Heterogeneous Nuclear Ribonucleoprotein F/H Proteins Modulate the Alternative Splicing of the Apoptotic Mediator Bcl-x*

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Bcl-x is a member of the Bcl-2 family of proteins that are key regulators of apoptosis. The Bcl-x pre-mRNA is alternatively spliced to yield Bcl-xS and Bcl-xL, two isoforms that have been associated, respectively, with the promotion and the prevention of apoptosis. We have investigated some of the elements and factors involved in the production of these two splice variants. Deletions mutagenesis using a human Bcl-x minigene identifies two regions in exon 2 that mediate Bcl-x 5′-splice site selection in human HeLa cells. One region (B3) is located upstream of the Bcl-xL 5′-splice site and enforces Bcl-xL production in cells and splicing extracts. The other region (B2) is located immediately downstream of the 5′-splice site of Bcl-xS and favors Bcl-xS production in vitro and in vivo. A 30-nucleotide G-rich element (B2G) is responsible for the activity of the B2 element. We show that recombinant heterogeneous nuclear ribonucleoprotein (hnRNP) F and H proteins bind to B2G, and mutating the G stretches abolishes binding. Moreover, the addition of hnRNP F to a HeLa extract improved the production of the Bcl-xS variant in a manner that was dependent on the integrity of the G stretches in B2G. Consistent with the in vitro results, small interfering RNA-mediated RNA interference targeting hnRNP F and H decreased the Bcl-xS/Bcl-xL ratio of plasmid-derived and endogenously produced Bcl-x transcripts. Our results document a positive role for the hnRNP F/H proteins in the production of the proapoptotic regulator Bcl-xS.

Alternative splicing is a powerful generator of proteomic diversity. It is estimated that as much as 74% of all human genes may use alternative splicing as part of their expression program (1). In the most remarkable example to date, the Drosophila DSCAM gene can potentially yield more than 38,000 different isoforms by alternative splicing (2). Alternative splicing has the potential to alter protein activity in many important ways. In some cases, the inclusion of sequences carrying a stop codon can trigger non-sense-mediated RNA decay, thereby down-regulating protein expression (3, 4). In other instances, alternative splicing yields protein variants with drastically different and sometimes antagonistic properties. This is the case with the Bcl-x pre-mRNA, which experiences alternative 5′-splice site utilization to produce the anti-apoptotic Bcl-xS protein or the proapoptotic Bcl-xL isoform (5).

Bcl-x is a member of the large bcl-2 family of apoptotic genes. Bcl-x proteins modulate mitochondrial protein release, an event associated with the induction of programmed cell death. In a number of cancers and cancer cell lines, the expression of the anti-apoptotic protein Bcl-xL is increased, and the ratio of the splice variants is frequently shifted to favor production of Bcl-xL (6–9). The overexpression of Bcl-xL is associated with decreased apoptosis in cancer cells, increased risk of metastasis, resistance to chemotherapeutic drugs, and poor clinical outcome (8, 10). In contrast, Bcl-xS can induce apoptosis and sensitize cells to chemotherapeutic agents (10–14). When cancer cells expressing high levels of Bcl-xL are treated with an antisense oligonucleotide complementary to the 5′-splice site of Bcl-xL, splicing shifts toward the 5′-splice site of Bcl-xS and cells undergo apoptosis (15).

Although perturbations in alternative splicing have been observed in neoplasia and metastasis (16–18), the identity of the factors that elicit these cancer-specific changes remains poorly documented. One study has uncovered that the progression from preneoplasia to metastasis in a mouse model of mammary tumors correlates with an increase in the abundance of specific SR proteins, a family of proteins involved in generic and alternative splicing (19). Although changes in SR protein expression also correlate with changes in CD44 splicing (19), alterations in SR proteins are likely to be only one of several alterations in the expression of splicing regulators during neoplasia and malignancy. For example, exclusion of the α-exon of fibroblast growth factor receptor 1 is controlled by intrinsic elements bound by the hnRNP 1/poly pyrimidine tract-binding protein (20), and poly pyrimidine tract-binding protein expression is increased in malignant glioblastomas (21). The situation with Bcl-x remains largely unexplored, and little is known about the processes that control Bcl-x alternative splicing in normal and cancer cells. A previous study in human adeno carcinoma A549 cells indicate that ceramide can shift the ratio of Bcl-x isoforms in favor of Bcl-xL (22). The activity of ceramide is antagonized by protein phosphatase-1 inhibitors, which are known to alter the phosphorylated state of SR proteins (23). Two elements flanking the Bcl-xL 5′-splice site, a purine-rich and a pyrimidine-rich region, are required for both basal and ceramide-induced use of the Bcl-xL site (24). However, the direct participation of trans-acting factors in the control of Bcl-x alternative splicing remains to be documented.

We have begun to dissect the elements that control the

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The abbreviations used are: hnRNP, heterogeneous nuclear ribonucleoprotein; siRNA, small interfering RNA; RT-PCR, reverse transcription–PCR; siF, siRNA targeting hnRNP F; siFH, siRNA targeting hnRNP F and H; snRNP, small nuclear ribonucleoprotein.
alternative splicing of human Bcl-x. Our study identifies two elements that affect the basal Bcl-x/Bcl-x splicing ratio in human HeLa cells. Although the B3 element stimulates Bcl-xL production, the B2 element activates Bcl-xS splicing. We show that hnrRNPF/H proteins mediate the activity of the B2 element in vitro and in vivo.

EXPERIMENTAL PROCEDURES

Plasmid Constructs—The human Bcl-x gene was obtained from Bacpac Resources. The wild-type minigene used for in vitro splicing assays was created by the sequential PCR amplification of the 5′-portion of the Bcl-x present in the BAC clone RPC1-11-243J16 using primers h1 (CTCGAGGAAACCGGGAGCTGG) and h2 (GACTGGCAGCTGGGCAATCT). This PCR fragment was cloned in pBlue-script K′ linearized at the HincII site to generate pBS5. The 3′-portion of Bcl-x was amplified by the PAC clone PAC1-5-57F17 using primers h1 (ATTATATACCAAGGGACCTCACT) and h2 (TCAATTTCGGACCTGAGTGA), and the resulting fragment was cloned in pBS5 linearized at the EcoRV site to generate pS2.13. pX2.13 was produced by cleaving pS2.13 with XhoI, treating with Klenow, and cutting with XbaI. The resulting Bcl-x fragment was then cloned into pcDNA1.1+ (Invitrogen) at the EcoRV and XbaI sites. More upstream portions of the Bcl-x gene were amplified from genomic DNA isolated from 293 cells using the same strategy using primers Ac2 (ATATATGTAGACCGGCCTCCCATGGCAGCAG) and Ac1 (ATATATGTCTACCATTCACTACC). The PCR fragment was digested with AccI and HincII and substituted for the wild-type HincII-AccI fragment of pS2.13. The pX2.13 construct was digested with AccI and HincII and substituted for the wild-type minigene used for in vitro splicing assays was created by the sequential PCR amplification of the 5′-portion of the Bcl-x present in the BAC clone RPC1-11-243J16 using primers h1 (CTCGAGGAAACCGGGAGCTGG) and h2 (GACTGGCAGCTGGGCAATCT). This PCR fragment was cloned in pBlue-script K′ linearized at the HincII site to generate pBS5. The 3′-portion of Bcl-x was amplified by the PAC clone PAC1-5-57F17 using primers h1 (ATTATATACCAAGGGACCTCACT) and h2 (TCAATTTCGGACCTGAGTGA), and the resulting fragment was cloned in pBS5 linearized at the EcoRV site to generate pS2.13. pX2.13 was produced by cleaving pS2.13 with XhoI, treating with Klenow, and cutting with XbaI. The resulting Bcl-x fragment was then cloned into pcDNA1.1+ (Invitro
RESULTS

Cis-acting Elements Controlling 5' Splice Site Selection in Bcl-x—As a first step toward the identification of elements involved in the control of 5’ splice site selection in human Bcl-x, we tested the effect of various deletions introduced in a cytomegalovirus promoter-driven Bcl-x minigene expressed in the human-transformed HeLa cell line. The Bcl-x minigene X1 was constructed by combining the upstream portion of the human gene with its downstream portion. Most of intron 2, which is greater than 55 kb, was left out for convenience (Fig. 1A). However, we kept the first 1.1 kb of intron 2, because this region contains sequences that are highly conserved between human and rodents. The first 110 nucleotides of intron 2 are actually coding and are used to produce the Bcl-xS isoform (28). Minigenes were transfected in HeLa cells, and the relative abundance of Bcl-xS and Bcl-xL mRNA isoforms was estimated by RT-PCR with one of the primers used to amplify mRNA products of lower mobility than endogenous Bcl-xL mRNA products in HeLa cells.

In contrast to endogenous Bcl-x expression, which predominantly produces the Bcl-xL mRNA variant in HeLa cells (Fig. 1B, lane 2), the X1 minigene yielded considerably more of the Bcl-xS variant (Fig. 1C, lane 2). Although the Bcl-x splicing profile from the minigene did not mimic the splicing profile of the endogenous transcripts, we nevertheless proceeded with our analysis of deletions with the goal of identifying sequences in the minigene that would alter the xS/xL ratio. The additional removal of exon 1 and intron 1

![Figure 1](image)

**Fig. 1. Splicing of Bcl-x in HeLa cells.** A, structure of Bcl-x and Bcl-x minigenes. The structure of the human Bcl-x gene (top) and different minigenes is drawn with exons (boxes) and introns (lines). Wavy lines indicate plasmid sequences. The size of introns and exons is indicated in nucleotides. The position of the 5’-splice sites of Bcl-xS and Bcl-xL is indicated, as well as the position of primers used to amplify mRNA products by RT-PCR. B, RT-PCR assay to amplify endogenous Bcl-x mRNA products in HeLa cells. The size of the RT-PCR amplified products is indicated and the ratio of Bcl-xS/Bcl-xL is shown at the bottom. C, RT-PCR assay to amplify mRNA products expressed from transfected minigenes. The size of the amplified products corresponding to plasmid-derived Bcl-xS and Bcl-xL is shown, as well as the ratio between these products. D, RT-PCR assays on minigenes carrying deletions located in between the Bcl-x 5’-splice sites. The names and sizes of the targeted regions are indicated on the left panel. The ratio between the Bcl-xS and Bcl-xL products is indicated below the lane numbers. The position of labeled DNA fragments used as markers (ladder) is indicated.
did not change the splicing ratio (Fig. 1C, lane 3, minigene X2).
Further removing the first half of exon 2 also did not significantly
alter the $x_S/x_L$ ratio (Fig. 1C, lane 4, X2.13). In different
experiments, the endogenous Bcl-xS/Bcl-xL ratio varied be-
tween 0.1 and 0.3, whereas the ratio from X2.13-derived tran-
scripts varied between 2 and 8 (not shown). These variations in
the Bcl-x splicing ratio are likely because of the status of the
cells at the time of the experiment. We next tested two different
exonic deletions in plasmid X2.13 (Fig. 1D). Each deletion had
a strong but completely different impact on the relative abun-
dance of the Bcl-x isoforms. The first deletion defined the B2
region, which occupies a 77-nucleotide portion downstream
from the 5′-splice site of Bcl-xL (position +11 to +87 relative
to the Bcl-xS splice junction). The deletion of B2 completely abro-
gated the production of Bcl-xS (Fig. 1D, lane 3), suggesting the
existence of an element that is essential for the use of the
5′-splice site of Bcl-xS. In contrast, the deletion of the B3
region, which is located upstream of the 5′-splice site of Bcl-xS
(position −10 to −95, relative to the Bcl-xS splice junction),
completely eliminated the production of Bcl-xS (Fig. 1D, lane 4).

The above results point to a role for specific exonic regions in
controlling the ratio of the Bcl-x isoforms. However, because the
deflections change the structure of the Bcl-x mRNA without
altering the structure of Bcl-xS mRNA, it is possible that the
deflections differentially alter the stability of the plasmid-de-
derived Bcl-x mRNAs.

Pre-mRNA S2.13 and derivatives were produced and incubated in splicing
extracts for 2 h. Although the efficiency of splicing for these large
introns pre-mRNAs was satisfactory (~5% of input pre-mRNA),
the analysis of splicing products in conventional denaturing
splicing gels was complicated by the production of large lariat
circles that migrate partially overlapped when some
deletions were tested (not shown). To simplify analysis we
relied on performing a RT-PCR assay to selectively amplify
the mRNAs produced from the synthetic pre-mRNA substrates.

The amplification results indicate that pre-mRNA S2.13 was
spliced to produce predominantly Bcl-xS (Fig. 2A, lane 2). Al-
though the Bcl-xS/Bcl-xL splicing ratio of pre-mRNA S2.13 varied
in different experiments and in different HeLa extracts, it
consistently produced more of the Bcl-xS isoform (e.g. Fig. 2B,
lane 2; Fig. 2C, lane 2; Fig. 3B, lane 2). In contrast, the B3
deletion was associated with a severe reduction in the relative
abundance of the Bcl-xS product (Fig. 2A, lane 4, S2.13ΔB3),
consistent with the in vivo splicing profile of X2.13ΔB3. The B2
deletion also reproduced the in vivo effect, resulting in no
detectable Bcl-xS mRNA product (Fig. 2A, lane 3, S2.13ΔB2).

To demonstrate that the 5′-splice site of Bcl-xS remains func-
tional in the ΔB2 construct, an antisense oligonucleotide (X5)
complementary to the 5′-splice site of Bcl-xS was added to
splicing mixtures. When pre-mRNA S2.13 was incubated with
increasing concentrations of oligonucleotide X5, Bcl-xL produc-
tion decreased to favor Bcl-xS (Fig. 2B, lanes 2–5). The X5
oligonucleotide mixed with the S2.13ΔB2 pre-mRNA also pro-
moted the production of Bcl-xS, although the shift was less
efficient at the lowest concentrations of X5 (Fig. 2B, lanes 6–9).

The 5′-splice site of Bcl-xS was also activated when we mutated
the 5′-splice site of Bcl-xL in the context of a ΔB2 deletion (data
not shown). Thus, deleting the B2 region did not irreversibly
inactivate the 5′-splice site of Bcl-xS. Moreover, the similar
impact of the deletions on in vitro and in vivo Bcl-x splicing
suggests that the B2 and B3 elements modulate 5′-splice site
selection rather than mRNA stability.

We also asked whether the effect of the deletions on alter-
native splicing could be caused by the juxtaposition of more
distal control elements in the vicinity of the Bcl-xS or Bcl-xL
5′-splice sites. To answer this question, we substituted the B2
and B3 elements for spacer sequences of similar length. Insert-
ing two different spacers as a replacement for B2 maintained
the deficiency in Bcl-xS production (Fig. 2C, compare S2.13 in
lane 2 with S2.13ΔB2+ and S2.13ΔB2− in lanes 3 and 4,
respectively). Likewise, substituting the B3 element for a
spacer of identical length did not restore the predominant

![Image](https://example.com/image.png)
Bcl-xL production (Fig. 2C, lane 5). Thus, our results suggest that the B2 and B3 regions contain sequences that directly affect 5′-splice site selection.

A Subregion of B2 Is Bound by hnRNP F and H Proteins—To investigate in more details the identity of the sequences and factors participating in the control of 5′-splice site selection in Bcl-x, we focused on the 77-nucleotide-long B2 element located immediately downstream of the Bcl-xS 5′-splice site. First, we produced pre-mRNAs containing deletions of the last 22 or 48 nucleotides of B2 (S2.13B2.1 and S2.13B2.2, respectively; see Fig. 3A). These substrates displayed an in vitro splicing profile that indicated an impairment in Bcl-xS production, although the Bcl-xS product was still detected (Fig. 3B, lanes 5 and 6). This result suggests that the 30-nucleotide portion located between positions +10 and +39 (B2G) might make the largest contribution toward enforcing the use of the Bcl-xS 5′-splice site. Consistent with this view, a pre-mRNA substrate carrying a deletion of the B2G element yielded no Bcl-xS products (Fig. 3B, lane 4), a splicing profile identical to S2.13ΔB2 (lane 3).

**Fig. 3.** The first 30 nucleotides of the B2 element (B2G) enforce Bcl-xS production. **A,** sequence of the region separating the Bcl-xS and Bcl-xL 5′-splice sites and structure of the deletions tested in splicing. The position of the Bcl-x 5′-splice sites is indicated. The sequences of the B2 and B3 elements are boxed (gray and white, respectively). The sequences deleted in ΔB2G, ΔB2.1, and ΔB2.2 are underlined. **B,** in vitro splicing assays of the deletion mutants. Pre-mRNAs S2.13 and derivatives were incubated in HeLa nuclear extracts for 2 h. RNA was extracted and submitted to RT-PCR amplification with a specific set of primers. The position of the amplified Bcl-xS and Bcl-xL products is indicated and the xS/xL ratio of the products is shown below the lane numbers. Lane 7 (t = 0) represents an amplification performed on a splicing mixture containing pre-mRNA S2.13 that was kept on ice.
The +10 to +39 region contains the sequence CGGGAUGGGUAAACUGGGGU (Fig. 3A). Similar G-rich sequences have been identified as signature binding sites for the hnRNP F and H proteins (29–36). To assess the ability of hnRNP F and H proteins to bind to this sequence, we monitored complex formation by mixing a 32P-labeled RNA oligonucleotide carrying the +16 to +42 sequence (B2G oligonucleotide) with recombinant His6-tagged hnRNP F or H produced from baculovirus-infected insect cells (Fig. 4A). As a control, we used a similarly produced His-tagged SRp30c protein (Fig. 4A, lane 3). In a gel shift assay, hnRNP F and H each retarded the migration of the B2G RNA, indicative of complex formation (Fig. 4B, lanes 2 and 22, respectively). In contrast, the baculovirus-produced His-tagged SRp30c protein did not significantly retard the migration of the B2G RNA (lane 23). The specificity of binding by hnRNP F and H was assessed by adding increasing amounts of cold oligonucleotides. The addition of a 100-fold excess of cold B2G RNA promoted the almost complete disappearance of the labeled complexes (Fig. 4B, lanes 5 and 14), whereas little dissociation was observed when a similar concentration of a control RNA oligonucleotide was used (lanes 9 and 18).

To address the role of hnRNP F/H proteins in the activity of the B2 element, we tested the impact of mutating the G-rich stretches in the B2G portion. We produced two mutated versions: the first one (Mut1) carried a GGGG to GCCG mutation in the 3′ terminal G run, whereas the second one (Mut2) contained the same mutation affecting both the middle and the 3′ terminal G runs. Each mutation compromised the binding of hnRNP F and hnRNP H, as measured by gel-shift assays on RNA oligonucleotides (Fig. 4C). However, a longer exposure of the gel showed the presence of a smear above the free Mut1 RNA band incubated with hnRNP F, suggesting that mutating the last G run in B2G did not completely eliminate hnRNP F binding (not shown). The mutations were next tested for their effect on Bcl-x splicing in vitro. In the context of the full B2 element, Mut1 partially reduced Bcl-x₅₉ usage, whereas...
Mut2 completely abrogated it (Fig. 4D, lanes 3 and 4, respectively). Our results therefore indicate that the two quadruple G stretches in B2G are important for stable hnRNP F and H binding. In the context of the B2 element however, mutating only the terminal G run is not sufficient to completely inactivate the element. The presence of another GGGG located 27 nucleotides downstream of the B2G element (see Fig. 3A) may explain why mutating only one G stretch in B2G is not sufficient to completely abolish Bcl-xL usage. Nevertheless, the fact that two intact GGGGs in B2G are important both for efficient hnRNP F/H binding and Bcl-xL splicing is consistent with the view that the hnRNP F/H proteins enforce Bcl-xL production by binding to the G-rich elements in B2G.

hnRNP F and H Stimulate Splicing to the 5′-Splice Site of Bcl-xL in Vitro—To further assess the role of hnRNP F/H proteins in Bcl-x splicing control, we tested the activity of the recombinant hnRNP F and H proteins on Bcl-x splicing in vitro. The addition of recombinant hnRNP F to a splicing mixture containing pre-mRNA S2.13 promoted a shift toward the production of Bcl-xL (Fig. 5A, lanes 6–9). The largest amount of recombinant hnRNP F used to promote this shift required a functional B2G element because adding His-tagged recombinant hnRNP F to a mixture containing the S-Mut2 pre-mRNA did not improve Bcl-xL production (Fig. 5C, lanes 9 and 10). Consistent with the fact that the Mut1 mutation only weakly affected Bcl-x splicing (Fig. 4D), the addition of recombinant hnRNP F to the S-Mut1 pre-mRNA improved the production of Bcl-xS (Fig. 5C, lanes 6 and 7). Overall, our results suggest that the binding of hnRNP F or hnRNP H to B2G is important for the activity of the B2 element.

hnRNP F/H Knock Down Reduces Bcl-xS Splicing in Vivo—To address whether the hnRNP F/H proteins affect the splicing of Bcl-x in vivo, we relied on siRNA-mediated RNA interference to decrease the levels of hnRNP F and H proteins in HeLa cells. For this experiment, we used siRNAs targeting hnRNP F alone (siF) or targeting both hnRNP F and H (siFH). Following two successive siRNA applications at a 24-h interval and at a concentration of 80 nM, we transfected minigene X2.13. Forty-eight hours after plasmid transfection, Western analysis was performed to assess the reduction in hnRNP F and H (Fig. 6A). When compared with the mock-transfected samples, protein extracts from cells that had been transfected with siF or siFH displayed a notable reduction in hnRNP F protein (Fig. 6A, lanes 1–3). siF did not affect the level of hnRNP H, and the reduction in hnRNP H provoked by siFH was partial (Fig. 6A, lanes 7–9). Specific RT-PCR assays were performed to assess the splicing of plasmid-derived Bcl-x transcripts following these treatments. The drop in hnRNP F expression was asso-
Fig. 6. hnRNPs F/H proteins modulate the alternative splicing of Bcl-x splicing in vivo. In A–C, HeLa cells were transfected twice at a 24-h interval with siRNA mixtures, followed by transfection with the Bcl-x minigene X2.13. In D and E, HeLa cells were transfected with the siRNA mixtures only. Control samples were treated similarly but in the absence of siRNA (Mock). Cells were collected 72 h after the first transfection to prepare protein and RNA samples. A and D represent Western blots performed with antibodies specific to hnRNPs F and hnRNPs H. To control for total protein loading, the hnRNPs A1 and A2 proteins were revealed with a polyclonal rabbit anti-A1/A2 antibody (48) and the proteins comigrated in most of these gels. B presents a RT-PCR analysis of Bcl-x transcripts derived from plasmid X2.13. The ratio of Bcl-xS/Bcl-xL isoform is given below the lane number. C and E summarize the RT-PCR results obtained from transfections with siRNA mixtures performed in triplicate. C monitors the Bcl-xS/Bcl-xL ratio on transcripts derived from plasmid X2.13, whereas E provides the same ratio for the endogenous Bcl-x RNAs.

associated with a shift toward the production of the Bcl-xL isoform (Fig. 6B, lane 3). A more important shift was observed when both hnRNPs F and hnRNPs H were targeted by RNA interference (lane 4). The impact of a knock down of hnRNPs F/H was confirmed by quantifying the Bcl-xS/Bcl-xL ratio in RNA interference assays performed in triplicate (Fig. 6C, left panel). In a
different experiment, we assessed the specificity of the RNA interference assay by using siFHm, a version of siFH containing two mismatches with respect to the wild-type hnRNP F and H mRNAs (AGAA to GAG). Although the impact of siFH on hnRNP F and H expression was less important in this experiment (Fig. 6A, lanes 5 and 11), a drop in Bcl-xS was observed (Fig. 6B, lane 6). In contrast, siFHm did not reduce the level of either hnRNP F or hnRNP H (Fig. 6A, lane 6 and 12, respectively), and no shift in the Bcl-x splicing ratio was observed (Fig. 6B, lane 7). Performing the experiment in triplicate confirmed the impact and specificity of the siFH treatment (Fig. 6C, right panel).

To determine whether the knock down of hnRNP F/H in HeLa cells also affected endogenous Bcl-x splicing, we performed RNA interference assays using siFH and siFHm in the absence of a transfected plasmid. Ninety-six hours after the first application, proteins were isolated, and the reduction in the abundance of hnRNP F and H proteins was confirmed by Western analysis (Fig. 6D). RT-PCR amplification assays indicated that siFH elicited a shift toward Bcl-xL, and this reduction was not seen with siFHm (Fig. 6E). Thus, the results of the RNA interference assays indicate that a reduction in the cellular levels of hnRNP F and hnRNP H proteins decreases the relative use of the x3 5’-splice site both in plasmid-derived and endogenous Bcl-x transcripts.

**DISCUSSION**

The regulation of Bcl-x alternative splicing is of critical importance to the apoptotic process and is highly relevant to cancer. In this study, we have identified two regions in exon 2 of Bcl-x that can modulate splicing in vitro and in vivo. The B2 and B3 elements are located in between the 5’-splice sites of Bcl-xS and Bcl-xL, where they respectively enforce the production of the Bcl-xS and Bcl-xL isoforms. The activity of the B2 element is mediated predominantly by a subregion, B2G, located 10–39 nucleotides downstream of the Bcl-xS donor site. Although the removal of B2G can replicate the effect of a deletion of the larger B2 element and abrogate Bcl-xS usage, sequences downstream of B2G also appear to have some modulatory activity. A more precise mutagenesis approach targeting these sequences will be required to elucidate the exact contribution of the 3’-portion of the B2 element. A recent study has identified two cis-acting elements (CRCE1 and CRCE2) that elicit the ceramide-induced switch toward Bcl-xL in the A549 human adenocarcinoma cell line (22). These elements also contribute to setting the basal Bcl-xS/Bcl-xL ratio in A549 cells. Although we have not tested the function of CRCE1 in the context of our minigene, CRCE2 was deleted when we tested the effect of sequences downstream of B2G (position +50 to +60, relative to the 5’-splice site of Bcl-xS). In contrast to A549 cells, removing the portion that contains CRCE2 only slightly reduced the production of Bcl-xS in a HeLa extract (Fig. 3B, S2.13AB2.2). The control of Bcl-x splicing therefore appears to be complex, and exon 2 may contain many control elements that allow Bcl-x splicing to respond differently to a variety of signals in distinct cellular contexts. The B3 element that we have uncovered fits in this category because deleting it severely reduced splicing to the Bcl-xL 5’-splice site. Future studies will focus on the mechanism used by B3 to control Bcl-x splicing. We have identified yet a different element in exon 2 that mediates a splicing switch in 293 cells, but not in HeLa cells, in response to the apoptotic inducer staurosporine.2

Notably, whereas Bcl-xS was the predominant species produced from the minigene, endogenous Bcl-x expression in HeLa cells favors the Bcl-xL isoform. This result indicates that the role of B2G is not as dominant in endogenously produced Bcl-x transcripts and suggests that the minigene lacks fundamental attributes that are essential to repress Bcl-xL and/or stimulate Bcl-xS usage. The splicing difference between endogenous and ectopic Bcl-x transcripts can result from at least three not mutually exclusive scenarios. First, the Bcl-x gene in HeLa cells may carry mutations that affect splicing control. Second, plasmid-derived Bcl-x transcripts may be overexpressed relative to the endogenous pre-mRNAs, a situation that could lead to the sequestration of limiting regulatory molecules with an impact on the Bcl-xS/Bcl-xL mRNA ratio. Third, the absence of most of the ~55-kb-long intron 2 sequence in the minigene may alter splicing regulation, because the shortened intron lacks specific control elements. Alternatively, the large size of intron 2 suggests that commitment between the 5’-splice sites of exon 2 and the 3’-splice site of exon 3 takes more time to occur than for an average length intron. Thus, a shortened intron may counteract the activity of weak control elements that normally benefit from this lag by being allowed to assemble more productive complexes that enforce Bcl-xS (or repress Bcl-xL) usage.

If this is the case, promoters that alter the processivity of the RNA polymerase II may have an impact on Bcl-x splice site selection. It will be interesting to examine this possibility by testing how Bcl-x is spliced when the speed of transcription is altered. Although alternative initiation sites in the mouse Bcl-x gene affect alternative splicing (37, 38), it is unclear whether these changes are mediated by different transcription rates or by the presence of distinct control elements located at the 5’-end of some of the pre-mRNAs.

**hnRNP F/H Proteins and the Control of Splice Site Selection in Bcl-x—**The 30-nucleotide B2G element involved in promoting the use of the Bcl-xS 5’-splice site has the following sequence: GAAACUCUCCGGGAUGGGGUAAACUGGGGU. Mutating the last two G stretches (underlined) abrogates the activity of the B2 element. Similar motifs have been identified as binding sites for members of the hnRNP F/H/2H9 family of proteins (29–35). We have shown that the recombinant hnRNP F and hnRNP H proteins can individually associate with the B2G RNA sequence, and that binding occurs in a G stretch-dependent manner. Moreover, recombinant hnRNP F and hnRNP H proteins can individually stimulate Bcl-xS splicing in vitro, and no stimulation by hnRNP F is observed when both GGGG are mutated. Most importantly, knocking down hnRNP F expression by RNA interference shifted the in vivo splicing of plasmid-derived transcript toward Bcl-xL. A simultaneous but partial reduction in hnRNP H affected Bcl-x splicing more robustly than the knock down of hnRNP F alone. Moreover, the combined knock down of hnRNP F and hnRNP H expression reduced endogenous Bcl-xS production. hnRNP F and hnRNP H may therefore have redundant activity with regard to Bcl-x alternative splicing. However, we cannot exclude the possibility that both proteins may be simultaneously required for the activity of the B2G element. The hnRNP F and H proteins have been documented to interact with a G-rich stretch located in the intrinsic DCS enhancer complex that favors exon N1 inclusion in the e-scr pre-mRNA in neuronal cells (31, 39). Although hnRNP H can interact with hnRNP F and is required for N1 exon splicing, hnRNP H-depleted extracts cannot be rescued by recombinant hnRNP F (31). Moreover, hnRNP F does not bind strongly to the DCS RNA element (35). Given that hnRNP F and hnRNP H each bind to the Bcl-x B2G element with similar efficiency, cooperation between hnRNP F and hnRNP H may not be as critical for Bcl-x splicing control. Our future work will assess the individual or cooperative nature of hnRNP F and H-mediated control of Bcl-x splicing.

The mechanism by which hnRNP F/H proteins modulate Bcl-x splicing remains unknown. The proximity of B2G to the

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2 D. Garneau, T. Revil, and B. Chabot, unpublished data.
5'-splice site of Bcl-xS suggests that hnRNP F/H directly stimulates the 5'-splice site of Bcl-xS. Notably, the binding of hnRNP H to an enhancer element in exon 6D of the human immunodeficiency virus pre-mRNA recruits U1 snRNP (33). If the B2G-hnRNP F/H complex directly enhances the recruitment of U1 snRNP to the 5'-splice site of Bcl-xS, this activity would be reminiscent of TIA-1, which binds very close to 5'-splice sites (40–42), facilitates the recruitment of U1 snRNP (43), and favors the production of the proapoptotic isoform of the Fas cell death receptor (44).

The antagonizing activities of Bcl-xS and Bcl-xL in apoptosis suggest that natural variations in the levels of hnRNP F and H proteins may influence how cells respond to apoptotic signals. The hnRNP F and H proteins display important differences in expression. Immunohistochemical analysis in normal tissues indicate that hnRNP F is often more abundant in the cytoplasm than in the nucleus, whereas the opposite is true for hnRNP H and the related hnRNP H'-protein (45). The nuclear abundance of hnRNP H is often increased in tumor tissues, such as liver carcinoma and pancreatic adenocarcinoma. hnRNP F expression appears generally high in tumors, except in liver carcinoma (45). Although these observations are in appearance inconsistent with the reduced production of Bcl-xS that would be expected in transformed tissues, the ratio between the Bcl-xS and Bcl-xL isoforms will likely be dictated by the coordinated contribution of several control elements and trans-acting factors. Also, posttranslational modification events may modulate the activity of hnRNP F/H proteins. Although the importance of posttranslational modifications in the activity of hnRNP F/H proteins has not yet been examined in detail, the observation that apoptotic HL60 cells display an increased in phosphorylated hnRNP H (46) is consistent with the proposed link between hnRNP F/H and apoptosis. The contribution of posttranslational modifications to the activity of hnRNP F/H and Bcl-x splicing awaits further investigation.

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