Heterogeneous Nuclear Ribonucleoprotein K Represses the Production of Pro-apoptotic Bcl-xS Splice Isoform

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The Bcl-x pre-mRNA is alternatively spliced to produce the anti-apoptotic Bcl-xL and the pro-apoptotic Bcl-xS isoforms. By performing deletion mutagenesis on a human Bcl-x minigene, we have identified a novel exonic element that controls the use of the 5’ splice site of Bcl-xS. The proximal portion of this element acts as a repressor and is located downstream of an enhancer. Further mutational analysis provided a detailed topological map of the regulatory activities revealing a sharp transition between enhancer and repressor sequences. Portions of the enhancer can function when transferred in another alternative splicing unit. Chromatography and immunoprecipitation assays indicate that the silencer element interacts with heterogeneous ribonucleoprotein particle (hnRNP) K, consistent with the presence of putative high affinity sites for this protein. Finally, down-regulation of hnRNP K by RNA interference enhanced splicing to Bcl-xS, an effect seen only when the sequences bound by hnRNP K are present. Our results therefore document a clear role for hnRNP K in preventing the production of the pro-apoptotic Bcl-xS splice isoform.

Alternative splicing is a major mechanism used to augment the number of proteins encoded by the genome. It is estimated that as many as 97% of multiple exon pre-mRNAs undergo alternative splicing (1, 2). Disruption of alternative splicing by mutating important regulatory sequences or by altering the expression or activity of proteins controlling splice site selection has been linked with different diseases, including cancer (3–7). Apoptosis is an important and complex cellular program involved in development and differentiation in higher organisms (8, 9). However, its aberrant control often contributes to cancer development and the resistance of cancer cells to drug therapy (10–13).

Genes implicated in the apoptotic pathway are alternatively spliced often to produce protein isoforms with distinct or even antagonistic activities (14, 15). A good example is the apoptotic regulator Bcl-x, which is alternatively spliced to produce two major isoforms, the anti-apoptotic Bcl-xL protein and the shorter pro-apoptotic Bcl-xS isoform (16). This alternative splicing decision involves a competition between two 5’ splice sites; the use of the downstream site creates Bcl-xL, and the use of the upstream one produces Bcl-xS (Fig. 1A). Bcl-xL is always the predominant form in cancer cells, and overexpressing it can confer resistance to chemotherapeutic agents (17–22). On the other hand, overexpression of the pro-apoptotic Bcl-xS isoform enhances sensitivity to the topoisomerase inhibitor etoposide and to taxol in a breast cancer cell line, while triggering apoptosis in melanoma cell lines (23, 24). Using antisense technologies to improve the production of the Bcl-xS splice variant can also induce apoptosis in cancer cells (25–27).

Alternative splicing is regulated by different proteins bound to sequence elements near splice sites. A variety of mechanisms is used to achieve regulation. Some splicing factors act by recruiting or inhibiting the binding of different components of the spliceosome. Others may change the conformation of the pre-mRNA to mask a splice site or to bring a pair of splice sites into closer proximity (28, 29).

Although individual factors can have a strong and specific effect on splicing decisions, alternative splicing often relies on a combination of factors to determine the appropriate levels of isoforms. The implication of multiple proteins likely provides additional levels of regulation that helps attuned splicing control to a variety of stresses, environmental cues, and growth conditions. In several cases, the interaction of regulatory factors can be antagonistic. For example, in the Drosophila male-specific-let-2 (msl-2) pre-mRNA, recruitment of SRP30c to the U-rich element on the avian myosin phosphatase targeting subunit-1 (MYPT1) pre-mRNA represses the binding of TIA-1 that is necessary for efficient U1 snRNP2 recruitment at the 5’ splice site (30). On the same pre-mRNA, SXL also diminishes U2AF recognition of the polypyrimidine tract at the 3’ splice site. TIA proteins bound to a U-rich element on the avian myosin phosphatase targeting subunit-1 (MYPT1) pre-mRNA repress the binding of PTB (31). PTB can also reduce the recruitment of ETR-3 to intronic elements near exon 5 of cardiac troponin T (32). In neurons, the binding of PTB to the introns surrounding the N1 exon of c-src is antagonized by nPTB protein, promoting exon inclusion. On the hnRNP A1 pre-mRNA, PTB diminishes the binding of SRp30c to the intronic CE9 element, reducing the inhibition by this protein on the use of the downstream 3’ splice site (33). SC35 and hnRNP A1 have partially overlapping binding sites on the human immunodeficiency virus 1 (HIV-1) tat exon 2. Preferential binding of SC35 enhances the inclusion

1 The online version of this article (available at http://www.jbc.org) contains supplemental Figs. 1-3.
2 The abbreviations used are: snRNP, small nuclear ribonucleoprotein; hnRNP, heterogeneous nuclear ribonucleoprotein particles; RT, reverse transcription; siRNA, small interfering RNA; siK, siRNA targeting hnRNP K; PTB, polypyrimidine tract-binding protein; nt, nucleotide; RNAi, RNA interference.
of the exon, whereas hnRNP A1, by reducing SC35 binding, increases exclusion (34). Thus, the competition provided by an overlapping or a closely abutting pair of enhancer/silencer represents a simple and frequent mechanism of splicing control.

The regulation of Bcl-x alternative splicing has received some attention in recent years leading to the discovery of several cis-acting elements and a few trans-acting control factors (Fig. 1B). Intronic regions downstream from the Bcl-x<sub>L</sub> 5′ splice site have been implicated as mediating signals from cytokines such as interleukin-6 and granulocyte-macrophage colony-stimulating factor (35). In addition, we have reported that an element located 187 nt upstream of the Bcl-x<sub>S</sub> splice site mediates a protein kinase C-dependent signal that represses splicing to the Bcl-x<sub>S</sub> donor site (36). On the other hand, ceramide enhances the use of the Bcl-x<sub>S</sub> 5′ splice site by lifting the repression mediated by two other elements (37, 38). The activity of one of these apparently involves SAP155 (39). The RNA-binding protein Sam68, under the control of the tyrosine kinase Fyn, can also increase the production of Bcl-x<sub>S</sub> in cooperation with

**FIGURE 1.** A, alternative splicing of Bcl-x produces two major isoforms, Bcl-x<sub>L</sub> and Bcl-x<sub>S</sub>. B, regulation of Bcl-x alternative splicing. The enhancer elements are shown as white boxes, and the repressors are black. The pointed and flat arrows indicate positive and negative regulation, respectively. Protein kinase C inhibition relieves repression caused by the SB1 element on the Bcl-x<sub>L</sub> splice site (36). The repressor elements CRCE1, recognized by SAP155, and CRCE2 mediate the production of Bcl-x<sub>S</sub> by ceramide as when induced by gemcitabine in A549 cells (38, 39), hnRNP F/H binds to the B2G element to enhance the production of the Bcl-x<sub>S</sub> isoform (41). RBM25, through an element located upstream of the Bcl-x<sub>S</sub> splice site, can also augment its use (44). A large intronic region (IRE) mediates the Bcl-x<sub>L</sub> increase caused by interleukin-6 (IL-6), granulocyte-macrophage colony-stimulating factor (GM-CSF), and 12-O-tetradecanoylphorbol-13-acetate (TPA) (35). Finally, the B3 region also enhances Bcl-x<sub>L</sub> formation through the binding of SRp30c to AM2 and ML2 and the U1 snRNP to two cryptic 5′ splice sites (42).
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hnRNP A1 (40), and this effect is inhibited by overexpression of ASF/SF2. The Bcl-x sequences bound by the above factors remain to be identified. We also uncovered enhancer elements for Bcl-xS and Bcl-xL, hnRNP F and H bind downstream of the Bcl-xL 5' splice site to stimulate splicing to that site (41). Enhancement of Bcl-xL is conferred by SRp30c, which binds upstream of the 5' splice site to antagonize the repressor activity of pseudo 5' splice sites (42). Recently, the SR protein SC35 was shown to increase the production of Bcl-xL (43). Finally, the binding of RBM25 to a sequence element upstream of the Bcl-xL 5' splice site stimulated its use, possibly by recruiting U1 snRNP through its interaction with the U1-associated protein hLuc7A (44). Thus, the region located between the two competing 5' splice sites of Bcl-x is densely populated by splicing control elements.

In this study, we have pursued our characterization of Bcl-x splicing control by examining the contribution of sequences directly upstream of the Bcl-x L donor site. Our mutational approach identified a region containing flanking enhancer and silencer activities. The activity of the repressor portion is mediated by hnRNP K, which makes this protein an anti-apoptotic regulator.

EXPERIMENTAL PROCEDURES

Cell Culture—The 293 cells used in this study were the EcR-293 cell line (Invitrogen). EcR-293 and HeLa cells were maintained in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum and 1% glutamine. PC-3 cells were maintained in Ham's F-12 medium containing 10% fetal bovine serum and 1% glutamine.

Plasmid Construction—The Bcl-x minigene X2.13 was constructed as described previously (41). The ΔB1 deletion was created by amplifying the X2.13 with primers XS-B1d (ATATTAGGGCATTCCTTTGAACAGGT) and Bcl-x AccI (ATCTCCTTGTCTACGCTT) using Pfu polymerase. The resulting insert was digested with BsmI and AccI and inserted in the S2.13 minigene digested with the same enzymes.

Protein A Beads—The Bcl-x minigene X2.13 was ligated in the S2.13 previously digested with the same enzymes. The same technique was used to obtain the elements using an equimolar ratio of DNA oligomers for the sense and antisense strands, incubated in 1× One-Phor-All buffer (GE Healthcare) at 100 °C for 10 min, and then slowly cooling down to 21 °C during 2 h. These mixtures were then ligated in minigene 45 previously cut with BseRI and blunt-ended with Klenow. All constructs were verified by digestion and sequencing.

Transfection of Plasmids or siRNA and RNA Extraction—All transfections of plasmids, RNA extractions, and consequent RT-PCR analysis were done as described previously (36) as were treatments with RNAi (41). The target sequences for siK1 and siK2 were GAGGCCAUUAUUGAUUAUC and UCUAGCAGGAGAAUAAUU, respectively.

Transcription and Splicing Assays—In vitro transcription and splicing of minigene 45 and derivatives, as well as RT-PCR analysis of the products, were done as described previously (45). Templates for the RNA transcripts used in the immunoprecipitation were amplified by PCR (Pfu Turbo, Stratagene) and gel-purified (Qiagen). Transcription was done using T3 RNA polymerase (Promega) and [α-32P]CTP (PerkinElmer Life Sciences).

RNA Chromatography—Coupling the RNA (IDT) to the beads and incubation of nuclear extracts in splicing conditions were done as described previously (33). After incubation, the beads were washed twice with 8 volumes of KCl-free buffer D (60 mM HEPES, pH 7.9, 0.2 mM EDTA, 0.5 mM phenylmethylsulfonyl fluoride, 0.5 mM dithiothreitol, and 20% glycerol) and then eluted twice with 4 volumes of buffer D containing 100 mM KCl. These were pooled and then precipitated by adding 1 volume of trichloroacetic acid, incubating for 20 min on ice, and spinning for 5 min at 10,000 × g. The pellets were resuspended in 0.1 N NaOH. These steps, starting with washing with the previous eluting KCl concentration, were repeated for buffer D containing 250, 500, and 1000 mM KCl. The various eluates were loaded on a 10% polyacrylamide gel and stained with silver nitrate (Invitrogen), and the bands of interest were analyzed by mass spectrometry.

RNA Immunoprecipitation—Sepharose-protein A beads (GE Healthcare) were incubated in buffer A (10 mM Tris-HCl, pH 7.4, 100 mM NaCl, 2.5 mM MgCl2) containing 0.5% Triton X-100 for 1 h at 25 °C, washed three times in the same buffer, and then resuspended for a final volume of 50% beads. One μl of 12G4 anti-hnRNP K antibodies (kindly provided by G. Dreyfus) was added per 50 μl of 50% slurry and incubated for 1 h at 4 °C. The beads were washed three times with cold buffer A. Equivalent quantities of transcripts were incubated in 12.5 μl of splicing mix (45) for 30 min at 4 °C and then washed five times with 1 ml of buffer A. Proteinase K and SDS were added to the beads and then incubated at 37 °C for 15 min. Following phenol extraction and ethanol precipitation, the RNA was resuspended in formamide dye and loaded onto a 6.5% denaturing acrylamide gel. The gels were then analyzed on a PhosphorImager and the results adjusted for relative amounts of radioactivity.
RESULTS

Mapping of Enhancer and Silencer Elements Upstream of the
5’ Splice Site of Bcl-xS—Previous deletion mutagenesis using
Bcl-x minigenes (36, 41) transfected in HeLa cells identified an
82-nt region (B1AU, Fig. 2A), starting 10 nt upstream of the 5’
splice site of Bcl-x that regulates Bcl-x splicing. When this
region is deleted from the X2.13 minigene carrying the two 5’
splice sites of Bcl-x, the Bcl-xL/Bcl-xS splicing ratio is increased
by 10-fold, as judged by RT-PCR analysis (Fig. 2B, lane 2, compare
lane 1 with lane 2). Thus, B1AU behaves as an enhancer for the
Bcl-xS 5’ splice site. Further dissection of B1AU was performed
by dividing the element into three parts. Deletion of the
upstream B1AG portion (31 nt) also decreased the relative
usage of the Bcl-xS site (Fig. 2B, compare lane 1 with lane 2). The
results presented are representative of many independent experiments.

Mutational Analysis of B1AG, B1d, and B1u—To identify nucleo-
tides important for the activity of the different elements, a collection
of 42 mutants was constructed, each one containing a dinucleotide mu-
tation in B1AG, B1d, or B1u (Fig. 3). Each mutant was transfected in
HeLa cells, and the xL/xS splicing ratio of plasmid-derived transcripts
was determined by RT-PCR. The analysis was performed at least
three times for most mutations (supplemental Fig. 2). The results of
one experiment are expressed relative to the ratio obtained with the wild-type X2.13 construct (Fig. 3A). Because most of the mutations in B1AG reduced
splicing to the Bcl-xS site (Fig. 3A, left panel), we may conclude
that B1AG is an enhancer. Disruptions anywhere in the contiguous
stretch of the central 16 nt (AACUGCGGUACCGGCG) shifted splicing toward
Bcl-xL. In silico analysis of these nucleotides using ESEfinder (46, 47) identified putative ASF/SF2-
binding sites (underlined in Fig. 3), and most of the mutations
that reduce the strength of the sites also compromised usage of
Bcl-xS. Intriguingly, mutating the nucleotides recently de-
scribed as important for the binding of RBM25 (44) (boxed in
Fig. 3) had no effect.

A similar mutational analysis of B1d yielded a more nuanced
conclusion (Fig. 3A, middle panel). The impact of mutating the first 14 nucleotides of B1d was consistent with the existence of
a splicing enhancer targeting Bcl-xS, and a contiguous stretch of
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Deleting both B1d and B1u (B1, Fig. 2B, lane 8) also
decreased the effect relative to the deletion of B1d alone (lane 8). Thus,
B1AU contains two antagonistic regions composed of at least three
elements as follows: B1AG and B1d acting as enhancers, and B1u behav-
ing as a repressor of Bcl-xS usage. The activities of the enhancer
(B1AC and B1d) and silencer (B1u) elements were similarly detected
when the analysis was carried out in human 293 and PC-3 cells (supple-
mental Fig. 1).

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a result that is more consistent with the presence of a silencer element. If a silencer element exists in B1d, it is not the dominant activity because deleting or transplanting B1d (see Fig. 4) indicates that its global activity is that of an enhancer.

Finally, dinucleotide mutations in B1u generally increased the use of Bcl-xS, consistent with this region being a silencer (Fig. 3A, right panel). The most important sequence was a stretch of eight nucleotides (AUAUCAGA) that represent putative binding sites for SRp40. However, mutations that did not affect the putative SRp40-binding site had a strong effect on Bcl-x splicing (Fig. 3A, right panel, mutation 9). Moreover, knocking down SRp40 in a variety of cell lines did not significantly affect Bcl-x splicing (data not shown).

Several mutants were also tested in 293 cells with an impact similar to what was observed in HeLa cells (supplemental Fig. 2). Globally, the results of the mutational analysis match very well the results of the deletions. Because splicing regulatory factors like SR and hnRNP proteins often have degenerate binding sites, not all mutations may alter in the same way the activity of a control element. Moreover, some mutations may create an element that imposes new splicing control. To facilitate the graphical representation of our results and to minimize the above caveats, we assigned to each mutation a value that is an average of the impact of the mutation and the two adjoining mutations, thus more accurately displaying the contribution of existing elements (Fig. 3B). The most important feature of this map is a transition from enhancer to silencer activities occurring in B1d. Although B1d globally behaves as an enhancer because of its 5’ portion, its 3’ portion appears to represent the extremity of a silencer that extends into B1u.

Enhancer Elements Can Function When Placed in a Different Context—Mutations may modify alternative splicing by changing the secondary structure near a splice site or because they compromise the interaction with a trans-acting regulatory factor. To assess the intrinsic modulatory activity of the elements on alternative splicing, we inserted each one into a previously characterized reporter gene (45) containing the 5’ splice sites of exon 7 and 7B of hnRNP A1, their surrounding sequences, and the 3’ splice site of the adenovirus major late exon L2 (Fig. 4A). The resulting minigenes were transcribed in vitro, and the resulting pre-mRNAs were incubated in HeLa extracts for 2 h. RT-PCR analysis was conducted to assess the splicing behavior of the inserted element. Placing B1AG upstream of the proximal 5’ splice site improved its use (Fig. 4B, compare lane 2 with lane 1). When the other enhancer element (B1d) was inserted at the same position, proximal 5’ splice site usage was also increased (Fig. 4B, lane 3). Finally, the silencer element B1u had very little impact on splicing (Fig. 4B, lane 4). These results were confirmed by testing the various transcripts in several batches of nuclear extracts (not shown). Our results therefore suggest
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To further analyze the interaction of hnRNP K with the Bcl-x pre-mRNA, we carried out an RNA immunoprecipitation assay using our model Bcl-x pre-mRNA (X2.13) and versions carrying various deletions. Radiolabeled transcripts were incubated in HeLa nuclear extracts under in vitro splicing conditions. hnRNP K antibody (a kind gift from G. Dreyfuss) conjugated to protein A-Sepharose was then added. After several washes, the labeled RNA was recovered and quantitated on the gel. This assay revealed that the binding of hnRNP K to X2.13 strongly decreased when B1, B1d, or B1u were deleted (Fig. 5). This interaction of hnRNP K with B1u may be explained by the presence of a contiguous stretch of four cytidines, a known recognition site for hnRNP K (49). Consistent with this possibility, the mutation B1u.4, which changes the last cytidine of that stretch and severely compromises the activity of the silencer, diminished the recovery of the RNA by anti-hnRNP K immunoprecipitation. Mutating the abutting CC into AA did not compromise recovery with the anti-hnRNP K antibody nor did it affect Bcl-x splicing (Fig. 3A). Notably, mutation B1d.7, which has an effect opposite that of deleting B1u (Fig. 2B and Fig. 3A), slightly but significantly increased recovery of the RNA by anti-hnRNP K immunoprecipitation. This mutation may enhance hnRNP K binding directly or indirectly by disrupting a binding site for an enhancer protein that competes with hnRNP K for binding in the region where the transition between enhancer and silencer activities occurs. We could not confirm direct binding of hnRNP K because our recombinant hnRNP K protein produced in bacteria lacked any type of binding activity.

Knockdown of hnRNP K Affects Bcl-x Splicing in a B1-dependent Manner—A few studies have documented a role for hnRNP K in splicing control (50–52). The related Nova proteins contain KH domains found in prototypical hnRNP K, and they regulate brain-specific alternative splicing events (52–54). To clarify the contribution of hnRNP K to Bcl-x splicing, we knocked down hnRNP K expression by RNA interference using two different nonoverlapping siRNAs (siK1 and siK2) in PC-3 and HeLa cells. The knockdown was successful in PC-3 cells but was less efficient in HeLa cells (Fig. 6A). Assessing the impact of the knockdowns on the endogenous Bcl-x splicing profiles in PC-3 cells revealed an increase in the use of the Bcl-x\(_S\) 5' splice site (Fig. 6B, lanes 1–3), consistent with the notion that hnRNP K represses the splicing of Bcl-x\(_S\). The effect was less dramatic in HeLa cells (Fig. 6B, lanes 4–6), as expected from a more limited depletion. We observed increased Bcl-x\(_S\) isoform formation upon siRNA treatment in several other cell lines (data not shown). To confirm that the modulation of splicing by hnRNP K requires the B1 element, we looked at the impact of the hnRNP K depletion on the splicing of transcripts produced from minigenes X2.13 containing or lacking the B1 element (Fig. 6C). Only the wild-type X2.13 minigene experienced a decrease in the Bcl-x\(_L\)/Bcl-x\(_S\) ratio when hnRNP K was depleted in PC-3 cells. Thus, our results indicate that the activity of hnRNP K is mediated through B1, most likely through the C-rich elements important for hnRNP K binding in B1d and B1u.

![Figure 4](https://example.com/figure4.png)

**FIGURE 4.** Insertion of the enhancer elements in the minigene 45 replicates their activity. A, each element was inserted upstream of the proximal 5' splice site (ss) of the minigene 45 as indicated. The primers used for subsequent RT-PCR analysis are shown. B, transcripts derived from minigene 45 were incubated in HeLa nuclear extracts in in vitro splicing conditions. RT-PCR analysis was done on total RNA. The splicing products are indicated as well as the proximal/distal (prox/dist) ratios. A new product presumably produced by a cryptic splice site is indicated with an asterisk.

that the enhancer activities of B1AG and B1d are mediated by trans-acting factor(s). The activity of B1u does not appear transplantable (Fig. 4B, lane 4), suggesting that B1u may promote splicing repression by preventing the activity of the upstream enhancers.

**hnRNP K Binds to the B1 Element**—To isolate factors that mediate the activity of these control elements, we carried out affinity chromatography with RNA portions covalently linked to agarose beads using HeLa nuclear extracts. The bound material was eluted with increasing amounts of KCl. The content was fractionated on acrylamide gels and revealed by silver staining; bands were cut, and proteins were analyzed by nanoliquid chromatography coupled on line with tandem mass spectrometry (supplemental Fig. 3). The strongest hit obtained came with B1d for which a 60-kDa protein that eluted at 250 mM KCl was identified as hnRNPK. Binding of hnRNPK may be explained by the presence of UCCCAU and UCCCAAU, which are sequences very similar to the high affinity RNA-binding site for hnRNPK (UC\(_{3–4}\)(AU)(UA)) identified through SELEX (48). Our previous analysis in Fig. 3A showed that mutating the two central cytidines of either sequence stimulated splicing to the 5' splice site of Bcl-x\(_S\), consistent with the view that hnRNPK might repress the production of Bcl-x\(_S\).
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**FIGURE 5. RNA co-immunoprecipitation of a portion of the Bcl-x pre-mRNA using hnRNP K antibodies is dependent on the B1 region.** The transcripts were incubated in HeLa nuclear extract in splicing conditions and then on beads coupled with hnRNP K antibodies (Anti-hnRNP K) or preimmune serum (Serum). After five washes, the RNA was precipitated, migrated on an acrylamide gel, and quantified on a PhosphorImager. The *left panel* is a graph of the values of radioactivity measured. The *right panel* shows the relative levels of a tenth of the input RNA. The sequence of the point mutations that were tested is indicated in the *bottom panel.*

**DISCUSSION**

Given the functional importance of the major Bcl-x splice isoforms in apoptosis and their antagonistic roles, it is not surprising that the splicing decisions that occur on the Bcl-x pre-mRNA are regulated by a variety of elements and factors (35–41, 44, 55). Many of these elements may help link splicing regulation with specific pathways that monitor the integrity of various cellular components and compartments as well as the availability of the nutrients and growth conditions.

In this study, we document the activity of an 82-nt region (B1AU) located immediately upstream of the 5′ splice site of Bcl-x\(_2\). The 5′ portion of this region displays enhancer activity because deleting or mutating specific nucleotides decreases the use of the Bcl-x\(_2\) site. Because portions of this region can function in a heterologous context, the activity of the enhancer likely requires trans-acting factor(s). The region contains three putative high affinity binding sites for ASF/SF2. However, a role for ASF/SF2 in the activity of these elements is unlikely because overexpressing recombinant ASF/SF2 in a variety of cell lines increases the production of Bcl-x\(_2\) rather than that of Bcl-x\(_S\) (40, 42, 56, 57). Recently, RBM25 was identified as binding to a six-nucleotide element (CGGGCA) in B1AG (44). Although the activity of RBM25 in HeLa cells is consistent with the enhancer activity of B1AG, our mutational analysis did not reveal a role for these sequences both in 293 and HeLa cells (Fig. 3 and data not shown). Perhaps mutating only two nucleotides at a time was not enough to destroy the activity of this sequence.

hnRNP K depletion, either enhancing or suppressing exon inclusion (58). This broad role in splicing regulation may come from its ability to interact with several splicing-related proteins, including other hnRNP proteins (49, 59). As with other hnRNP proteins, hnRNP K has also been implicated in other steps of gene expression, including transcription and translation (49). For example, hnRNP K activates transcription of c-myc and serves as a cofactor for p53-dependent transcriptional activation following DNA damage (60, 61), but it can also repress transcription of thymidine kinase and CD43 (62, 63). hnRNP K can also bind to the Nova1 pre-mRNA and can decrease the inclusion of alternative exon 4 (52). Recently, it was shown that hnRNP K can play a prominent role in alternative splicing control because nearly half of 56 alternative splicing events in apoptotic genes were affected upon

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is present in B1u (ACCCCA) (Fig. 7A). Mutating the last two nucleotides strongly diminished the repression of Bcl-xS, as well as decreased binding of hnRNP K. Another high affinity binding site that was previously identified using yeast three-hybrid screens is CCAUCN_{2-7}(A/U)CC-(A/U)N_{7-18}UCAC(C/U)C (66, 67). B1d and B1u contain ACAUC- CAGUCCACA|UCACCCCA (the vertical line indicates the division between both elements). Thus, three putative hnRNP K-binding sites exist within a stretch of 25 nucleotides, and cooperative interactions may stabilize the binding of hnRNP K and antagonize the binding of positive factors in the central portion of B1d. The fact that B1u displays little activity when transplanted in a different pre-mRNA is consistent with a model in which the role of the silencer would be to antagonize the binding or activity of flanking activators. The silencer element is likely to be more complex than the binding of hnRNP K alone because the 3′ end of B1u is the most active silencer region and yet it lacks putative hnRNP K-binding sites. A simple working model for the antagonizing activities associated with B1AU is presented in Fig. 7B. The enhancer activity would be mediated by factor(s) interacting with the 5′ half, perhaps RBM25. The central portion contains sequences bound by hnRNP K, but this region may overlap with binding sites for the enhancing factors. Two putative hnRNP K-binding sites already exist in B1d, and a third one is likely present in B1u. As indicated in the Introduction, overlapping binding sites for factors with different activities are often used to control splicing decisions in other pre-mRNAs (33, 34, 68, 69).

hnRNP K can interact with Sam68 in vitro and in vivo (70, 71), a protein known to regulate Bcl-x splicing (40). However, although we have shown that hnRNP K represses the production of Bcl-xS, Sam68 stimulates it. Although it is unclear where Sam68 binds, hnRNP K may neutralize Sam68 by interacting with it when these proteins are in close proximity. However, performing a knockdown of Sam68 had no effect in our conditions and cell lines (data not shown).
hnRNP K Represses the Bcl-x<sub>S</sub> Isoform

The ability of hnRNP K to repress the production of the pro-apoptotic Bcl-x<sub>S</sub> isoform would confer to hnRNP K an anti-apoptotic function that may help cancer cells escape death signals. As shown recently, the knockdown of hnRNP K can influence the alternative splicing of several apoptotic genes (58), including favoring the production of the pro-apoptotic splice form of MCL1. This MCL1 is another BCL2 family member, and its pro-apoptotic variant is consistently underproduced in breast cancer tissues (72). An anti-cell death function for hnRNP K is also suggested by the increase in the nuclear concentration of hnRNP K that occurs in proliferating cells as well as in tumors (73) and the higher level of hnRNP K observed in a variety of cancers compared with the corresponding healthy tissues (74 – 80). Overexpression of hnRNP K also led to the transformation of Rat1A cells (81). Because DNA damage transiently increases the production of hnRNP K in many human cancer cell lines (60), this response may be used to further repress the production of apoptotic isoforms, hence creating conditions to implement DNA repair without triggering apoptosis. Through its role in splicing, hnRNP K may therefore help coordinate the DNA damage response with apoptotic regulation.

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