Antagonistic Effects of the SRp30c Protein and Cryptic 5′ Splice Sites on the Alternative Splicing of the Apoptotic Regulator Bcl-x*S

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Alternative 5′ splice site selection allows Bcl-x to produce two isoforms with opposite effects on apoptosis. The pro-apoptotic Bcl-xS variant is up-regulated by ceramide and down-regulated by protein kinase C through specific cis-acting exonic elements, one of which is bound by SAP155. Splicing to the Bcl-xL 5′ splice site is also enforced by heterogeneous nuclear ribonucleoprotein (hnRNP) F/H proteins and by Sam68 in cooperation with hnRNP A1. Here, we have characterized exon elements that influence splicing to the 5′ splice site of the anti-apoptotic Bcl-xL isoform. Within a 86-nucleotide region (B3) located immediately upstream of the Bcl-xL donor site we have identified two elements (ML2 and AM2) that stimulate splicing to the Bcl-xL 5′ splice site. SRp30c binds to these elements and can shift splicing to the 5′ splice site of Bcl-xL in an ML2/AM2-dependent manner in vitro and in vivo. The B3 region also contains an element that represses the use of Bcl-xS. This element is bound by U1 small nuclear ribonucleoprotein and contains two 5′ splice sites that can be used when the Bcl-xL 5′ splice site is mutated or the ML2/AM2 elements are deleted. Conversely, mutating the cryptic 5′ splice sites stimulates splicing to the Bcl-xL site. Thus, SRp30c stimulates splicing to the downstream 5′ splice site of Bcl-xL, thereby attenuating the repressive effect of upstream U1 snRNP binding sites.

Alternative splicing is used by most metazoans from plants to vertebrates to expand the repertoire of proteins produced from a limited set of genes (1, 2). Humans in particular make ample use of this process since nearly 70% of our multi-exon genes are alternatively spliced (3).

A great diversity of factors are implicated in the control of splice site selection. In mammals, hnRNP2 and SR proteins form two families of RNA binding proteins that play a variety of functions in this process (4–6). For example, the binding of hnRNP A1 and hnRNP H in introns can stimulate intron definition or promote exon skipping (6, 7), a situation that may be relevant to the mechanism of action of other hnRNP-like proteins such as Nova-1 (8). On the other hand, the binding of SR proteins in alternative exons often promotes exon inclusion by antagonizing the repressing activity of nearby-bound hnRNP proteins (5). SR proteins can also have dual functions in splicing control. For example, although the interaction of SRp30c in the alternative exons of SMN2 and gonadotropin-release hormone stimulates its inclusion (9, 10), it also associates with exonic silencer elements in tau exons 2 and 10 (11) and intronic silencer element in the hnRNP A1 pre-mRNA (12, 13).

Although the contribution of alternative splicing to mammalian evolution, animal development, and human diseases is becoming better understood (14–20), the functional impact of the multitude of splice variants is still poorly documented. The contribution of alternative splicing to protein function has been studied in more detail in the field of apoptosis where splicing variants for receptors (e.g. Fas, LARD), adaptors (e.g. TRAF2), and several caspases display antagonistic functions (21). Bcl-x remains a popular example because alternative splicing produces a long anti-apoptotic splice variant (Bcl-xL) and a short pro-apoptotic isoform (Bcl-xS). The relative proportion of the Bcl-x splice isoforms is often altered in cancer cells (22–26), and several reports suggest that the overexpression of Bcl-xL contributes to the resistance of cancer cells to chemotherapeutic agents (27, 28).

The mechanism controlling the alternative splicing of Bcl-x has received some attention recently. In A549 cells, the production of the Bcl-xL isoform is increased by ceramide (29). The effect of ceramide requires PP1 (30), a phosphatase known to act on SR proteins. Two cis-acting elements, CRCE1 and CRCE2, that flank the Bcl-xS 5′ splice site participate in the ceramide-mediated response (31). The U2 snRNP protein SAP155 was shown to associate with CRCE1, and its genetic depletion improved the production of Bcl-xL (32). Cytokines also affect the ratio of the Bcl-x isoforms, and an intronic region downstream of the Bcl-xS 5′ splice site has been implicated in mediating these effects (33). The phosphorylation of Sam68 by Fyn favors the production of Bcl-xL in cooperation with hnRNP A1 (34). Recently, we have identified an element in exon 2 (SB1) that represses splicing to the downstream Bcl-xS 5′ splice site (35). The repressor activity of SB1 requires active protein kinase C in 293 cells. Finally, we also observed that the Bcl-xS 5′ splice
site is stimulated by downstream G-rich elements bound by hnRNP F/H proteins (36). The same study also reported that deleting a 86-nt portion (B3) upstream of the 5′ splice site of Bcl-xL decreased the production of Bcl-xL in vitro and in vivo. Here we show that B3 contains three elements that modulate the Bcl-x splicing ratio. Two elements stimulate the production of Bcl-xL, and their activity is mediated specifically by SRp30c. The third element contains two 5′ splice site sequences and has an opposite effect on Bcl-xL usage. Our results highlight the combinatorial nature of the B3 element and the complexity of effectors controlling the alternative splicing of Bcl-x.

**EXPERIMENTAL PROCEDURES**

**Plasmids**—pS2.13, pX2.13, and ΔB3 derivatives have been described previously (36). pS2.13ΔAM results from a religation after cleavage of pS2.13 with Accl and MscI and Klenow treatment. pS2.13ΔML was produced by amplifying with oligos HincII and XL-MscI. Products were cleaved with HincII and MscI and reintroduced in pS2.13 cut with the same enzymes. Most of the other deletion mutants were produced in a similar way; the sense oligo carrying the mutation was paired with oligo HincII, whereas in a second reaction the antisense oligo carrying the mutation was paired with the NcoI oligonucleotide (supplemental Table 1). Both PCR products derived from these sets of oligos were mixed for a second round of amplification with the terminal oligos. The resulting fragments were cut with HincII and NcoI and ligated in pS2.13 cut with the same enzymes. The individual mutations were transferred in the pX2.13 background by purifying the XhoI (Klenow-filled)-XbaI fragments and inserting it at the EcoRV and XbaI sites of pX2.13. pVSBA and pVSBAΔB3 were constructed by amplifying the Bcl-x vector (from exon 2 to 52 bp of intron sequence) using either pX2.13 or pX2.13ΔB3 with primers XNF (GCTGGCTAGCGTTAAAACTTAAAGCTTA) and XR (TACACGTGCAAGGATGATCCTGACCAAGAG). Plasmid pSPad and primers AXAF (TCAAGATCCACCGTGCTGCTGTCGCGAG) and A3R (TAAATCTAGAGCCGGATAGTTTTCCGGATCCA) were used for amplification of 3′ adenovirus portion that contained 77 and 66 bp of adenovirus intron and exon sequences, respectively. The final PCR product was amplified using the first and second PCR fragments and primers XNF and A3R. Insertion of final PCR products into NotI-blunted pcDNA3+ vector produced plasmids pVSBA and pVSBAΔB3. Primers T1187-203F (CTGTGCGTGGAAAGCG) and A3R were used on pVSBA and pVSBAΔB3 to amplify fragments that were inserted into SacII/XbaI sites of pKS, thereby providing a shortened exon 2 fragment that lacked the 5′ splice site for Bcl-xL. The Smal-linearized plasmids pKVSBA noS and pKVSBAΔB3 noS were transcribed with T7 RNA polymerase. All plasmids were extensively digested with restriction enzymes and sequenced for confirmation. Plasmids used for the production of recombinant SRp30c have been described previously (12).

**Transfection Assays**—Plasmid transfections were carried out with polycarbonateimide (Polysciences Inc., Warrenton, PA). Five µg of polycarbonateimide were mixed with 1 µg of DNA in 200 µl of Opti-MEM I (Invitrogen). The mixture was applied to cells for 6 h at 37 °C. Dulbecco’s modified Eagle’s medium supplemented with 10% of fetal bovine serum (Wisent, St-Bruno, Québec, Canada) was then added, and the cells incubated for 24 to 48 h. In all cases RNA was extracted from cells or transfected cells using TRizol (Invitrogen) using the procedure described by the manufacturer. Splicing was assessed by RT-PCR. Reverse transcription was carried out with oligonucleotide RT3, and PCR was carried out with oligonucleotides X3 and RT2.

**In Vitro Transcription**—Pre-mRNA substrates for in vitro splicing assays were prepared by PCR amplification using the polymerase Pfu Turbo (Stratagene, La Jolla, CA) and oligos AvF3 and X2B (0.1 µg/µl) on plasmid S2.13 and derivatives. The PCR fragments were gel-purified and transcribed using homemade T3 RNA polymerase in the presence of traces of [α-32P]UTP (800 Ci/mmol; PerkinElmer Life Sciences) using standard protocols (37). Transcripts were fractionated on denaturing acrylamide gels and gel-purified. Transcripts were resuspended at a final concentration of 2 fmol/µl. In some cases transcripts were prepared by direct transcription from the plasmid, and the RNA was purified as above.

For the B3+ transcribed and its derivatives, PCR fragments were produced using oligos T3ΔB3 (bearing the T3 promoter sequence) and X-5L and plasmid S2.13 or its 2Δ derivative. Hot transcripts were produced from these templates using [α-32P]UTP as described in Chabot (1994). RNAs were resuspended at 100,000 cpm/µl.

**In Vitro Splicing**—Two fmol of transcript were incubated for 2 h at 30 °C in a mixture containing 5 µl of HeLa nuclear extract (38), 2.5 µl of polyvinyl alcohol 13%, 0.5 µl of ATP 12.5 mM, 0.5 µl of MgCl₂ 80 mM, 0.25 µl of RNAGuard, 0.25 µl of dithiothreitol 100 mM, 0.5 µl of creatine phosphate 0.5 M, and 0.5 µl of creatine kinase 2 units/µl (Roche Applied Science). After incubation, the reactions were stopped by adding 450 µl of sodium acetate 0.3 M and 0.1% SDS. After an extraction with phenol/chloroform/isoamyl alcohol, nucleic acids were precipitated with ethanol in the presence of 35 µg of glycogen. The final pellet was resuspended in 10 µl of water.

To monitor splicing, 2 µl of the above final RNA mixtures were reverse-transcribed for 1 h at 37 °C with the Omniscript reverse transcriptase in a final volume of 10 µl using the Qiagen kit (Qiagen Corp., Valencia, CA), oligo X2B, and RNAGuard. One µl of the mixture was then incubated with 0.5 µl each of oligos X2 and X3 (0.1 µg/µl), 0.2 µl of Taq DNA polymerase (5 units/µl), 2.5 µl of buffer (100 mM Tris-HCl, pH 9.0, 50 mM
Control of Bcl-x Splicing

KCl, 15 mM MgCl$_2$, 20 µl of H$_2$O, 0.2 µl of a mixture of dNTPs at 10 mM each, and 0.1 µl of [α-32P]dCTP 3000 Ci/mmol (PerkinElmer Life Sciences). The PCR cycle consisted in a denaturation for 3 min at 95 °C followed by 35 cycles of 15 s at 95 °C, 30 s at 60 °C, and 30 s at 72 °C. The incubation was terminated with 3 min at 72 °C.

Samples were fractionated on a native acrylamide gel which was exposed for 2 h on a film with a screen. The gel was also analyzed on a PhosphorImager Storm 860 (GE Healthcare) for quantification.

Gel-shift Assay—RNA oligos (Integrated DNA Technologies, Coralville, IA) were 5’ end-labeled with polynucleotide kinase and [γ-32P]ATP (3000 Ci/mmol; PerkinElmer Life Sciences), and the labeled material was purified on a MicroSpin G-25 column (GE Healthcare). Recombinant SRp30c (in D buffer) was incubated with 5000 cpm of the labeled RNAs, as in splicing experiments. The labeled material was purified on a MicroSpin G-25 column (GE Healthcare). Recombinant SRp30c (in D buffer) was incubated with 5000 cpm of the labeled RNAs, as in splicing experiments. The labeled material was purified on a MicroSpin G-25 column (GE Healthcare).

UV Cross-linking and Immunoprecipitation Assays—Fifty thousand cpm of B3+ transcript or derivative were incubated in an in vitro splicing mixture (without RNAGuard) for 10 min at 30 °C. The sample was then submitted to 500 µl of UV radiation using the UV Stratalinker 2400 (Stratagene). We then proceeded to an RNase A digestion (1 µg/µl) for 30 min at 37 °C. Immunoprecipitation was carried out by adding 2.5 µl of anti-SRp30c antibody (13) followed with 50 µl of activated protein A CL-4B beads (GE Healthcare) and by washing four times in a NET2 buffer (50 mM Tris-HCl pH 7.5, 150 mM NaCl, 0.05% Nonidet P-40, 0.5 µl diithiothreitol). All samples were resuspended in Laemmli buffer and separated by SDS-PAGE (10%). Gels were exposed 72 h on XAR film.

Affinity Chromatography—The midAM RNA oligo was coupled to agarose-adipic acid beads (Sigma) according to the manufacturer’s recommendations. Twenty-five µl of these beads were then incubated in 93.75 µl of in vitro splicing mixture including the nuclear extract but without polyvinylalcohol for 10 min at 30 °C. To assess the role of U1 snRNP, the nuclear extract was incubated with a 2’-O-methyl oligonucleotide complementary to the 5’ end of U1 snRNA. The beads were eluted twice with 200 µl of buffer D then washed twice in 400 µl of the same buffer. The elution and washing steps were repeated with increasing amounts of KC1 (0.1, 0.25, 0.5, and 1.0 M) followed by a final elution in Laemmli buffer. The eluted proteins were precipitated in trichloroacetic acid and separated by SDS-PAGE. The gel was silver-stained, and the bands of interest were cut out and destained. In-gel trypsin digestion and liquid chromatography-tandem mass spectrometry analysis was performed at the Genome Quebec Innovation Center at McGill University.

RESULTS

The B3 Element Acts as an Enhancer for the 5’ Splice Site of Bcl-x$_S$—We have shown previously that the deletion of a 86-nt region upstream of the 5’ splice site of Bcl-x$_S$ decreases the use of this splice site in vivo and in vitro (36). We constructed a variety of deletion mutants (Fig. 1, A and B) to identify regions within B3 that are responsible for this activity in vitro and in vivo. Transcripts were produced (S2.13 and derivatives) and incubated in a HeLa nuclear extract for 2 h at 30 °C. Using RT-PCR to assess the production of the xS and xL splice isoforms, we confirmed that B3 is important for Bcl-xL usage in a HeLa extract since its removal shifts splicing to the Bcl-xS 5’ splice site (Fig. 1C, compare lane 4 with lane 3). Removing the 3’ half of B3 (ΔML) had an effect that was almost as strong as ΔB3 (Fig. 1C, compare lane 6 with lane 4). Deleting the upstream and downstream halves of ML (ΔML1 and ΔML2, respectively) indicated that ML2 was the active portion that enforced splicing to the Bcl-x$_S$ 5’ splice site (lanes 9 and 10). In contrast, removing the first half of B3 (ΔAM) did not affect Bcl-x splicing (Fig. 1C, lane 5). However, although removing the upstream portion of AM (ΔAM1) had no effect, removing the downstream half (ΔAM2) slightly decreased the relative level of Bcl-x$_L$ (lane 8). A deletion encompassing AM2 and the inactive

FIGURE 2. B3 enhances splicing complex assembly on the 5’ splice site of Bcl-x$_S$. A, a splicing complex formation assay was performed on native acrylamide gels using uniformly labeled pre-mRNAs containing or lacking the B3 element. Transcripts were incubated at 30 °C in HeLa nuclear extracts, and aliquots were taken at different times (0, 15, and 30 min). Heparin was added at a final concentration of 0.6 µg/µl. The position of nonspecific (NS) complexes, complex A, and the origin of the wells are indicated. B, to confirm the spliceosomal origin of complex A, splicing mixtures were made with the B3 containing-transcript were co-incubated with a 2’-O-Me oligonucleotide complementary to U2 snRNA at the indicated concentrations. C, the structure of the transcripts used in the above assays are depicted. The size in nucleotides of different portions of the pre-mRNAs is indicated. Note that both transcripts lack the Bcl-x$_L$ 5’ splice site region.
ML1 region (ΔmidB3, lane 11) confirmed the positive contribution of AM2 to Bcl-xL splicing. Deleting both ML2 and AM2 (2xΔ/H9004) almost completely eliminated splicing to the Bcl-xL 5′/H11032 splice site in vitro (Fig. 1D, lane 5). The deletion of midAM did not decrease the relative production of Bcl-xL (Fig. 1D, lane 6), whereas ΔmidMID did (lane 7), suggesting that the active portion in AM2 is at the 3′ end of this element. The most active portion of ML2 also appeared to be near its 3′ end since the magnitude of the effect of ΔmidML was not as important as that observed with ΔmlML (Fig. 1, D, lanes 8, and C, lane 10, respectively).

The impact of these deletions on Bcl-x splicing was verified in vitro by transfecting CMV promoter-driven minigenes in HeLa cells (Fig. 1E). The parent minigene X2.13 contains the same Bcl-x portion as the S2.13 transcript used in vitro. Although transcripts produced from the parent minigene are spliced preferentially to the Bcl-xS 5′/H11032 splice site (36), the deletion of B3 abrogates the production of Bcl-xL, an outcome also observed with ΔML and ΔML2 (Fig. 1E, lanes 2, 4, and 8). The in vitro impact of all the other deletions was confirmed in vivo except for ΔAM1 and ΔmidAM, which stimulated the use of Bcl-xS (Fig. 1E, lanes 5 and 11), a situation not observed in vitro (Fig. 1C, lane 7, and D, lane 6). This result suggests the existence of a silencer element (see below). A silencer in the AM1-midAM region could explain why deleting AM (which removes the AM2 enhancer) had no effect (Fig. 1E, lane 3).

Thus, three regions in B3 contribute to splicing control. Although we cannot differentiate between effects on Bcl-xL or Bcl-xS donor sites, the argument of proximity would suggest that ML2 and the downstream portion of AM2 stimulate splicing to the Bcl-xL 5′ splice site, whereas a sequence in midAM represses this event. To confirm the overall enhancer activity of B3 on the Bcl-xL 5′ splice site, we tested if B3 could stimulate complex formation on a simple pre-mRNA carrying the 5′ splice site of Bcl-xL. Because complex formation is more easily observed with a strong 3′ splice site, we produced hybrid pre-mRNAs carrying the 3′ splice site of the adenovirus major late transcript. As seen in Fig. 2, deletion of B3 eliminated the U2 snRNP-dependent assembly of splicing complex A. This result, therefore, indicates

**Control of Bcl-x Splicing**

FIGURE 3. SRp30c binds to B3. **A**, gel-shift assay using recombinant GST-SRp30c protein and 5′ end-labeled RNA oligonucleotides containing the AM2 and ML2 elements of B3 as well as the mutated ML2-2 derivative. Oligonucleotides S21 and its mutated derivative S21-cccc act as a positive and negative control, respectively, for SRp30c binding (13). Contiguous sequences matching the high affinity site determined by SELEX (AGGA(G/C)) are indicated in bold. B, UV cross-linking assays in nuclear extracts. Uniformly radiolabeled transcripts containing the B3 element and 20 nt of downstream sequence including the Bcl-xL 5′ splice site (B3+) or lacking the AM2 and ML2 elements (2xΔB3+) were incubated in HeLa nuclear extracts with two concentrations of His-SRp30c. The position of molecular mass markers (in kDa) and of His-SRp30c is shown. C, to confirm the identity of the SRp30c, UV cross-linking was followed by an immunoprecipitation step using the anti-SRp30c antibody. The position of His-SRp30c and of endogenous SRp30c is shown.
that B3 can stimulate early spliceosome assembly on the 5' splice site of Bcl-xL.

A Role for SRp30c in the Activity of B3—We noted that AM2 and ML2 share the sequence AUUGUAU. However, this sequence does not appear essential because mutating the GG in the ML2 sequence, and binding was compromised by the GG→UU mutation (Fig. 3A, lanes 9 and 10). Recombinant SRp30c also bound to AM2 (lane 8) even though it lacks a perfect match to AGGAG. To ask whether SRp30c could bind to these elements in splicing conditions, we carried out a UV cross-linking assay in a HeLa extract incubated with a uniformly labeled RNA transcript (B3+) containing the B3 element and the Bcl-xL splice site. The cross-linking profile was compared with that of an RNA molecule lacking the AM2 and ML2 regions (2xΔB3+). Increasing amounts of recombinant His-SRp30c were added, and a cross-linking product was detected with B3+ at the position expected for His-SRp30c (Fig. 3B, lane 1–3). The intensity of this band was reduced considerably when the 2xΔB3+ RNA was used, and this was the only difference noted in the cross-linking profile (Fig. 3B, lanes 4–6). The identity of this band was confirmed by performing an immunoprecipitation with anti-SRp30c antibodies on the cross-linked material (Fig. 3C, lane 5). When B3+ was incubated in an extract not supplemented with His-SRp30c, we observed a band migrating slightly faster than His-SRp30c (Fig. 2C, lane 1). This band is weaker or is absent when the assay is performed with 2xΔB3+ (lane 3). The immunoprecipitation assay indicates that a product with this behavior is recovered with the anti-SRp30c antibody (lanes 4 and 6). Thus, both recombinant and endogenous SRp30c proteins can interact with B3, and this interaction requires the AM2 and ML2 elements.

Next, we tested the role of SRp30c in the activity of B3 in vitro. Two concentrations (2.5 and 5.0 μM) of His-SRp30c were added to the extract. The lowest amount corresponds approximately to the amount found in 5 μl of extract (one splicing reaction) (Fig. 4B). The addition of His-SRp30c to the wild-type S2.13 pre-mRNA increased the production of Bcl-xL (Fig. 4C, lanes 1–3), whereas it had a moderate effect when ML2 was deleted (lanes 6–8), possibly reflecting AM2-mediated activity. Indeed, removing both ML2 and AM2 nearly completely elim-

![Image](Image 60x319 to 396x733)
Control of Bcl-x Splicing

The binding of U1 snRNP to midAM was not totally surprising because the deletion of ML2 and AM2 (2xΔ) promoted the use of a cryptic 5’ splice site (xM1) mapping in the midAM portion of B3 (see below). We also tested the addition of equivalent amounts of hTra2β, an SR-related protein often associated with splicing enhancer activity (41–44). hTra2β did not significantly affect Bcl-x splicing in vitro, although it did stimulate the use of a distal 5’ splice site on an unrelated reporter pre-mRNA (Fig. 4). We also tested the impact of exogenously expressing SRp30c in HeLa cells. SRp30c increased the production of the Bcl-xL RNA isoform derived from the X2.13 minigene (Fig. 5, A and B). No improvement in Bcl-xL usage was detected when SRp30c was co-expressed with the variant lacking B3. The stable expression of a tagged SRp30c in HeLa and 293 cells was also associated with an increase in endogenous Bcl-xL levels (data not shown). Thus, SRp30c can stimulate the use of the Bcl-xL 5’ splice site in a B3-dependent manner both in vivo and in vitro. We also tested the activity of the SR protein ASF/SF2, which is 74% identical to SRp30c (47). Although ASF/SF2 improved splicing to the Bcl-xL site, consistent with previous results (34), this effect was independent of B3 (Fig. 5, C and D).

The Silencer Element Contains Cryptic 5’ Splice Sites—Our deletion analysis suggested the existence of a splicing silencer in the midAM portion of B3 since removing this region increased Bcl-xL usage in vivo (Fig. 1E, lane 1). With the hope of identifying a factor responsible for this activity, we carried out affinity

Figure 5 Impact of overexpressing SRp30c in vivo. A, HeLa cells were transfected with a SRp30c expression vector and the X2.13 Bcl-x minigene or its ΔB3 counterpart. Triplicate transfections were processed for RT-PCR analysis. B, graphical representation of the results compiled from panel A. The change in the percentage of Bcl-xL products elicited on X2.13 transcripts by SRp30c overexpression is highly significant (p < 0.005). C and D, HeLa cells were transfected with a His-tagged ASF/SF2 expression vector and the X2.13 Bcl-x minigene or its ΔB3 counterpart.

The current study links two observations made previously; specifically, that the 86-nt-long B3 element is required for the optimal use of the Bcl-xL 5’ splice site and that SRp30c can stimulate Bcl-xL splicing in vitro (36). We have shown here that SRp30c can stimulate Bcl-xL usage in vivo (Fig. 1E, lane 1). With the hope of identifying a factor responsible for this activity, we carried out affinity

chromatography using an RNA that contained the midAM sequence. Factors from a HeLa nuclear extract were loaded and eluted successively with 0.1, 0.25, 0.5, and 1.0 M KCl. Compared with a column lacking RNA, ~15 and 7 distinct proteins were eluted at 0.25 and 0.5 M KCl, respectively (not shown). The identity of these bands is presently being investigated. Although no bands were observed in the 1.0 M KCl eluate, specific low molecular proteins remained bound to the column after the 1.0 M KCl wash (Fig. 6A, lane 6). Mass spectrometry analysis of these bands revealed that the largest one was the U1 snRNP-specific polypeptide A (7 peptides covering 43% of the protein). A faster migrating protein was B/B’ (7 peptides covering 24% of the protein), but the analysis of the smallest one was inconclusive even though it yielded two peptides present in U1A. When we used an extract in which the 5’ end of U1 snRNA was blocked by a specific 2’-O-methyl oligonucleotide, these proteins were not recovered (Fig. 6A, lane 7), suggesting that U1 snRNP binds to midAM.
Control of Bcl-x Splicing

FIGURE 6. **The silencer element midAM contains pseudo 5′′ splice sites.** A, affinity chromatography using a HeLa extract was performed on beads containing an RNA oligonucleotide containing the midAM sequence. Mock-treated beads lacking RNA were also used (mock). We also used a HeLa extract that was previously incubated with a 2′-O-methyl oligonucleotide complementary to the 5′ end of U1 snRNA (U1). After loading, several washing steps were performed with increasing concentrations of KCl. Only the fractions eluted with 1.0 M KCl and then with Laemmli buffer are shown. The bands indicated by asterisks were cut out, destained, and sent for mass spectrometry analysis. Their identity is shown when known. B, RT-PCR analysis of in vitro splicing assays performed in a HeLa extract with the S2.13 Bcl-x pre-mRNA and a derivative containing a mutated Bcl-xL 5′′ splice site (noXL). The identity of the new PCR product (xL) was confirmed by sequencing. C, diagram representing the mutations used in panels B and D. Mutations to create strongXM improve the match to the consensus 5′′ splice site, whereas mutations in weakXM reduce it. D, RT-PCR analysis of transcripts derived from the Bcl-x plasmid X2.13 and derivatives that were transfected in HeLa cells. The experiment was done in triplicate, and the percentage of xL product is shown below the lane numbers. E, graphical representation of the results compiled from the experiment shown in panel D. The differences in splicing observed with both the strongXM and the weakXM mutations are significant (p < 0.005). F, sequences of the Bcl-x authentic and cryptic 5′′ splice sites. A base-pairing score was calculated based on the complementarity of the sequences with U1 snRNA (3 points for a GC or a CG base pair, 2 points for AU, UA, Y/A, and AY, and 1 point for GU, UG, GY, and YA).
Bcl-xL. U1 snRNP binding to the pseudo sites is proposed to repress splicing at the cryptic Bcl-xL splice site, and the AM2 and ML2 elements were explored through obstruction of a portion of the ML2 silencer. Retrospectively, the impact of this oligonucleotide can be explained through the results of Taylor et al. (48) who used an RNA oligonucleotide complementary to an exonic portion upstream of the Bcl-xL 5' splice site to shift splicing toward the Bcl-xS site in vivo by destroying or when the AM2 and ML2 enhancers are deleted. Although we cannot rule out the possibility that these mutations destroy binding sites for other control factors. A silencer model for the activity of AM2 and ML2 elements bound by SRp30c and the pseudo donor sites. SRp30c binding stimulates the use of the Bcl-xL 5' splice site, possibly by stabilizing U1 snRNP at the 5' splice site of Bcl-xL. U1 snRNP binding to the pseudo sites was proposed to repress splicing at the Bcl-xL 5' splice site.

Consistent with an enhancer function for ML2 and AM2, search engines designed to find putative enhancer motifs (RESCUE-ESE (45), PESX (46), ESR-Search (47)) identify such enhancer sequences predicted with the search engines RESCUE-ESE, PESX, and ESR-Search. Access to these engines was obtained on the World Wide Web through RESCUE-ESE, PESX, ESR-Search. The portions corresponding to midAM, AM2, and ML2 are shown. The complementary sequence of the ISIS 22783 oligonucleotide used to inhibit the use of Bcl-xL is also shown.

Supplementing HeLa extracts with recombinant SRp30c stimulated Bcl-xL splicing, and the AM2 and ML2 elements were required for this effect. This observation was confirmed in vivo by showing that the SRp30c-mediated stimulation in the production of Bcl-xL was not observed when the minigenome lacked B3. Recombinant SRp30c could bind to naked AM2 and ML2 RNAs. ML2 contains a sequence that fits the high affinity binding site for SRp30c (AGGA(G/C) Ref. 13). For AM2, it is possible that the two GGA and the GAG triplets contribute to the binding of SRp30c. Our UV cross-linking assays indicate that SRp30c can bind to the enhancer elements in the context of a nuclear extract. SRp30c binding to these sites may then help stabilize the binding of U1 snRNP to the downstream 5' splice site of Bcl-xL (Fig. 8).

FIGURE 7. Nucleotide sequence of the B3 element and alignment of enhancer sequences predicted with the search engines RESCUE-ESE, PESX, and ESR-Search. Access to these engines was obtained on the World Wide Web through RESCUE-ESE, PESX, ESR-Search. The portions corresponding to midAM, AM2, and ML2 are shown. The complementary sequence of the ISIS 22783 oligonucleotide used to inhibit the use of Bcl-xL (59) is also shown.

FIGURE 8. Model for the activity of AM2 and ML2 elements bound by SRp30c and the pseudo donor sites. SRp30c binding stimulates the use of the Bcl-xL 5' splice site, possibly by stabilizing U1 snRNP at the 5' splice site of Bcl-xL. U1 snRNP binding to the pseudo sites was proposed to repress splicing at the Bcl-xL 5' splice site.

Our characterization of B3 also identified the upstream midAM silencer. However, SRp30c also stimulated Bcl-xL usage when the silencer element was mutated (∆midAM and weakX1; data not shown), indicating that the stimulatory activity of SRp30c on the Bcl-xL 5' splice site is probably direct and does not strictly rely on a possible repression of the upstream silencer. A direct stimulation by SRp30c is compatible with current models of action for SR proteins, which are known to facilitate U1 snRNP recruitment (49–51). More recently, the RS domain of SR proteins has also been proposed to contact directly the 5' splice site/U1 snRNA duplex to enhance binding (52). However, it remains to be confirmed if SRp30c, which contains the shortest RS domain of the mammalian SR proteins, can stimulate the binding of U1 snRNP at the Bcl-xL donor site.

Although increasing the levels of SRp30c in HeLa extracts and cells improved the production of Bcl-xL, we have been unable to detect a change in the Bcl-x splicing ratio after a reduction in SRp30c levels using siRNA-mediated assays (data not shown). Although a complete knockdown may be required to observe an effect on Bcl-x splicing, this result may suggest that another factor mediates the activity of B3 in HeLa cells, a situation that would explain why endogenous SRp30c did not cross-link strongly to B3.

A role of SRp30c in Bcl-x splicing may be limited to situations that increase the level of SRp30c. For instance, SRp30c is overexpressed in activated T cells (53). The antigenic stimulation of T cells strongly predisposes cells to apoptosis, and cell survival depends on the ratio of anti- and pro-apoptotic members of the Bcl-2 family (54). Thus, an increase in the level of SRp30c in activated T cells may facilitate the production of the anti-apoptotic Bcl-xL isofrom to help antagonize the impact of specific stimuli that would otherwise trigger apoptosis.

We showed that the midAM silencer element was bound by U1 snRNP, an observation consistent with the existence of two adjacent 5' splice site sequences (CAG/GUAUG/GUGAGU; where/indicates the splice junctions). We noted that these sites could be activated weakly when the Bcl-xL 5' splice site is destroyed or when the AM2 and ML2 enhancers are deleted. Why these 5' splice site sequences are not used more efficiently is intriguing. Even improving the match of both sites such that each one was now a “perfect” 5' splice site did not activate cryptic splicing. One possibility is that their close proximity may create strong mutual interference, as seen previously with authentic 5' splice sites (55).

Improving the match of the cryptic sites eliminated Bcl-xL usage, whereas weakening them strongly stimulated it. These results suggest that the binding of U1 snRNP to these sites can repress splicing to the authentic Bcl-xL donor site (Fig. 8), although we cannot rule out the possibility that these mutations destroyed binding sites for other control factors. A silencer function for U1 snRNPs bound upstream of an authentic 5' splice site is not unprecedented. Indeed, in the well described case of the P element transposase in Drosophila (56), two pseudo 5' splice sites repress splicing to the authentic 5' splice site located ~25 nt downstream. Repression is mediated by a multicomponent complex containing U1 snRNP, P-element somatic inhibitor, and hrp48 (57). Although the xM1 and xM2
junctions in Bcl-x are farther away from the downstream 5’ splice site (~80 nt), they are separated by 6 nt, as in transposase. It will be interesting to assess whether any of the proteins that we found interacting with midAM plays a role in building a U1 snRNP-containing repressor complex.

Thus, B3 contains enhancer and silencer activities, but the dominant impact is that of an enhancer. This enhancement can be mediated by SRp30c and appears to counteract the repressive activity of upstream U1 snRNP binding sites. It is intriguing to speculate that this organization may act as a checkpoint that helps the cell gauge the ability of its splicing machinery to distinguish authentic from aberrant 5’ splice sites. A general reduction in this discriminatory ability would decrease the production of Bcl-xL, thereby predisposing cells to apoptosis.

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REFERENCES
1. Gravely, B. R. (2001) Trends Genet. 17, 100–107
2. Blencowe, B. J. (2000) Trends Biochem. Sci. 25, 106–110
3. Johnson, J. M., Castle, J., Garrett-Engele, P., Kan, Z., Loerch, P. M., Ao, C. D., Santos, R., Schadt, E. E., Stoughton, R., and Shoemaker, D. D. (2003) Proc. Natl. Acad. Sci. USA 100, 1049–1054
4. Black, D. L. (2003) Trends Biochem. Sci. 28, 1067–1074
5. Sanford, J. R., Longman, D., and Caceres, J. F. (2003) J. Biol. Chem. 278, 10491–10499
6. Shen, H., and Green, M. R. (2006) J. Mol. Biol. 360, 1061–1070
7. Guo, Y., Zhang, Q. Q., Zhang, X. H., and Chasin, L. A. (2004) Cell 118, 419–430