Differential splicing from the bcl-X gene generates several isoforms with opposite effects on the apoptotic response. To explore the mechanism controlling the balance between the various isoforms, we have characterized the 5′ region of the mouse bcl-x gene. We identified three new promoters in addition to the two previously described (Grillot, D. A., M., G.-G., Ekhterae, D., Duan, L., Ionoara, N., Ohta, S., Seldin, M. F., and Núñez, G. (1997) J. Immunol. 158, 4750–4757). These five promoters (P1-P5) would give rise to at least five mRNAs with different 5′-untranslated region, all sharing the same translation initiation site. Except for the product of the most proximal promoter (P1), the other mRNAs are generated by alternative splicing of noncoding exons to a common acceptor site located in the first translated exon. Reverse transcriptase-polymerase chain reaction, primer extension, and RNase protection assays demonstrate a tissue-specific pattern of promoter usage. P1 and P2 are active in all tissues analyzed, whereas the other three promoters show tissue-specific activities. P3 is active in spleen, liver, and kidney, P4 is active in uterus and spleen, and P5 is active in spleen, liver, brain, and thymus. We present evidence suggesting that promoter selection influences the outcome of the splice process. Transcripts from P1 generate mainly the mRNA for the long isoform Bcl-XL, whereas transcripts from P2 generate mRNAs for the isoforms Bcl-XM, Bcl-XS, and Bcl-xy and transcripts from P3 yield mainly mRNAs for the isoform Bcl-XM. Our results suggest a key role of promoter choice in determining alternative splicing and, thus, the balance of Bcl-X isoforms.

The term apoptosis refers to a physiologic and genetically controlled program for cells to commit suicide. Control of cell survival is of central importance for the formation of various organs during embryogenesis as well as in adult tissues with high cell turnover such as reproductive glands and the lymphoid system. Moreover, disabling programmed cell death may be a critical step in tumorigenesis. Cells undergo apoptosis not only in response to external signals but also by a cell autonomous genetic program controlled at several checkpoints. One crucial control relies on the ability of the cell to sense changes in the ratio between members of the Bcl-2 family. The bcl-2 oncogene was first identified in human B-cell lymphomas in the context of a chromosomal translocation, which placed it within the immunoglobulin locus (2). bcl-2 promotes cell survival by inhibiting apoptosis induced under a wide variety of circumstances, suggesting that it is a ubiquitous inhibitor of cell death triggered by multiple pathways (3). bcl-2 is the founding member of a growing multigene family with many representatives in mammals, including bcl-X, bax, bak, and mcl-1, which can either promote or prevent apoptosis (4–7). Bcl-2-related proteins have a putative transmembrane domain at their carboxyl terminus and are found associated with mitochondrial, endoplasmic reticulum, and nuclear membranes (8). The various members of the Bcl-2 family can form homo- and heterodimers, which exert opposite effects on apoptosis. For instance, whereby Bax homodimers are formed upon death stimuli and promote apoptosis, Bax/Bcl-2 heterodimers or Bcl-2 homodimers prevent apoptosis (9).

It has been suggested that members of the bcl-2 gene family control mitochondrial membrane permeability during apoptosis by regulating the electrical and osmotic homeostasis of mitochondria (10, 11). Those that prevent apoptosis inhibit release of cytochrome c, whereas those that promote programmed cell death induce this release. According to the mechanism of action suggested by Shimizu et al. (10), proapoptotic bcl-2 family members accelerate the opening of the mitochondrial porin channel (also named voltage-dependent anion channel), whereas the anti-apoptotic members close this channel by binding directly to it (10).

Bcl-X is highly related to Bcl-2 and exists in several isoforms generated by alternative splicing (4) (Fig. 1). Some of these isoforms seem to have a ubiquitous expression, whereas others are expressed in a tissue-specific manner or in response to specific stimuli. The large isoform, Bcl-XL, protects cells against apoptosis, whereas a short isoform, Bcl-XS, antagonizes cell death inhibition by interacting with Bcl-XL and Bcl-2 (4). The cDNA of a third Bcl-X isoform, Bcl-XSTM, was cloned in mouse and shown to be generated by alternative splicing of the carboxyl-terminal transmembrane domain of bcl-xL (12). As a consequence, the protein localizes diffusely throughout the cytosol. Bcl-XSTM was found to prevent programmed cell death (12). A fourth isoform Bcl-XL¶, promotes apoptosis and seems to be specifically expressed in cerebellum, heart, and thymus (13). Finally, a fifth isoform named Bcl-X was recently identified as
an antiapoptotic protein, which is induced in lymphocytes and correlates with T-receptor expression (14).

Although Bcl-2 and Bcl-XL are both apoptotic inhibitors, their functions are not redundant since they are not found in the same cell types nor at the same developmental stages (15). Moreover, contrary to the bcl-2 knockout mice, mice lacking bcl-X die around embryonic day 13 due to extensive postmitotic neuronal death (16).

The genomic structure of the human and mouse bcl-X genes have been recently described (1). The gene has been shown to contain at least one additional promoter (8, 14).

Here we present a detailed analysis of the 5′-flanking region of the mouse bcl-X gene, including several kilobases upstream of the previously described promoters P1 and P2 (1). Three TATA consensus regions were found located at positions −1886, −2721, and −3412 upstream of the translation initiation site. They named these regions as promoter P3, P4, and P5, respectively. Each of these promoters shows tissue-selective expression, suggesting that tissue-specific factors may be involved in controlling bcl-X transcription. The significance of the utilization of different promoters is not clear, but promoter choice could play a key role in the control of bcl-X expression in response to different stimuli by influencing the splicing process and, thus, leading to the synthesis of specific Bcl-X isoforms.

**MATERIALS AND METHODS**

**Plasmid Constructions and DNA Sequencing**—pNM1–9 SalI vector contains a 10-kilobase DNA fragment from the 5′ region of the mouse bcl-X gene cut from the plasmid pNM1–9 (kindly provided by N. Toyama) with SalI enzyme and subcloned into SalI site of pBluescriptKS plasmid. pNM1–9EagI is a subclone containing a 9.6-kilobase fragment from pNH–9 cut with EagI and religated. DNA sequencing was performed by SeqLab (Gottingen, Germany).

**Isolation of Total RNA and RNA Analysis**—CF1 male and female mice were sacrificed by cervical dislocation, and total RNA was prepared from fresh tissues with the guanidinium thiocyanate–phenol–chloroform extraction method (18). RNase protection assays were performed as described below (19). For preparing the P1 riboprobe, plasmid pNM1–9SalI was digested with MmeII and transcribed by T3 RNA polymerase. The full-length transcribed riboprobe was 558 bp. For preparing the P2 riboprobe, plasmid pNM1–9EagI was digested with EcoRI and transcribed by T7 RNA polymerase; the full-length transcribed riboprobe was 495 bp. [a-32P]CTP (Amersham Pharmacia Biotech)-radiolabeled RNA probes were prepared using a kit according to the instructions of the manufacturer (Promega, Madison, WI) and as described before (20). The probes were coprecipitated with RNA samples, dissolved in hybridization buffer, denatured at 95 °C for 10 min, and hybridized at 52 °C for 18 h. After digestion with RNase A and T1, followed by digestion with proteinase K, the samples were precipitated, denatured, and subjected to electrophoresis on a 6% denaturing acrylamide gel.

For primer extension analysis primers, E-rev (5′-TAGATCGGATGAGACCTGGTGCT-3′), D-rev (5′-AAATGGACTATAACCTGACATTTTCTG-3′), and C-rev (5′-GAAGAGGACTGGCACTGAGAC-3′), corresponding to the reverse complement of nt −3223 to −3244 (primer E-rev), nt −2548 to −2573 (primer D-rev), and nt −1754 to −1775 (primer C-rev), were used. RNA (30 μg) was precipitated and resuspended in 12 μl of annealing buffer (120 mM KCl, 24 mM Tris-HCl, pH 8, 5 × 10^−6 cm of 32P-labeled primer), denatured at 75 °C for 10 min, and hybridized at 39 °C for 25 min. After hybridization, the reaction was extended with 50 units of Moloney murine leukemia virus reverse transcriptase (Life Technologies, Inc.) in 30 μl of reaction mixture (60 mM Tris-HCl, pH 8, 20 mM KCl, 10 mM MgCl2, 1.5 mM dithiothreitol, 0.6 mM dNTP, 0.05% SDS) followed by phenol–chloroform extraction and ethanol precipitation. The radiolabeled DNA was electrophoresed through a 6% polyacrylamide/urea gel in parallel with sequencing reactions from the genomic bcl-X cloned into pBluescript or with radiolabeled pBR322 MspI size marker.

**RT-PCR and Sequence of the 5′-Untranslated Region of bcl-X cDNAs**—For reverse transcription 4 μg of total RNA was used. The first cDNA strand was synthesized with Moloney murine leukemia virus reverse transcriptase (Life Technologies, Inc.) in 30 μl of reaction mixture (60 mM Tris-HCl, pH 8, 20 mM KCl, 10 mM MgCl2, 1.5 mM dithiothreitol, 0.6 mM each dNTP) at 37 °C for 1 h. The reaction was stopped by the addition of 80 μl of stop mixture (150 mM NaCl, 15 mM Tris-HCl, pH 8, 0.5% SDS) followed by phenol–chloroform extraction and ethanol precipitation. The radiolabeled DNA was electrophoresed through a 6% polyacrylamide/urea gel in parallel with sequencing reactions from the genomic bcl-X cloned into pBluescript or with radiolabeled pBR322 MspI size marker.

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Evidence for Transcription Initiation Upstream of the P2 Promoter—An RNase protection assay with a probe encompassing the P1 promoter (Fig. 2A) yielded two protected fragments: a doublet of 176 nt and another of 147 nt. The 176-nt fragments originate from transcripts initiated at the P1 promoter, which were detected in all tissues tested, including uterus, spleen, heart, and liver (Fig. 2A, lanes 2–5) as well as in lung, thymus, brain, and kidney (data not shown). The 147-nt fragments could correspond to transcripts initiated upstream of P1 and spliced to a consensus 3'-acceptor splice site located at position −112 from the ATG. In principle these transcripts could also originate from initiation around this region, but there is no obvious consensus site, and previous studies using primer extension assays did not detect transcripts initiating around −112 (1). A comparison of the intensity of the 147-nt and the 176-bp bands suggests that most of the transcripts hybridizing with this probe are initiated upstream of the P1 promoter. The origin of the minor 125-nt fragments is unclear, but they could correspond to another previously described start site at position −96 (1) or to exon B.

RNase protection assays with a probe encompassing the P2 promoter were performed with three main bands (Fig. 2B). Two of them, the 104-nt and 124-nt bands, represent protected products starting at the P2 promoter (nt 1870) and extending to the 5'-splice donor site at 207; see the nucleotide sequence in Fig. 6A). Transcripts from P2 were detected in spleen and heart (Fig. 2B: lanes 3 and 4) and in very low amounts in uterus and liver (lanes 2 and 5). The P2 promoter was also active in other tissues, such as thymus and kidney (data not shown). The protected 400-nt band could correspond to transcripts of exon B initiated upstream of P2 and extending to the 5'-splice donor site at −553. This signal was stronger than the transcripts initiated at P2 in uterus, heart, and liver but weaker in spleen, suggesting differential promoter usage in different tissues. The existence of these kind of transcripts was supported by RT-PCR cloning and sequencing of a fragment extending from −1310 to −460 (data not shown). The larger fragments at the top of the gel correspond to the residual intact probe. Other minor bands were not reproducibly observed.

Three Novel Promoter Regions—To explore the presence of additional promoter regions upstream of P2, we sequenced 3.42 kilobases upstream of the ATG start codon in mouse bcl-X gene (Fig. 3). Recently, a nucleotide sequence of the 5'-region (6447 nt) of the mouse bcl-X gene was deposited at the GenBank (accession number AF088904) that is virtually identical to the one that we determined. Inspection of the nucleotide sequence revealed a putative 3'-acceptor splice site at position −468 from the ATG start codon, suggesting a maximal length for exon B of 1271 bp (from nt −1468 to −197; see the nucleotide sequence in Fig. 3 and the scheme in Fig. 5). In addition, several putative TATA boxes were observed between the nt −3420 to −1870, of which only three, located at −1886 (P3), −2721 (P4), and −3415 (P5), showed activity in transcript analysis. Primer extension assays were performed to map the initiation sites of transcripts corresponding to these three TATA boxes. Three reverse complementary oligonucleotides with sequences located downstream P3 (primer C-rev), P4 (primer D-rev), and P5 (primer E-rev) were used as primers.

FIG. 2. RNase protection assays from total RNA were performed to map transcripts from the P1 (A) and P2 (B) promoters in uterus (lane 2), spleen (lane 3), heart (lane 4), and liver (lane 5). RNA (lane 1) was used as a negative control. The length in nucleotides of the protected fragments is indicated on the right, and a schematic interpretation of the position of the corresponding transcripts along with the radioactive probes used are shown at the top.

AA-3'), and P5s (5'-CATTCGGATCCATTAGAGTTGCT-3') (nt −1779 to −1754; nt −2690 to −2577; and nt −3293 to −3269, respectively). Primer Al-rev (5'-CCGAGATCCAAAGGACCAAGATAGGTT-3') was used as the reverse primer. PCR was performed by 30 cycles (96 °C for 35 s, 60 °C for 20 s, and 72 °C for 1 min) and a final extension at 72 °C for 10 min.

PCR products were purified by electrophoresis in 1.6% agarose gel, and the different bands were extracted from agarose and purified with clean up kit (DNA Clean-up system, Promega). Sequence analysis was performed by SeqLab (Göttingen, Germany).

RT-PCR and Southern Blots of the Specific bcl-X-spliced cDNA. cDNAs were prepared as described above. For PCR amplification, the oligonucleotides were used as forward primers: P1 (5'-CTGAAAGCT-GTCGTCGTCGTCG-3') P2L (5'-GACTGCTGAGTTGATGGATCCACAAA-3'), and P3L. The oligonucleotides S/L-rev (5'-GTAGAGTGGATGGTCAG-3') and γ-rev (5'-TTGACCTCCCGATCCTCGTTGTTCC-3') (corresponding to the 5'-end of each specific second translated exon) were used as reverse primers in order to amplify bcl-XsA and bcl-X, isoforms, respectively. For amplification of the bcl-X isoform it was necessary to perform a semi-nested PCR using, in the second round, the oligonucleotide Al-rev (5'-ACACCACGTGTTGCTGTAGGACAT-3') (corresponding from nt +1 to +26 from the translation initiation site) as the reverse primer. For amplification of bcl-XsA isoforms (corresponding to the samples belonging to spleen, brain, and liver), it was also necessary to perform a semi-nested PCR using, in the second round, the oligonucleotide S/L(rev) (5'-CCGCCAGTGGAGTGGATGGTCAG-3') as the reverse primer. PCR products were resolved by electrophoresis in 1.6% agarose gel and transferred to a nylon plus membrane (Qiabane) with NaOH (0.4 M) as the transfer solution. Southern blots were performed using a fragment containing exon A of mouse bcl-X as the hybridization probe. The membranes were incubated in the prehybridization buffer (potassium phosphate (120 mM), pH 7.2, 10% polyethylene glycol (M, 8000), 7% SDS, NaCl (250 mM), 50% formamide, and 100 μg/ml calf thymus DNA) at 42 °C for 2 h. Then membranes were hybridized in the same solution with the 32P-labeled probe 42°C overnight. A wash was carried out 2× SSC at 55°C for 5 min at room temperature in 2× SSC (1× SSC = 0.15 M NaCl and 0.15 M sodium citrate), 0.5% SDS followed by 2× 5 min at 50 °C in 2× SSC, 0.1% SDS. Membranes were exposed between 2 and 5 days.

RESULTS

Promoter Choice and Alternative Splicing of bcl-X Gene

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RESULTS

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Reverse transcriptase extended fragments using primer C-rev (Fig. 4A) placed a main initiation site at position −1856 (30 bp downstream of the P3 TATA box) and two other minor start sites 4 and 6 nucleotides farther upstream. The P3 transcripts were found in spleen (lane 3), liver (lane 5), and kidney (lane 6) but not in uterus (lane 2) or thymus (lane 4).

Reverse transcriptase-extended fragments using primer D-rev (Fig. 4B) placed another initiation site at position −21596 (25 bp downstream of the P4 TATA box). The existence of this transcript was confirmed by RT-PCR and sequencing of a 146-bp fragment generated by using the P4L and D-rev oligonucleotides as forward and reverse primers, respectively (data not shown). The P4 transcripts were detected only in uterus (lane 2) and spleen (lane 3), whereas no detectable transcripts were observed in liver (lane 4) or very little, if any, in kidney (lane 5).

Finally, reverse-extended fragments using primer E-rev (Fig. 4C) located a major initiation site at position −23387 (24 nt downstream of the P5 TATA box). The existence of this transcript was confirmed by RT-PCR, cloning, and sequencing of a 90-bp fragment generated by using the P5L and E-rev oligonucleotides as forward and reverse primers, respectively (data not shown). Again, the P5 transcripts were observed only in some tissues, such as spleen (lane 3), liver (lane 5), and brain (lane 6), but not in others, such as uterus (lane 2) and thymus (lane 4). Very small amounts of P5 transcripts were detected in thymus (lane 4).

**Multiple mRNAs Derived from the bcl-X Gene**—The structure of the bcl-X 5′-flanking region is shown schematically in Fig. 5. If all five promoters were used and the transcripts spliced to the coding region, at least five mRNAs differing in the 5′-noncoding leader exons (exons A-E) would be expected. Until now, mRNAs containing exons D and E had not been detected by cloning. However, we have detected four mRNAs, one derived from the promoter P1, two containing exon B, and one containing exon C. The 5′ structures of these mRNAs are shown in Fig. 6, along with the nucleotide sequence around the start site. As previously described (1), we detected mRNA A, derived from the P1 promoter in all tissues analyzed.

There are at least three different mRNAs containing exon B sequences of various lengths, which originate from the use of three alternative 5′-donor splice sites. All these three mRNAs share the same 3′-acceptor splice at position −21112 in exon A. Using cDNA cloning with RT-PCR, we detected only two of these mRNAs, one of which uses nt −2555 as the 5′ splice site (mRNA B1) and the other of which splices at nt −2397 (mRNA B2). mRNA B1 was obtained from total RNA of mouse uterus and spleen by RT-PCR with the oligonucleotides P2L and ATG-rev (Fig. 5) as forward and reverse primers, respectively. mRNA B2 was also previously described (1). Fang et al. (12) report evidence of the existence in B and T lymphocytes of a third mRNA containing exon B sequences with a 5′-splice site located at nt −2197 (mRNA B3) (GenBankTM accession number MMU10102). We did not detect this mRNA in any of the tissues tested.

mRNA transcripts containing exon C were detected by nested RT-PCR of total RNA from mouse spleen using primers P3L and ATG-rev for the first PCR and P3S and AI-rev in the second round (Fig. 6, mRNA C). The nucleotide sequence of all the transcripts we have detected did not show any open reading frames located upstream of the known coding region (Fig. 6) (4). We could not detect any mRNA transcripts containing exon D or exon E when we performed RT-PCR with primers P4L/P5L and ATG-rev followed by nested PCR with primers P4S/P5S and AI-rev from total RNA of tissues in which P4 and P5 were active as judged by primer extension assays (uterus and spleen).
for P4 and spleen, liver and brain for P5). These results suggest that either the proportion of these mRNAs is very low in the total RNA or that the PCR products were too long to be efficiently amplified in our assays. Moreover, RT-PCR products were generated with the primers P5/L/E-rev and P4/L/D-rev, and their sequences corresponded to exons E and D, respectively (data not shown).

Use of Different bcl-X Promoters Generates Specific Spliced Products and Bcl-X mRNA Isoforms—The results described above showed tissue-dependent activities of the multiple promoter regions present in the bcl-X gene (Figs. 2 and 4). P1, P2, and P4 are active in uterus and heart; only P1 and P2 are active in thymus; P1, P2, P3, and P5 are active in liver; and all promoters are active in spleen. The question arises as to the physiological relevance of alternative and tissue-specific promoter usage. Our results suggest that the selection of promoter usage would be involved in the regulation of the levels of the various bcl-X mRNAs. The differences in the 5’-noncoding exons of the transcripts may stabilize different secondary structures, which could play a role in generating specific spliced products. To test this possibility, we performed RT-PCR using forward primer oligonucleotides corresponding to each of the noncoding regions (primers P1, P2L, and P3L for exons A, B, and C, respectively) and a common reverse primer that hybridized specifically with the second translated exon (primer S/L-rev) (Fig. 7). We choose RNA from four tissues in which the different transcripts are expressed (spleen, liver, brain, and thymus). Activation of P1 generated mainly bcl-X₇, and very little, if any, bcl-X₈ (Fig. 7A, lanes 2, 3, 4, and 8). Transcripts containing exon B generated both bcl-X₇ and bcl-X₈ mRNAs, but with a higher proportion of the former in all tissues tested (Fig. 7B, lanes 2, 3, 4, and 6). Transcripts containing exon C were only detected in thymus and corresponded to both isoforms but with a higher proportion of bcl-X₈ relative to bcl-X₇ (Fig. 7C, lane 8). This was unexpected, as P3 is active in spleen, liver, and brain (Fig. 4A). Thus, a bcl-X isoform different from bcl-X₇, bcl-X₈ is likely generated from these transcripts. RT-PCR using oligonucleotide P3L as forward primer and a reverse primer whose sequence hybridized specifically with bcl-X₈ (primer γ-rev) revealed the presence of bcl-X₈ mRNAs containing exon C in all the tissues analyzed (Fig. 8B). In addition, bcl-X₇ was found as a spliced product of transcripts containing exon B (Fig. 8A) in all tissues tested, whereas no amplified product of this isoform was detected with transcripts derived from the P1 promoter (data not shown). Taken together the results suggest that independently of the cell type, the use of a specific promoter generates spliced transcripts specific for different Bcl-X isoforms.

DISCUSSION

The Bcl-2 family is known to control apoptosis induced by a wide range of agents. Some members of the family like Bcl-2 and Bcl-X₇ prevent apoptosis, whereas others, like Bax and Bcl-X₈, induce cell death by dimerizing with Bcl-2 or Bcl-X₇, or by forming Bax-Bax dimers (4). In this way, the ratio between proapoptotic and antiapoptotic dimers acts as a “rheostat”
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which determines whether a given cell is going to die or to survive. Members of the Bcl-2 family with similar functions are not found in the same cell at the same time (15). Thus, a pair of proteins with opposite effect, either Bcl-2/Bax or Bcl-Xs/Bcl-Xl depending on the cell type or developmental stage, forms the rheostat. To ensure a correct control of apoptosis, the levels of the proteins present in a given specific rheostat must be precisely regulated. Bcl-Xs and Bcl-Xl are not the unique splice products generated by a given gene. In mice and rat, five different isoforms of the mouse bcl-X gene have been described, with opposite effects on programmed cell death (4, 12–14). The control of apoptosis requires expression of the correct amount of each isoform, suggesting not only an accurate regulation of transcription but also of splicing of the bcl-X gene.

The experiments summarized in this paper demonstrate that the mouse bcl-X gene exhibits a complex structure, in particular around its 5'-UTR, which contains at least four different exons located upstream the unique open reading frame. Grillot et al. (1) report that the mouse bcl-X gene is transcribed from two TATA-less promoters located between −149 and −142 (P1) and between −655 and −727 (P2) (1). Our present study demonstrates that there are at least three additional promoters farther upstream: P3 located at −1886, P4 at −2721, and P5 at −3412 upstream of the translation initiation codon. These three novel promoters contain a TATA consensus sequence according to the weight matrix descriptions of eukaryotic RNA polymerase II promoter elements (21). Very recently, the existence of transcripts originating from a promoter corresponding to our P3 has been reported for the human bcl-X gene and seems to be conserved in the mouse (22).

Using immunohistochemical analysis, Krajewski et al. (15) report a strong Bcl-X expression in neurons from brain, in epithelial and cortical cells of the thymus, in red pulp granulocytes from spleen, in hepatocytes, in the loop of Henle of the kidney, and in endometrial cells (15). We did not analyze all of these cell types, but we found bcl-X expression in all the tissues tested. In addition we have observed that the use of the different promoters varied markedly in the tissues analyzed. We found that P1 and P2 were active in all tissues tested, whereas P3, P4, and P5 showed a tissue-dependent pattern of activity. According to RNase protection and primer extension assays, P3 was active in spleen, liver, and kidney, P4 in uterus and spleen, and P5 in spleen, thymus, liver, and brain.

Analysis of the nucleotide sequence of the 5'-flanking region of the mouse bcl-X gene led to the identification of several consensus motifs for the binding of transcription factors. The physiological significance of these motifs remains to be determined. It has been described that expression of the different isoforms of bcl-X gene is controlled by several stimuli acting via various transcription factors, including Ets-1 and 2, GATA-1, NF-kB, AP-1, and STAT (signal transducers and activators of transcription) factors (23). For instance, the Ets-2 transcription factor inhibits apoptosis induced by colony-stimulating factor 1 deprivation of macrophages through a Bcl-Xl-dependent mechanism (23). In particular, several reports show that the 5'-flanking NF-kB-like sites in regulation of bcl-X gene expression has been recently reported (30).

We have previously shown that steroid hormones dexamethasone and progesterone induce bcl-X expression and increase the bcl-Xs/bcl-Xl ratio in endometrial cells (20). Studies from other authors confirm that steroid hormones control bcl-X expression in different tissues (31–33). Transient transfection experiments with a reporter gene under the control of the mouse bcl-X P1, P2, or P3 promoters have demonstrated a hormone-dependent expression, although no consensus hormone regulatory element motifs are found in the neighborhood of these promoters. This suggest that steroid receptors might control bcl-X expression by interacting with other transcription factors, such as AP1, NF-kB, and STAT5, rather than by a specific interaction with hormone regulatory element, as has been recently shown in other systems (34–36). The activation of the five promoters described in this study would generate at least five mRNAs with different 5'-UTRs. Multiple promoter usage is a mechanism that offers the possibility of responding to distinct combinations of factors present in the different tissues. Using RT-PCR, we have found only some of these transcripts. The mRNA A, which is generated by activation of P1, starts at nt −144 in exon A and is expressed in all analyzed tissues. We also identified two different types of mRNAs containing exon B sequences that differ in the selection of the 5'-donor splice site. We cloned mRNA B1 from the spleen, whereas mRNA B2 had been described in several reports and corresponds to transcripts generating three isoforms, long, short, and γ (GenBank accession numbers U51278, U51279, and U51277, respectively), as confirmed here by RT-PCR. A third type of transcripts containing exon B sequences (mRNA B3) can be found in GenBank (accession number MMU10102). Its sequence corresponds to bcl-XSTM, a bcl-X isoform lacking the transmembrane domain (Fig. 1A) (12). In our study we did not detect this mRNA, but we found an mRNA containing exon C sequences (mRNA C). mRNA C generated only bcl-XS, in spleen, brain, and liver, whereas in thymus it generated the two ubiquitous isoforms, bcl-Xs and bcl-Xl. According to the primer extension assays, P3 is not active in

A. Pecchi and M. Beato, unpublished observation.
thymus, suggesting that the detected amplification products are derived from promoter regions located farther upstream.

Given the important role of bcl-X in controlling programmed cell death, its expression should be precisely regulated. We suggest that multiple promoter usage in the mouse bcl-X gene provides for tissue specificity and variations in expression levels of the different spliced products. The existence of multiple transcripts differing in their 5'-UTR and likely in their secondary structure could determine the generation of specific spliced products. In this way, external signals, by influencing promoter selection in a specific tissue, would determine the formation of specific Bcl-X isoforms. It has been described that promoter sequences influence the selection of the splice site through an interaction between the transcription machinery and serine-arginine proteins (SR) involved in the splicing process (17).

Although far from being complete, our results describe for the first time the complexity of the structure of the 5'-flanking region of the bcl-X gene and provide the basis for future studies on the physiological relevance of multiple promoter usage and alternative splicing in controlling programmed cell death.

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REFERENCES
5. Chittenden, T., Harrington, E. A., O’connor, R., Flemington, C., Lutz, R. J.,
Promoter Choice and Alternative Splicing of bcl-X Gene

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