Oxidative stress promotes cardiac myocyte apoptosis through the mitochondrial death pathway. Since Bcl-2 family proteins are key regulators of apoptosis, we examined the effects of H$_2$O$_2$ on the expression of principal Bcl-2 family proteins (Bcl-2, Bcl-xL, Bax, Bad) in neonatal rat cardiac myocytes. Protein expression was assessed by immunoblotting. Bcl-2, Bax, and Bad were all down-regulated in myocytes exposed to 0.2 mM H$_2$O$_2$, a concentration that induces apoptosis. In contrast, although Bcl-xL levels initially declined, the protein was re-expressed from 4–6 h. Bcl-xL mRNA was up-regulated from 2 to 4 h in neonatal rat or mouse cardiac myocytes exposed to H$_2$O$_2$, consistent with the re-expression of protein. Four different untranscribed first exons have been identified for the Bcl-x gene (exons 1, 1B, 1C, and 1D), where exon 1 is the most proximal and exon 1D the most distal to the coding region. All were detected in mouse or rat neonatal cardiac myocytes, but exon 1D was not expressed in adult mouse hearts. In neonatal mouse or rat cardiac myocytes, H$_2$O$_2$ induced the expression of exons 1B, 1C, and 1D, but not exon 1. These data demonstrate that the Bcl-x gene is selectively responsive to oxidative stress, and the response is mediated through distal promoter regions.

Cardiac myocytes, the contractile cells of the heart, are terminally differentiated cells, which withdraw from the cell cycle in the perinatal period. Consequently, myocyte cell death, as occurs (for example) following ischemia and/or reperfusion, significantly affects cardiac function. One of the principal insults that may promote cardiac myocyte death is oxidative stress, and the levels of oxidative stress are increased in ischemic hearts (1, 2) with higher levels being produced following reperfusion (3). H$_2$O$_2$ (0.1–0.5 mM), as an example of oxidative stress, promotes cardiac myocyte apoptosis through the mitochondrial death pathway (4). However, lower concentrations are non-toxic and may have cytoprotective and/or growth promoting effects (5–8).

Bcl-2 family proteins are key regulatory components of the mitochondrial cell death pathway (9, 10). Some family members are cytoprotective (e.g. Bcl-2, Bcl-xL, Bcl-w, Mcl-1), whereas others promote apoptosis (e.g. Bad, Bak, Bax, Bid, Bim, Bmf). These proteins act at the mitochondria to regulate cytochrome c release. Bcl-2 family proteins act either as heterodimers or as oligomers and the dynamic equilibrium between such complexes appears to determine the predisposition to apoptosis. Translocation of Bax to the mitochondria and oligomerization of Bax and/or Bak promotes permeabilization of the outer mitochondrial membrane and release of cytochrome c and other apoptosis-inducing factors, possibly by forming a pore in the membrane. Heterodimerization with either Bcl-2 or Bcl-xL prevents oligomerization and protects from apoptosis. Other pro-apoptotic proteins, such as Bad, Bim, or Bmf compete for binding to Bcl-2/Bcl-xL causing release of Bax/Bak, which can then form oligomers and induce apoptosis. Thus, the balance between pro- and anti-apoptotic Bcl-2 family proteins influences the rate of apoptosis.

Bcl-2 and Bcl-xL are the principal Bcl-2 family proteins that protect cells from apoptosis. The regulation of the Bcl-x gene appears particularly complex. Initial studies of the mouse Bcl-x gene demonstrated that, like Bcl-2, the Bcl-x gene consisted of three separate exons (11). Exon 1 is untranslated and is separated from the first coding exon, exon 2, by a short facultative intron. Exon 3 codes for the C terminus of Bcl-xL and is separated from exon 2 by a large intron of at least 9 kb. At least four proteins potentially derive from alternatively spliced Bcl-x mRNAs. Exons 1, 2, and 3 are spliced together to produce Bcl-xL. Splicing from an alternative donor site within exon 2 to exon 3 results in a shorter form of the protein (Bcl-xS), which lacks a central region present in Bcl-xL, but the reading frame is maintained and the C terminus is identical (11). In contrast to Bcl-xL, Bcl-xS induces apoptosis. Read-through of the donor site at the end of exon 2 produces Bcl-xδ (12), and splicing of exon 2 to a novel exon 4 generates Bcl-xy (13). Apart from alternative splicing of the coding exons of the Bcl-x gene, there is additional complexity in the regulation of the 5’ non-coding region. Grillot et al. (11) reported the presence of at least two principal transcription initiation sites for exon 1 at −655 and −727, in addition to a cluster of initiation sites at the start of exon 2. Subsequently, an alternative first exon upstream of exon 1 (exon 1B) was identified in human lymphoma cells (14). A third study suggested that at least five different promoters (P1–P5) operate in the mouse Bcl-x gene, each of which is associated with a different first exon (exons A–E) (15). This terminology is confusing, since exons A and B correspond, respectively, to exons 2 and 1 identified by Grillot et al. (11), and exon C corresponds to the region identified as exon 1B (14). The different exons of the Bcl-x gene are summarized in Fig. 1. Despite the identification of potential promoters and transcriptional start sites further upstream in the Bcl-x gene (i.e. exons D and E (15)), expression of these exons was not detected in erythroid progenitor cells or differentiating erythroblasts in which Bcl-xL is up-regulated (16), and their significance in a biological system remains to be established.
A number of Bcl-2 family proteins are expressed in cardiac myocytes including Bcl-2, Bcl-xL, Bad, Bax, and Bid (4, 17, 18). As in other cells, overexpression of Bcl-2 in cardiac myocytes is cytoprotective (19–21). H2O2-induced apoptosis in neonatal myocytes is associated with rapid translocation of Bad and Bax to the mitochondria (<5 min), an event which precedes cytochrome c release (4, 17), suggesting that, in the context of oxidative stress, Bad/Bax translocation may be one of the initiating events. Bcl-2 family proteins are therefore key regulators of apoptosis in cardiac myocytes, as in other cells, and the expression of the various family members is likely to influence the rate of myocyte death. In this study, we examined the effects of H2O2 on the expression of four key family members which are readily detected in cardiac myocytes (Bcl-2, Bcl-xL, Bax, and Bad).

**EXPERIMENTAL PROCEDURES**

**Preparation of Neonatal Rat or Mouse Cardiac Myocytes**—Myocytes were dissociated from the ventricles of 1–3-day Sprague-Dawley rat hearts as described previously (22) and plated at a density of 1.4 × 10⁶ cells/mm² in 35-mm Primaria tissue culture dishes precoated with 1% (w/v) gelatin. Neonatal mouse myocytes were prepared from the ventricles of C57Bl6 mice using essentially the same procedure as for neonatal rat myocytes, and cells were plated at a density of 2.0 × 10⁶ cells/mm². Myocytes were plated in Dulbecco’s modified Eagle’s medium/M199 (4:1) containing 5% (v/v) fetal calf serum and 10% (v/v) horse serum (16 h, 37 °C), and then incubated in serum-free medium for 24 h prior to experimentation. Myocytes were exposed to H2O2 (0.02 mM, 0.2 mM) for the times indicated.

**Immunoblot Analysis**—Myocytes were washed in Ca²⁺-/Mg²⁺-free Dulbecco’s phosphate-buffered saline (Invitrogen), extracted in Buffer A (20 mM glycerophosphate, pH 7.5, 50 mM NaF, 2 mM microcystin LR, 2 mM EDTA, 0.2 mM Na₃VO₄, 10 mM benzamidine, 200 μM leupeptin, 10 μM trans-epoxysuccinyl-l-leucylamido-(4-guanidino)butane, 5 mM dithiothreitol, 300 μM phenylsulfonyl fluoride, 1% (v/v) Triton X-100) and then incubated in serum-free medium for 24 h prior to experimentation. Myocytes were exposed to H2O2 (0.02 mM, 0.2 mM, 2 mM) for the times indicated.

**RT-PCR Analysis**—Cardiac myocytes were exposed to H2O2 (0.02 mM) for up to 24 h, and the levels of Bcl-2, Bcl-xL, Bax, and Bad were quantified by scanning densitometry.

**Total RNA Isolation and cDNA Synthesis**—Myocytes were homogenized in 0.5 ml of RNAzol B (AMS Biotech) and total RNA prepared according to the manufacturer’s instructions. RNA pellets were dissolved in diethyl pyrocarbonate-treated water, and the concentrations were calculated from the absorbance at 260 nm. First strand CDNA synthesis was performed from total RNA (2 μg) in a reaction mixture containing 500 units of (dT) and 1 mM dNTP mix. Tubes were heated (65 °C, 5 min). 5 × first-strand buffer, 0.1 M diethoxtetrol, recombinant ribonuclease inhibitor (40 units) and SuperScript II RNase H reverse transcriptase (200 U) (Invitrogen) were added, and CDNA synthesis was performed (50 min, 42 °C).

**Preparation of Genomic DNA**—Rat spleen (stored at –80 °C) was powdered under liquid N₂, homogenized in Buffer AT (Qiagen DNeasy tissue DNA extraction kit), and passed through a 21-gauge needle 5–20 times. DNA was extracted according to the manufacturer’s instructions, eluted in 100 μl, and the concentration was calculated from the absorbance at 260 nm.

**Ratiometric RT-PCR Analysis**—Ratiometric RT-PCR was performed as previously described, and in our hands, results from this method are comparable with those obtained by quantitative “real-time” PCR (24). Primers were designed for the coding regions of rat Bcl-2, Bcl-L, Bax and for mouse or rat Bcl-xL coding region. Primers for the Bcl-xL coding region (exon 2) were designed not to detect Bcl-xS. Primers were designed for each of the potential first exons upstream of exon 2 of the Bcl-x gene and to amplify the region across exons 1 and 2 (Table I and Fig. 1). To avoid confusion, we have used the Grillot/MacCarthy-Morrogh (11, 14) terminology referring to exons D and E of the Pecci et al. (15) shown below. Exons and introns are not shown (for further details see Table I). bp numbering extends from the first coding ATG. Exons and introns are not shown to scale.

**RESULTS**

H2O2 Promotes Down-regulation of Bcl-2 Family Proteins in Cardiac Myocytes—We examined the effects of different concentrations of H2O2 (2, 0.2, and 0.02 mM) on the expression of four principal Bcl-2 family proteins, which are expressed in cardiac myocytes: Bcl-2, Bcl-xL, Bax, and Bad. 0.2 mM H2O2 results in myocyte death associated with features of apoptosis (4), whereas higher concentrations induce non-apoptotic cell death. 0.02 mM H2O2 is non-toxic, but has effects on gene expression. ² Neonatal rat cardiac myocytes were exposed to H2O2 for up to 24 h, and the levels of Bcl-2, Bcl-xL, Bax, and

¹ The abbreviations used are: RT, reverse transcriptase; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

² T. J. Kemp and A. Clerk, unpublished data.
Bcl-x Expression in Cardiac Myocytes

To determine which of the untranslated exons are expressed in response to H$_2$O$_2$, neonatal mouse cardiac myocytes were exposed to 0.2 mM H$_2$O$_2$, whereas exons 1B, 1C, and 1D were all signifi-
cantly down-regulated in neonatal mouse cardiac myocytes in response to 0.2 mM H$_2$O$_2$. As in rat myocytes, Bcl-xL mRNA expression was increased at 2 and 4 h, and this was sustained over the 8-h period studied (Fig. 3A). In contrast, there was no change in expression of Bcl-2 or Bax mRNA (Fig. 3, B and C), and Bax was marginally down-regulated at the mRNA level (Fig. 3D). Since more detailed analysis of the regulation of Bcl-x has been performed for the mouse gene than for the rat gene (15), we confirmed that Bcl-xL mRNA was up-regulated in neonatal mouse cardiac myocytes in response to 0.2 mM H$_2$O$_2$. As in rat myocytes, Bcl-xL mRNA expression was increased at 2 and 4 h (Fig. 3F). Thus, 0.2 mM H$_2$O$_2$ increases the expression of Bcl-xL mRNA in cardiac myocytes, and this probably accounts for the selective re-expression of Bcl-xL protein.

Up-regulation of Bcl-xL mRNA by H$_2$O$_2$ Is Mediated by Selective Promoter Activation—As illustrated in Fig. 1, four putative untranslated first exons have been identified for the mouse Bcl-x gene (15). To avoid confusion we have extended the terminology used by MacCarthy-Morrogh et al. (14) and refer to exons 1 (the original first exon identified by Grillot et al. (11)), 1B, 1C, and 1D (which correlate to exons C, D, and E described by Pecci et al. (15)). Transcripts containing exons 1 and 1B had been previously detected in heart extracts, but whether exons 1C and 1D are also expressed had not been studied. Using specific primers to each of the untranslated exons of the mouse Bcl-x gene for ratiometric RT-PCR analysis, we confirmed that exons 1 and 1B were expressed in adult mouse heart and all other tissues studied (spleen, kidney, liver, and brain) (Fig. 4A). Exon 1C, but not exon 1D, was also detected in all adult mouse tissues (Fig. 4A and results not shown). Bcl-x exons 1, 1B, and 1C were all readily detected in neonatal mouse cardiac myocyte cultures confirming expression in the myocytes themselves. However, although it was not detected in adult hearts, exon 1D was expressed in neonatal cardiac myocytes (Fig. 4B), suggesting that expression of this region is down-regulated during postnatal development of the heart. Since all primers were designed to amplify regions within individual predicted exons, we considered it necessary to confirm that there was no contamination of samples with genomic DNA and primers were designed for RT-PCR to amplify the region across the intron containing exons 1B, 1C, and 1D (which correlate to exons C, D, and E described by MacCarthy-Morrogh et al. (15)). Transcripts containing exons 1B, 1C, and 1D (which correlate to exons C, D, and E described by Pecci et al. (15)). Transcripts containing exons 1 and 1B had been previously detected in heart extracts, but whether exons 1C and 1D are also expressed had not been studied. Using specific primers to each of the untranslated exons of the mouse Bcl-x gene for ratiometric RT-PCR analysis, we confirmed that exons 1 and 1B were expressed in adult mouse heart and all other tissues studied (spleen, kidney, liver, and brain) (Fig. 4A). Exon 1C, but not exon 1D, was also detected in all adult mouse tissues (Fig. 4A and results not shown). Bcl-x exons 1, 1B, and 1C were all readily detected in neonatal mouse cardiac myocyte cultures confirming expression in the myocytes themselves. However, although it was not detected in adult hearts, exon 1D was expressed in neonatal cardiac myocytes (Fig. 4B), suggesting that expression of this region is down-regulated during postnatal development of the heart. Since all primers were designed to amplify regions within individual predicted exons, we considered it necessary to confirm that there was no contamination of samples with genomic DNA and primers were designed for RT-PCR to amplify the region across the intron containing exons 1B, 1C, and 1D (which correlate to exons C, D, and E described by Pecci et al. (15)).

Quantitative RT-PCR was performed to determine whether re-expression of Bcl-xL protein in response to 0.2 mM H$_2$O$_2$ is dependent upon the selective re-expression of Bcl-xL protein.

To determine which of the untranslated exons are expressed in response to H$_2$O$_2$, neonatal mouse cardiac myocytes were exposed to 0.2 mM H$_2$O$_2$, and the different exons were analyzed by ratiometric RT-PCR. The expression of exon 1 did not change in response H$_2$O$_2$, whereas exons 1B, 1C, and 1D were all signif-

**Fig. 2.** H$_2$O$_2$ induces down-regulation of Bax, Bad, Bcl-2, and Bcl-xL in rat cardiac myocytes. Rat neonatal cardiac myocytes were exposed to 2 mM (A–D) or 0.2 mM (E–H) H$_2$O$_2$ for the times indicated. Extracts were immunoblotted for Bax (A and E), Bad (B and F), Bcl-2 (C and G), or Bcl-xL (D and H). Representative blots are shown in the upper panels; densitometric analysis of the data is provided in the lower panels expressed relative to controls. Results are means ± S.E. for 3 (B and D), 4 (G and H), 5 (A, C and F), or 7 (E) independent experiments.
significant increased (Fig. 5A). The sequence for the region 5’ to the rat Bcl-x gene has recently become available and comparison of the region encompassing all potential untranslated first exons with the mouse sequence indicates a high degree of conservation from some distance upstream of exon 1D through to the first coding ATG of the Bcl-x gene (see on-line Supplemental Material). Using primers specific for the rat sequence (Table I), we demonstrated that, consistent with the data for mouse myocytes (Fig. 5A), 0.2 mM H$_2$O$_2$ selectively increased the expression of exons 1B, 1C, and 1D in neonatal rat cardiac myocytes with no change in expression of exon 1 (Fig. 5, B and C). These data suggest that oxidative stress results in specific expression of the distal untranslated exons of the Bcl-x gene in cardiac myocytes rather than the most proximal exon 1.

![Table I](https://example.com/table1.png)

### Table I

| mRNA    | Size | Forward 5’ - 3’ | Reverse 5’ - 3’ |
|---------|------|-----------------|-----------------|
| GAPDH (M) | 452  | ACCACAGTCATGCCATCAC (590–610) | TCCACCACCTGTTGCTGTA (1022–1042) |
| Bad (R)  | 340  | CAGTGTCCGGCTGATCATACT (612–632) | TCCAGCTAATGATAGAGAC (952–932) |
| Bcl-2 (R) | 472  | GCTAGCGAGGAGATGATCGAG (77–98) | GTTCGAGATGCGGTGCTTA (551–532) |
| Bcl-xl (R, coding) | 417  | AGGATACAGCTGGAGTACAG (77–97) | TCCCTGGACGTACCCCTTCCT (494–474) |
| exon 1 (M) | 195  | GATGGAGGAACCAGGTTGTG (628 to 608) | CCCGGAAAGTCTTTGTAT (434 to 454) |
| exon 1B (M) | 206  | GCCTGAGACTCCTCAAATCG (1823 to 1803) | TGACTGGTGGAGAGGAGG (322 to 305) |
| exon 1C (M) | 193  | CCCGGAGCTGAGTCTCCACT (2695 to 2675) | GATGGAGGAACCAGGTTGTG (628 to 608) |
| exon 1 (R) | 162  | CGATGGTCCGGGAGGTTGTG (628 to 608) | CCCGGAAAGTCTTTGTAT (434 to 454) |
| exon 1B (R) | 201  | GCCTGAGACTCCTCAAATCG (1823 to 1803) | TGACTGGTGGAGAGGAGG (322 to 305) |
| exon 1C (R) | 273  | AGGATACAGCTGGAGTACAG (77–97) | TCCCTGGACGTACCCCTTCCT (494–474) |
| exon 1D (R) | 264  | AGGATACAGCTGGAGTACAG (77–97) | TCCCTGGACGTACCCCTTCCT (494–474) |

**Fig. 3.** H$_2$O$_2$ (0.2 mM) promotes selective up-regulation of Bcl-xL mRNA in rat or mouse cardiac myocytes. Rat neonatal cardiac myocytes were exposed to 0.2 mM H$_2$O$_2$ for the times indicated. The expression of Bcl-xL (exon 2) (A), Bcl-2 (B), Bax (C), or Bad (D) mRNA was analyzed by ratiometric RT-PCR. Representative images from a single experiment are shown in the upper panels. The expression of GAPDH in the samples from the same experiment is also shown (E). Densitometric analysis of the data is provided in the lower panels expressed relative to controls. Results are means ± S.E. for 4 (B) or 5 (A, C, and D) independent experiments.
DISCUSSION

Down-regulation of Bcl-2 Family Proteins during Cardiac Myocyte Death—H2O2, as an example of oxidative stress, induces cardiac myocyte death. In response to 2 mM H2O2, which should induce a more necrotic phenotype, all four Bcl-2 family proteins studied were profoundly down-regulated (Fig. 2, A–D). In contrast, in response to 0.2 mM H2O2, which induces apoptosis, although the down-regulation of Bcl-2 was similar to that seen in response to 2 mM H2O2, the effect on Bax and Bad was less pronounced (Fig. 2, E–G). Furthermore, although Bcl-xL decreased initially, the protein was re-expressed (Fig. 2, H). The effects of the two concentrations on Bcl-2 family proteins are therefore distinct and may be a reflection of the type of cell death. The mechanisms involved in the down-regulation of these Bcl-2 family proteins were not investigated in this study. However, the down-regulation of Bcl-2, Bax, and Bad in response to 0.2 mM H2O2 is likely to be primarily due to increased protein degradation and/or reduced protein synthesis, since the mRNA levels for Bcl-2 and Bax did not change and, although there was a decrease in Bad mRNA, this was relatively modest (Fig. 3, B–D). The initial decline in Bcl-xL protein also probably reflects increased degradation, since Bcl-xL mRNA expression did not change significantly before ~2 h (Fig. 3A). The subsequent increase in Bcl-xL mRNA presumably accounts for the re-expression of Bcl-xL protein from ~4–6 h (Fig. 2H). There was some variability in the time at which Bcl-xL protein was re-expressed, which may be due to a conflict between the rate of protein degradation and synthesis of new protein. The significance of Bcl-xL re-expression in

Fig. 4. Expression of Bcl-x non-coding exons 1, 1B, 1C, and 1D in mouse tissues, and cardiac myocytes. RNA was extracted from adult mouse heart, spleen, kidney, liver, and brain (A and B) or from mouse neonatal cardiac myocytes (B and C), or genomic DNA was prepared (A and C). The expression of Bcl-x exons 1, 1B, 1C, and 1D, and the region encompassing the intron between exon 1 and exon 2 was examined by ratiometric RT-PCR analysis using the primers detailed in Table I.

Bcl-xL Expression in Cardiac Myocytes
response to 0.2 mM H$_2$O$_2$ is not clear. However, this concentration does not induce apoptosis in all myocytes over 24 h (4), and re-expression of Bel-xL in surviving cells may be a significant component of the survival program.

Regulation of Bel-xL mRNA Expression in Cardiac Myocytes—Although Bel-x transcripts have been reported to derive from at least five different promoters (i.e. the region between exons 1 and 2 and regions upstream of exons 1, 1B, 1C, and 1D), only the three most proximal (regions upstream of exons 1, 1B, and 1D) were previously shown to be operative in the heart (15). In this study, we demonstrated that exon 1C was expressed in adult mouse heart and neonatal cardiac myocytes, and although it was not detected in adult hearts, exon 1D was expressed in neonatal cardiac myocytes (Fig. 4A). These data suggest that there is developmental regulation of the upstream promoters such that exon 1D is no longer expressed in adult hearts. Our preliminary data suggest that, in neonatal cardiac myocytes, exons 1D and 1C are expressed as a single exon that incorporates the intervening sequence (results not shown). This suggests that during development there is a switch in the transcriptional initiation site that is used. Exons 1C/1D do not appear to be continuous with exon 1B (results not shown). Consistent with this, comparison of the mouse and rat sequences indicates that there is a region of low homology between exon 1C and exon 1B (see on-line Supplemental Material).

Although all four untranslated exons were detected in neonatal cardiac myocytes, only the expression of exons 1B, 1C, and 1D was significantly increased by 0.2 mM H$_2$O$_2$, with no increase in exon 1 (Fig. 5). The reasons for multiple promoter usage and differential expression of untranslatable exons are unclear, although previous studies suggest that the promoter may influence the isoform of Bel-x which is produced (15). In cardiac myocytes, Bel-xL (Fig. 2) and Bel-xS (results not shown) were up-regulated concomitantly, and it is possible that, for example, exon 1B may preferentially induce Bel-xL whereas exons 1C/1D may induce Bel-xS. However, there is minimal evidence in support of such a scenario. Alternatively, switching of non-coding exons to produce different 5′-untranslated regions may influence the mode and rate of translation. In cardiac myocytes, H$_2$O$_2$ (>0.1 mM) suppresses global protein synthesis by ~95% over at least 4 h, probably because of repression of cap-dependent initiation of translation (25). However, a small group of proteins continues to be synthesized, including p21$^{CIP1/WAF1}$ (26), presumably through cap-independent mechanisms. An increasing number of eukaryotic mRNAs have been identified which contain internal ribosome entry sites required for translation to occur in the context of global inhibition of protein synthesis. Most studies so far have focused on the regulation of expression of Bel-xL from the promoter regions upstream of exons 1 and 2, and binding sites for a number of transcription factors have been identified in this region (11). It is now clear that further analysis of sequences upstream from exons 1B, 1C, and 1D is required to identify the regions that are responsive to H$_2$O$_2$ and establish which transcription factors are involved.

It is of note that the homology between rat, mouse and human Bel-x gene is extremely high across the region encompassing exons 1B through to exon 3. However, although the homology between the rat and mouse sequences across exons 1C and 1D is high (see on-line Supplemental Material), we have had problems aligning this region with the human sequence (42% identity at best). It is possible exons 1C and 1D represent a different gene from Bel-x, but the homology between rat and mouse extends almost continuously from some distance 5′ to exon 1D through the first coding ATG of Bel-x, and the regulation of exons 1C and 1D in response to H$_2$O$_2$ is similar to that of exons 1B and Bel-xL exon 2. Furthermore, the homology between the rat and mouse sequences is lost abruptly some distance 5′ to exon 1D (see on-line Supplemental Material), suggesting that this may be the extreme 5′ end of the gene. The reasons for the lack of homology with the human sequence are not clear. If exons 1C and 1D are part of another gene, it is possible that this gene is elsewhere on the human genome, but we have been unable to find any human sequences with significant homology to the rat/mouse sequences. It seems more probable that evolutionary constraints on the primary sequence were not high. Motifs for internal ribosome entry sites are not conserved at the primary sequence level, and it is the secondary structure that appears to be of prime importance (29). If the upstream region of the Bel-x gene produces a mRNA capable of promoting cap-independent translation, the constraints would therefore be expected to occur at the level of secondary structure rather than primary sequence. Further analysis is required to determine whether the upstream region is relevant to expression of human Bel-x gene products and if this region does indeed promote translation through cap-independent mechanisms.

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Regulation of Bcl-xL Expression by H$_2$O$_2$ in Cardiac Myocytes

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