrid DUP exon 2 is shown as a black and white box. β-globin intron sequences are represented by the solid black lines. The positions of restriction enzyme sites used to make the deletion clones are shown above DUP4-1+, and the oligonucleotide used for primer extension is depicted by the arrow under the last exon of DUP4-1+. DUP4-139+ has a single copy of the N1 exon 3′ splice site, while DUP4-140+ has 2 copies, depicted as shaded boxes. DUP4-160+ and DUP4-161+ have the N1 exon 3′ splice site with the conserved CUCUCU elements changed to either all C-residues or all U-residues, respectively. DUP4-162+ uses the *src* exon 4 3′ splice site, shown with dark diagonal lines. DUP4-138D+ has a deletion from the *Bbv*I site in the first intron to the *Ppu*MI site in the third intron, removing the entire second exon. This provides a size marker for the splicing of exon 1 to exon 3. **B**: Primer extension analysis of the DUP constructs depicted in **A**. Clone numbers are shown above the lanes. Because of the disparate sizes of the products, samples were separated on 4% polyacrylamide gels and the two relevant sections of the gel are shown. The bands in lane M are DNA size markers corresponding to 622, 527, and 242 nt. Splicing of exons 1–2–3 results in a 468-nt primer extension product (top panel). Splicing between exons 2 and 3, with retention of the first intron, produces products ranging from 600–680 nt in size. These can be seen in lanes 10–15. Splicing of exon 1 directly to exon 3 gives a 244-nt product (bottom panel). Clones 4-139+, 4-140+, and 4-161+ also generate an approximately 500-nt product that apparently derives from a cryptic splice site generated in the plasmid construction. Primer extension analysis of RNA from mock-transfected LA-N-5 cells and HEK293 cells is shown in lanes 9 and 18. A nonspecific band can be seen in the LA-N-5 lanes, and is the only band seen in the mock-transfected lane 9.



three copies of the GGGGGCUG sequence, separated by a 9-nt spacer had no effect on splicing (DUP4-107, Fig. 7B, lanes 8 and 17). However, when this repeated intronic element was combined with the exonic element, exon inclusion increased in both cell types (Fig. 7B; lanes 9 and 18). Therefore, the exonic purine-rich element can enhance splicing if the appropriate intronic elements are present downstream.

Individual splicing regulatory elements are highly variable in activity between cell lines

The region surrounding *src* exon N1 is crowded with sequence elements that affect N1 splicing but vary in activity between the two cell lines tested, LA-N-5 and HEK293. These differences in activity presumably reflect differences in the regulatory protein content of the

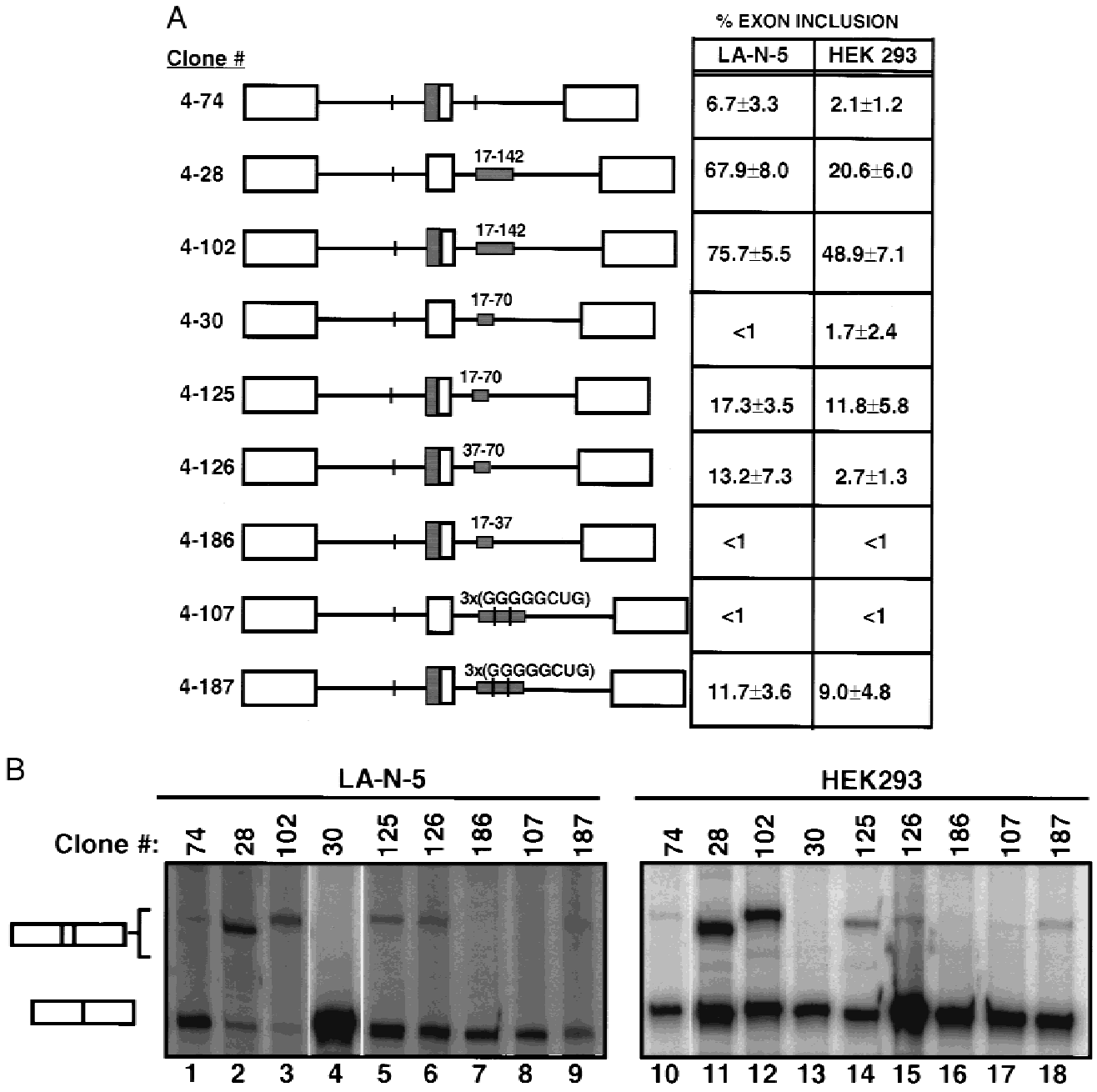


FIGURE 7. The purine-rich exon element increases activation by the downstream enhancer elements. **A**: Globin and *src* sequences are as indicated in Figure 1. Clones contain either the DUP middle exon or a chimeric *src*/DUP exon containing the purine-rich element. The downstream enhancer sequences from src with their nucleotide numbers are shown. DUP4-107 and DUP4-187 contain the sequence GGGGGCUG in three copies separated by a 9-nt globin intron derived spacer sequence. The level of exon inclusion is shown to the right of each diagram. **B**: Primer extension analysis of the plasmids depicted in **A**. Clone numbers are shown above the lanes. The size of the exon-included product varies because the middle DUP exon is 33 nt, whereas the chimeric *src*/DUP exon is 34 nt. Samples from LA-N-5 transfections are in lanes 1–9 and samples from HEK293 transfections in lanes 10–18.

cells. To further examine this variability between different cell types, we tested minigenes containing different combinations of the N1 regulatory elements in three additional cell lines: N1E-115, HeLa, and NIH3T3. The mouse neuroblastoma cell line N1E-115 exhibits low levels of exon N1 inclusion in the endogenous *src* mRNA. Neither HeLa human cervical carcinoma cells nor 3T3 mouse fibroblasts show N1 exon splicing (Black, 1991, and unpubl. data). None of the cell lines spliced the original DUP4-1 test exon to a significant level (DUP4-1, Fig. 8B).

Sixteen different plasmid constructs were each transfected into each of the five cell lines. The transfections were repeated two to five times to assure the repro-

ducibility of the level of exon inclusion. These data are extensive and are shown numerically in Figure 8A and as autoradiographs in Figure 8B. For simplicity, the data for selected clones is also shown as bar graphs in Figure 8C.

DUP4-5 contains the N1 exon and all of the flanking intronic sequence necessary for neuron specific splicing (Fig. 8B, lane 2). This clone shows a high level of exon inclusion only in the LA-N-5 cells (29% inclusion). The other neuronally derived cell line, N1E-115, showed less N1 exon splicing (8%). HeLa cells gave a low level of N1 splicing (1.4%), whereas HEK and 3T3 cells did not splice the N1 exon. The levels of N1 exon splicing with this construct parallel the splicing of the endogenous src RNA in these cells, demonstrating that the *cis*-acting sequences responsible for regulating N1 splicing are contained within this fragment of the *src* gene.

The largest intronic splicing enhancer sequence and the upstream repressor sequence behaved as expected from previous results. Intron nt 17–142 (DUP4-28) are known to activate test exon inclusion in either LA-N-5 cells (68%) or in HEK293 cells (21%). When this plasmid was expressed in the three new cell lines, test exon splicing was highest in HeLa cells (77%), and somewhat lower in N1E-115 (67%) and 3T3 cells (43%) (Fig. 8B, lanes 3, Fig. 8C). Thus, the 17–142 sequence functions as an intronic splicing enhancer in all five cell lines tested. When the N1 3′ splice site was combined with the enhancer in DUP4-29 or DUP4-75, splicing was repressed (Fig. 8B, lanes 4 and 6, Fig. 8C). As expected, this effect was stronger in the nonneuronal cells. Compared to DUP4-28, exon inclusion with DUP4-75 decreased about 10-fold in HeLa, HEK293, and 3T3 cells, whereas in the neuronal cells there was a 1.5- to 3.5-fold decrease.

More interesting results were obtained when combinations of smaller elements were tested. The purine-rich exon sequence had only small effects on splicing when combined with both the 3′ splice site and the downstream enhancer (compare clones 4-29 and 4-75). However, this element had a different pattern of activity when present by itself (clone DUP4-74), showing the most exon inclusion in N1E-115 and HeLa cells (10–11%), and lower levels in LA-N-5, HEK293, and 3T3 cells (Fig. 8C). Moreover, when this exon element was combined with just the downstream enhancer (DUP4-102), splicing is increased over the downstream enhancer alone and high levels of exon inclusion were seen in all of the cells (Fig. 8C). In particular, with these combined enhancer elements, HeLa cells showed almost complete splicing of the test exon (98.5%).

We also tested two smaller elements of the intronic splicing enhancer as simple triply repeated elements. The first triple element, GGGGGCUG, did not activate splicing by itself in any of these cells (DUP4-107, Fig. 8C). In contrast, when this element was combined with the N1 3′ splice site, with the exonic element, or with both elements (DUP4-155, DUP4-187, and DUP4-141), splicing was enhanced over that seen with any of the individual elements. The triple GGGGGCUG-repeat most strongly potentiated the exonic element, inducing up to 48% exon inclusion in HeLa cells (DUP4-187, Fig. 8C). Interestingly, with this combination of elements, the LA-N-5 cells showed lower levels of exon inclusion than other cells. This is the opposite pattern from that seen with the downstream enhancer/3′ splice site combination (DUP4-29), where LA-N-5 cells showed the highest exon inclusion.

A triple repeat of the other short intronic enhancer element, UGCAUG (DUP4-108), also had variable activity in the different cell lines. With this element alone, the LA-N-5 cells showed the highest levels of exon inclusion (48%), whereas the 3T3 and HEK293 cells had the lowest (2.5–5%; Fig. 8C). Most interestingly, combining the N1 exon 3′ splice site with the UGCAUG repeat did not repress splicing. Instead, the 3′ splice site sequence increased the level of exon inclusion in all of the cells (DUP4-156, Fig. 8C). Evidently, the repression of splicing by the N1 3′ splice site does not function with the UGCAUG element. Finally, the purine-rich exonic element also cooperated with the repeated UGCAUG element. This combination again showed strong exon inclusion in all cell types (DUP4-183, Fig. 8C).

One issue that is not addressed in these studies is how the expression level of the pre-mRNA affects its splicing pattern. Most of the variability in the level of total RNA that we observe is due to differences in the number of cells that take up DNA and express the plasmid (data not shown). Thus, HEK cells are much more transfectable than LA-N-5 and many more HEK cells express the transfected plasmid. However, there is likely to also be some variability in the activity of the CMV promoter in the different cell lines, leading to differences in the per cell concentration of pre-mRNA. In a system dependent on subtle differences in regulatory protein concentration, changes in promoter activity are likely to affect changes in pre-mRNA splicing pattern. Similarly, changing the promoter itself from the endogenous src promoter to MLV (Black, 1992) or CMV might also affect changes in splicing (Cramer et al., 1997).

This analysis of the individual splicing regulatory elements points to the difficulty in determining the origins of tissue specificity in splicing. The individual sequences are highly variable in their activity between the different cells, and only in combination do they direct cell-type specific splicing similar to that of the whole N1 exon.

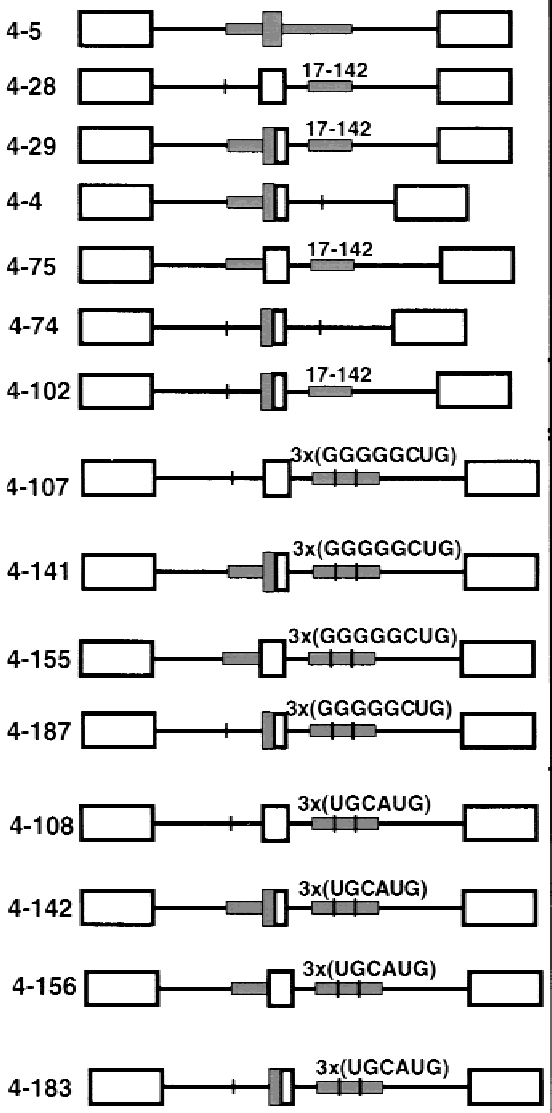
DISCUSSION

The *c-src* N1 exon provides an interesting model for studying tissue-specific regulation of splicing. N1 is a simple short exon, with good matches to the splice site

consensus sequences, but its splicing is highly cell-type specific; only in mature neurons is the N1 exon spliced into the *src* mRNA. In this study, we attempted to dissect all of the features of the N1 exon that determine its cell-type specificity.

After the enhancer, the most significant regulatory sequence was the polypyrimidine tract in the 3′ splice site upstream of the N1 exon. When placed adjacent to a test exon whose inclusion was activated by the downstream enhancer, the N1 3′ splice site strongly inhib-

A



	% EXON INCLUSION				
	LA-N-5	N1e115	HEK293	HeLa	3T3
4-5	28.9±3.9	7.8±3.2	<1	1.4±0.2	<1
4-28	67.9±8	66.7±1.9	20.6±6	77.2±8.5	43±5.4
4-29	43.3±5	13.3±1.9	2.3±0.6	8.5±1.1	2.8±0.1
4-4	8.1±1.9	7.8±3.2	<1	1.7±0.3	<1
4-75	35.2±5.4	19.9±4.9	1.9±0.7	7.7±1.1	3.6±1.2
4-74	6.7±3.3	10.9±6.9	2.1 ± 1.2	10.4±3.1	4.4±0.6
4-102	75.7±5.5	71.1±5.5	48.9±7.1	98.5±1.2	46.8±1.5
4-107	<1	1.6±0.9	<1	<1	<1
4-141	26.2±3.1	23.2±5.1	2.6 ± 0.6	11.4±2.1	<1
4-155	14.8±0.9	13.1±4.9	<1	10.7±2.1	5.6±2.2
4-187	11.7±3.6	33.7±10.9	9.0±4.8	47.8±3.1	19.3±8.6
4-108	48.6 ±4.2	24.6±5.5	2.5 ± 1.8	17.7±5.1	5.3±1
4-142	80.4±4.4	43.1±4.3	5.9 ± 2.2	18.1±8.8	14.5±3.3
4-156	83.1±6.5	65.0±7.3	4.2±0.3	21.6±7.1	11.4±3.7
4-183	67.5±8.3	73.3±8.1	19.5± 0.6	83.1±11.6	34.6±9.5

FIGURE 8. The activity of individual splicing regulatory elements is highly variable between different cell lines. **A**: Globin and *src* sequences are as indicated in Figure 1A. Clones contain either *src* exon N1 (DUP4-5), the DUP middle exon (DUP4-28, DUP4-75, DUP4-107, DUP4-155, DUP4-108, DUP4-156) or a chimeric *src*/DUP exon (DUP4-29, DUP4-4, DUP4-74, DUP4-102, DUP4-141, DUP4-187, DUP4-142, DUP4-183). The downstream enhancer sequences from *src* with their nucleotide numbers are shown. DUP4-107, DUP4-141, DUP4-155, and DUP4-187 contain the triple GGGGGCUG sequence, whereas DUP4-108, DUP4-142, DUP4-156, and DUP4-183 contain the triple UGCAUG sequence. Clone numbers are to the left of each diagram and the level of exon inclusion is shown to the right. Each column shows the percentage of exon inclusion for a different cell line. **B**: Primer extension analysis of the plasmids depicted in **A**. The size of the exon-included product varies since the middle exon is 23 nt for DUP4-5, 33 nt for clones containing the DUP exon, and 34 nt for the chimeric *src*/DUP exon. Each panel shows the results for a different cell line labeled on the right. LA-N-5 cells are from a human neuroblastoma, whereas the N1E-115 cells are from a mouse neuroblastoma. HEK293 cells are human embryonic kidney cells, HeLa cells are human cervical carcinoma cells, and 3T3 cells are mouse fibroblasts. **C**: The data for selected clones in **A** presented in bar graphs. The individual clones are given as clone numbers along the bottom. Each bar represents the exon inclusion for that clone in a particular cell line as indicated. (*Figure continues on facing page.*)

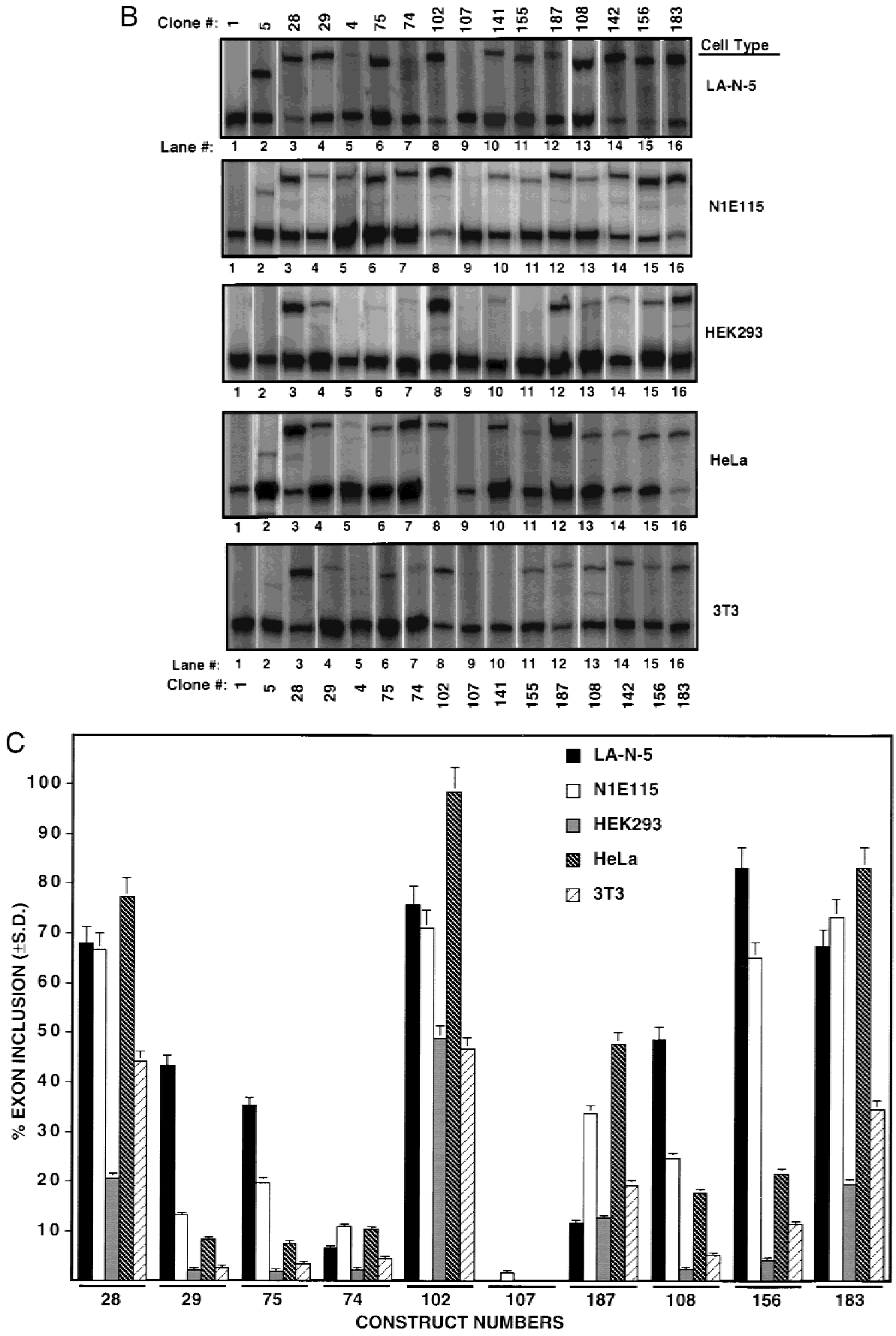


FIGURE 8. (*Continued.*)

ited splicing. Most interestingly, this splicing inhibition was much stronger in nonneural HEK293 cells than in LA-N-5 neuroblastoma cells. The combination of partially neural specific activation from the enhancer and predominantly nonneural repression from the 3′ splice site generated a highly neural specific exon. With these two regulatory elements, the test exon was included in the mRNA 20 times more frequently in LA-N-5 cells than in HEK293 cells, a level of specificity approaching that of the whole N1 exon. Thus, the enhancer and the 3′ splice site are the primary elements controlling N1 exon splicing.

Functional differences between 3′ splice sites

The N1 3′ splice site sequence does not repress the splicing of all exons. When this sequence was placed adjacent to the longer constitutive β-globin exon 2 (DUP exon 3), splicing was not inhibited in either cell type (Fig. 6). This repressor may function only with short exons that are activated by an enhancer. On the other hand, splicing activated by three copies of the 37–70 sequence or by the triple repeat of the UGCAUG element was not repressed by the N1 3′ splice site. This argues that this splice site sequence cannot repress all enhancers but must make specific interactions with features downstream to inhibit splicing. Although we found no enhancer mutations that blocked the repression, these features may be redundant, preventing their easy identification through mutagenesis. In the in vitro splicing system, inhibition of splicing by the N1 3′ splice site requires CUCUCU elements in both the polypyrimidine tract and the enhancer region (Chan & Black, 1995, 1997), again implying an interaction of upstream and downstream elements. In vitro, these inhibitory exon bridging interactions apparently involve the PTB protein (Chan & Black, 1997).

Although the inhibition of splicing by the N1 3′ splice site was previously observed in vitro, it was not anticipated that the activation of splicing also required specific features of the upstream 3′ splice site. The enhancer activates the splicing of short exons carrying 3′ splice sites from either *src* exon N1 or β-globin exon 2 (Modafferi & Black, 1997). However, the enhancer did not induce splicing of exons with either the 3′ splice site from *src* exon 4 or the exon N1 site carrying U to C mutations (Fig. 5). In contrast, both of these 3′ splice sites functioned in the context of a large exon (Fig. 6). Thus, not all 3′ splice sites allow enhancer activation of the test exon.

In the course of these experiments, the different 3′ splice site sequences exhibited four different behaviors (Table 2). First, there was the adenovirus exon 2 splice site that functioned with any exon or in any cell line. Second, there were sites that spliced in the context of a large exon but were not used in a small exon, regardless of the presence of a downstream enhancer.

TABLE 2. Activities of various 3′ splice sites.

	Splicing activity in LA-N-5 cells			
3′ splice site	Test exon (33–34nt)	Test exon + downstream enhancer	Long exon (265 nt)	Test exon repressed in HEK293 cells
Adeno ML exon 2	++++	++++	N.D.	no
src exon 4	−	−	++++	no
src exon N1 U → C	−	−	++++	no
β globin exon 2	−	+++	++++	no
src exon N1 C → U	−	+++	++++	no
src exon N1	−	++	++++	yes

++++: 90–100% exon inclusion
+++: 50–90% exon inclusion
++: 20–50% exon inclusion
−: <1% exon inclusion
N.D.: not determined

These sequences included the *src* exon 4 splice site and the N1 exon splice site carrying the U to C mutations. Third, there were sites that functioned in a short exon when activated by the downstream enhancer, but did not show repression of splicing in nonneural cells. These included the β-globin exon 2 splice site and the N1 exon site carrying C-to-U mutations. Finally, there was the wild-type N1 exon 3′ splice site that was activated by the enhancer but repressed in nonneural cells. These results are summarized in Table 2 and indicate that the variable sequences of splice sites determine more than their relative ability to bind the general splicing apparatus. There are important functional differences between 3′ splice sites that are presumably determined by the proteins and regulatory sequences with which they interact.

Cooperation between exonic and intronic enhancers

In addition to the 3′ splice site and the multiple elements of the downstream enhancer, there is another element affecting splicing of the N1 exon. This sequence is at the 5′ end of the N1 exon itself and includes the element GAGGAAGG. Similar purine-rich elements act as exonic splicing enhancers in other exons (Lavigueur et al., 1993; Sun et al., 1993; Tian & Maniatis, 1993; Staknis & Reed, 1994; Ramchatesingh et al., 1995; Yeakley et al., 1996). When placed by itself in the test exon, this sequence had only a small effect on splicing. However, activation of the test exon by an intronic enhancer always increased in the presence of this exonic element (Fig. 7). This was most striking for clone 4-187, containing the triple GGGGGCUG repeat, in 3T3 and HEK293 cells (Fig. 8). With this combination of exonic and intronic elements, splicing increased four- to fivefold over either element alone. The identification of this N1 exonic element, which was missed in earlier mutagenesis studies of the *c-src* gene, underscores

the redundant nature of splicing regulatory sequences. With multiple sequence elements each having moderate effects on splicing, the mutation of any one element may not alter the overall level of exon inclusion. Only by isolating an element and examining its activity by itself or with other individual elements could its activity be observed.

The combination of exonic and intronic enhancer elements has also been described in the regulation of exon 5 of the cardiac troponin T transcript (Ryan & Cooper, 1996). In this case again, the exonic element is a general splicing enhancer, whereas the intronic elements determine the tissue specificity. The intronic GGGGGCUG element in the *src* transcript is thought to bind to hnRNPs H and F in vitro (Min et al., 1997; Chou et al., 1999), whereas the exonic element is similar to sequences known to bind to the proteins SF2/ASF and Tra-2 (Sun et al., 1993; Tacke & Manley, 1995; Liu et al., 1998; Tacke et al., 1998). It is not clear whether the proteins bound to these two RNA elements directly contact each other or instead independently interact with components of the spliceosome to alter their assembly. It will be interesting to examine the proteins binding to these src regulatory elements to determine their interactions with each other and to look at their effect on the assembly of other splicing factors.

Cell type specificity and combinatorial control of splicing

The cell specific use of the N1 exon is mediated by a variety of sequence elements surrounding the exon. The combination of the N1 3′ splice site and the downstream enhancer (DUP4-75) conferred regulation on the test exon that was close to that of the wild-type N1 exon (DUP4-5); however, it was not identical. N1E115 and HeLa cells in particular showed more exon inclusion with DUP4-75 than with DUP4-5, indicating that additional sequence elements must affect exon inclusion in these cells. Other combinations of splicing regulatory elements placed in the DUP test exon diverged widely in activity from the normal N1 exon. HeLa cells often gave high levels of test exon splicing in response to individual sequence elements, even though HeLa cells normally exclude the N1 exon (clone 4-5, Fig. 8). This was most apparent in clones 4-28 and 4-102, where the intronic enhancer induced higher splicing in HeLa cells than in LA-N-5 cells. The splicing induced by the enhancer in HeLa cells was highly suppressed by the N1 3′ splice site. Thus in HeLa cells, the repressor is particularly important in maintaining the proper tissue specificity of splicing.

The idea that cells are variable in their use of the individual regulatory elements is underscored by the two triple repeat elements. The triple UGCAUG element activated splicing most strongly in LA-N-5 cells with lower effects in N1E115 and HeLa cells. This element has also been shown to affect splicing of the fibronectin and nonmuscle myosin transcripts (Huh & Hynes, 1994; Kawamoto, 1996; Lim & Sharp, 1998). In contrast, the triple GGGGGCUG element combined with the purine rich exonic element enhanced splicing most strongly in HeLa and N1E115 cells but only weakly in LA-N-5. Although the whole enhancer functions in all the cells, the importance of any single element in the enhancer evidently depends on the cell type being examined.

The differing effects of these sequences are presumably due to variable concentrations of the regulatory proteins that bind to them. Each cell line has a distinct combination of regulatory proteins that defines its response to a set of elements. Proteins that act through the exonic element and some that act through the intronic enhancer may be broadly distributed, as these elements function in all the cells. Other enhancer proteins, as well as proteins that govern repression by the 3′ splice site, are restricted in their expression to certain cells. In this model, the level of exon inclusion will be determined by the particular combination of regulatory elements present in the exon and by the relative concentrations of the cognate regulatory proteins present in a particular cell.

Most systems of regulated splicing employ multiple regulatory elements distributed along the RNA transcript. Repression of an exon in the *Drosophila sxl* transcript by the sex lethal protein requires multiple Sxl protein binding sites present on both sides of the regulated exon (Horabin & Schedl, 1993; Samuels et al., 1994; Deshpande et al., 1996). Activation of a female-specific exon in the *Drosophila doublesex* (*dsx*) transcript requires multiple positive-acting elements that make up a complex exonic splicing enhancer (Hertel & Maniatis, 1998, and references therein).

In mammalian cells, systems of tissue-specific splicing appear even more complex, employing diverse sequence elements and a combination of positive and negative control. The neuronally regulated exon in the $\gamma 2$ subunit of the $GABA_A$ receptor contains regulatory elements in the upstream 3′ splice site and within the exon itself (Zhang et al., 1996). Like the *src* N1 exon, these elements act both positively and negatively; some sequences are thought to allow splicing in neural cells and others to prevent splicing elsewhere. Similarly, the neural specific Y exon of the Agrin transcript contains both positive and negative regulatory elements, but in this case they are present in the downstream intron (Wei et al., 1997). A combination of positive and negative control is also seen for exons regulated in muscle and other cell types (Helfman, 1994; Y. Wang et al., 1997).

One protein implicated in the repression of several different tissue-specific splicing patterns is the polypyrimidine tract binding protein (Patton et al., 1991; Mulligan et al., 1992; Gooding et al., 1994; Singh et al., 1995). It appears that repression of the neuron-specific

exons *src* N1 and GABA$_A$ γ2 is mediated by PTB (Ashiya & Grabowski, 1997; Chan & Black, 1997). PTB is ubiquitously expressed and appears to mediate a general repression of splicing that is released in specific cell types. The protein factors determining the tissue-specific release from inhibition have not been identified. However, in both the *src* N1 and γ 2 exons, a neural-specific protein related to PTB is seen binding to the repressor elements in neural extracts (Ashiya & Grabowski, 1997; Chan & Black, 1997). This protein may relieve the repression of splicing in neural cells through the displacement of normal PTB. Although by no means proven, this model agrees with the data shown here that the 3′ splice site engages in a negative interaction with the downstream enhancer that is somehow relieved in the neural cells.

The proteins that bind to these splicing regulatory elements must be densely packed along the RNA forming a complex RNP structure. Several proteins have been identified that bind to the core of the downstream enhancer and are needed for N1 splicing in vitro, including hnRNP H, hnRNP F, and the KH type splicing regulatory protein (KSRP) (Min et al., 1995, 1997; Chou et al., 1999). Although these proteins are all widely expressed, they assemble onto an enhancer RNA more strongly in neural extracts and the enhancer shows higher activity in neural cells. The source of this partial neural specificity is not clear. These and other splicing regulatory proteins, such as hnRNPs and SR proteins do show some variation in concentration between different cell types (Dreyfuss et al., 1993; Zahler et al., 1993; Kamma et al., 1995). This variation will produce highly tissue-specific changes in the ratios of particular proteins, which could lead to very specific changes in splicing patterns. Indeed, the ratio of hnRNP A1 to the SR protein SF2/ASF has strong effects on certain splice site choices (Mayeda et al., 1992; Caceres et al., 1994). Such a model of combinatorial control, where splicing is dependent on relative concentrations of generally expressed proteins, does not preclude highly tissue-specific factors acting in concert with it. The neural specific Elav (Koushika et al., 1996), Nova (Buckanovich & Darnell, 1997), and PTB-related proteins (Ashiya & Grabowski, 1997; Chan & Black, 1997) may be examples of such factors.

This combinatorial model of splicing regulation is similar to the control of transcription (Ernst & Smale, 1995; Hertel et al., 1997). In the regulation of promoter activity, multiple DNA sequence elements bind to proteins of varying tissue specificity, contributing to an overall promoter strength. Instead of one or a few highly tissue-specific factors, the interaction of various factors creates the specificity. Although the contribution of any one protein may be small, the whole system can be highly tissue specific. Unfortunately, this combinatorial control often makes it difficult to identify the sources of tissue specificity in transcription. This may also prove true in splicing.

MATERIALS AND METHODS

Plasmid construction

All DNA constructs were made using standard cloning procedures and confirmed by sequencing (Ausubel et al., 1987; Sambrook et al., 1989). As described previously, constructs containing *src* sequences from the intron downstream of exon N1 were PCR amplified from a *src* minigene construct to place restriction sites on the ends of the resulting products (Modafferi & Black, 1997). These fragments were then digested with the appropriate restriction enzymes and ligated into linearized DUP vectors (Dominski & Kole, 1991; Modafferi & Black, 1997).

Plasmids containing the *src* N1 exon 3′ splice site with or without the exon sequence were described previously (Modafferi & Black, 1997). The downstream enhancer fragments were placed in these clones by isolating *Bgl*II–*Dra*III fragments from the DUP enhancer plasmids. These fragments were ligated into DUP plasmids (containing the various 3′ splice sites) digested with the same enzymes.

In Figure 3, DUP4-104 was made by annealing two phosphorylated complementary oligonucleotides (DUP127 5′-CTGTCTTCGCACCTCAGCCTCTCCTTCTCTCTGCTTCTCTCTCGCTGGGCC and DUP128 5′-CAGCGAGAGAGAAGCAGAGAGAAGGAGAGGCTGAGGTGCGAAGACAGGGCC) and ligating them into DUP plasmids that had been linearized at the Apa site in the first intron.

The Adenovirus and *src* exon 4 3′ splice sites in Figure 5 were subcloned from plasmids S16 and S17 (Black, 1991). These 3′ splice site fragments were gel purified after *Cla*I cleavage, T4 DNA polymerase treatment, and finally digestion with *Apa*I. The short 3′ splice site fragments were ligated into DUP4-1 plasmid that had been cleaved with *Nco*I, filled, and cleaved with *Apa*I. DUP clones 4-105 and 4-106 were made by PCR of DUP4-4, containing the *src* 3′ splice site and 5′ half of exon N1, using oligonucleotides DUP125 (5′-TCCGGGCCCTGTCTTCGCACCTCAGCCTCTCCTTCCCCCCGCTTCCCCCCGCTGGCCCT) and DUP126 (5′-TCCGGGCCCTGTCTTCGCACCTCAGCCTCTCCTTTTTTTTGCTTTTTTTTGCTGGCCCT) as upstream primers (the underlined letters indicate the altered nucleotides), and DUP3 (5′-CACATGCCCAGATCTATTGGT) as the downstream primer. The resulting product was digested with *Apa*I and *Bgl*II and subcloned into DUP4-1 digested with the same enzymes.

In Figure 6, all of the deletion clones except DUP4-162 were made by digesting the plasmid DNA with *Bsu*36I and religating the plasmid DNA to precisely delete DUP exon 2 and the downstream intron. DUP4-162 was constructed using opposing oligonucleotides complementary to the 3′ splice site (DUP139 5′-CTGAGCAGGAGGGAGGGAGCAAAGAATGAGG) and the third DUP exon (DUP140 5′-GCTGCTGGTGGGTCTACCCTTGGACCC) to perform inverse PCR (ExSite Mutagenesis Kit, Stratagene) and generate the deletion in the final product. After digesting the DNA with *Dpn*I, the product was gel isolated and ligated. The third globin exon was inserted into the CMV DUP clones using a *Bam*HI fragment from the SP6 DUP33 clone (Dominski & Kole, 1991).

All plasmids with the β-globin 3′ splice site upstream of the short exon used in Figure 7 were described previously except DUP4-127, which was constructed by annealing oligo-

nucleotides *Bgl*17 (5′-CCAGATCTGGTAGAGGGGGATG CTT) and *Bam*37 (5′-GATGGATCCAGCGAAGCATCCCC CTC). The double-stranded product was digested with *Bgl*II and *Bam*HI, and inserted into DUP4-1 linearized at the *Bgl*II site. The *Bgl*II–*Dra*III fragments from these plasmids were subcloned into either DUP4-4 (src N1 3′ splice site/exon fragment), DUP4-75 (src N1 3′ splice site), or DUP4-74 (src N1 exon fragment) all digested with *Bgl*II and *Dra*III, as described above.

Transfection, cell culture, and primer extension

Most of these procedures were described previously (Modafferi & Black, 1997). Briefly, plasmid DNA was transfected using a modified HBS/$CaCl_2$ method. After 36–48 h, cytoplasmic RNA was isolated by the NP-40 lysis method, followed by proteinase K digestion and acid phenol extraction. RNA was precipitated for the primer extension reactions (30 μg for the HEK293 samples, and 50 μg for the LA-N-5, HeLa, N1E-115, and 3T3 samples). DUPRT#3 was used as a primer for reverse transcription in all experiments, except in Figure 6 where a primer complementary to sequences in globin exon 3, DUPRT#7 (5′-CACTTTCTGATAGGCAGCCT GCACTGG), was used. For this primer, preincubating the oligonucleotide with 5% DMSO at 85 °C for 2 min and then quick chilling on ice was necessary for efficient labeling with γ-^{32}P ATP by T4 polynucleotide kinase. All primer extension samples were electrophoresed on denaturing 8% polyacrylamide gels, except those in Figure 6 which were run on a 4% gel. The dried gels were exposed to PhosphorImager screens and the bands quantified using ImageQuant Software (Molecular Dynamics). To control for lane-to-lane variation in the amount of product, the splicing of the test exon is measured relative to the amount of exon excluded product rather than as an absolute band intensity. The percentage of exon inclusion was defined as [cpm exon included product/(exon included product + exon skipped product)] × 100. The average inclusion levels from at least three independent transfections are shown with the standard deviation calculated using DeltaGraph Software.

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