**bcl-x\_L** is the major **bcl-x** mRNA form expressed during murine development and its product localizes to mitochondria

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**SUMMARY**

Most examples of cell death in animals are controlled by a genetic program that is activated within the dying cell. The apoptotic process is further regulated by a set of genes that act as repressors of cell death. Of these, **bcl-2** is expressed in a variety of embryonic and postnatal tissues which suggests a critical role for **bcl-2** in organogenesis and tissue homeostasis. Surprisingly, mutant mice with targeted disruption of **bcl-2** appear normal at birth and complete maturation of lymphoid tissues before succumbing to fulminant lymphopenia and polycystic renal disease by 2-5 weeks of age. This suggests that there may be genes other than **bcl-2** that can regulate apoptosis during development. To begin to investigate this possibility, we have cloned and characterized the murine **bcl-x** gene, whose human counterpart displays striking homology to **bcl-2**. The predicted murine **bcl-x** gene product exhibits a high level of amino acid identity (97%) to its human counterpart. Just like **Bcl-2**, the murine **bcl-x\_L** gene product can act as a dominant inhibitor of cell death upon growth factor withdrawal. In addition, the bulk of the **bcl-x\_L** product localizes to the periphery of mitochondria as assessed by a **bcl-x\_L**-tag expression system, suggesting that both Bcl-2 and Bcl-x\_L proteins prevent cell death by a similar mechanism. **bcl-x\_L** is the most abundant **bcl-x** mRNA species expressed in embryonic and adult tissues. The levels of **bcl-x\_L** mRNA appear higher than those of **bcl-2** during embryonal development and in several adult organs including bone marrow, brain, kidney and thymus. In addition to **bcl-x\_L**, we have identified another form of **bcl-x** mRNA, **bcl-x\_S**, that results from an unspliced **bcl-x** transcript. **bcl-x\_S** mRNA is expressed in various embryonic and postnatal tissues. Surprisingly, the expression of **bcl-x\_S** (a negative regulator of programmed cell death) was undetectable by a sensitive S1-nuclease assay and polymerase chain reaction analysis of mouse tissues. Based on its tissue and developmental patterns of expression, it appears that **bcl-x** may play an important role in the regulation of cell death during development and tissue homeostasis.

Key words: Apoptosis, **bcl-x**, **bcl-2**, cell death

**INTRODUCTION**

Cellular homeostasis and development in vertebrates is regulated by several processes that include cell proliferation, differentiation and cell death. The death of cells can be conceptually divided into two main categories: accidental cell death (necrosis) and naturally occurring cell death or Programmed Cell Death (PCD) that is often accomplished by apoptosis. PCD is widespread during embryogenesis, endocrine-dependent cellular atrophy, normal cellular turnover, and clonal selection in the immune system (Ellis et al., 1991). For example, during development of the central nervous system in vertebrates, as much as 85% of certain populations of neurons undergo cell death (reviewed by Oppenheim, 1991). The death mechanism appears to occur through competition for trophic factors derived from target tissues, which may serve to select the proper set of neuronal connections (Oppenheim, 1991). Similarly, in postnatal tissues, terminal differentiation of complex epithelia such as that from the skin is coupled to PCD (McCall and Cohen, 1991). In the immune system, PCD plays a critical role in the selection of appropriate lymphoid populations (Cohen, 1991). Thus, PCD plays an important role during development and tissue turnover by reinforcing appropriate cellular patterns or removing cells that are harmful or no longer needed.

PCD appears to be a genetically regulated process. PCD can be induced by a variety of stimuli, including deprivation of essential growth factors, signalling via certain cell surface receptors or exposure to hormones or drugs such as corticosteroids (Ellis et al., 1991; Williams, 1991). Although the mechanisms of PCD are poorly understood, it is generally thought that dying cells participate in their own demise by activating a genetically programmed suicide pathway (Cohen and Duke, 1984; Martin et al., 1988). In the nematode *Caenorhabditis elegans*, fourteen genes have been identified that are involved in the death of somatic cells during development (reviewed by
Elliott et al., 1991). Some of these genes act at an early stage of the pathway, whereas other genes are directly responsible for the cell death mechanism. For example, mutations that inactivate two autosomal recessive genes ced-3 and ced-4, prevented normal patterns of cell death during development (Ellis and Horvitz, 1986). The ced-3 and ced-4 genes encode proteins that may display killing activity themselves or regulate such activity in cells (Yuan and Horvitz, 1990; Yuan et al., 1993). In contrast to ced-3 and ced-4, ced-9 acts by suppressing cell death. Inactivation of ced-9 by mutation causes death in many different cells during Caenorhabditis elegans development (Hengartner et al., 1992). Similarly, genes that control the cell death pathway in the developing eye of Drosophila melanogaster have been identified recently (Bonini et al., 1993).

In mammals, several genes have been isolated recently that are induced upon activation of the PCD pathway (Baughman et al., 1991; Owens et al., 1991; Ishida et al., 1992). Wild-type p53 has been shown to induce apoptosis (Yonish-Rouach et al., 1991; Ryan et al., 1993) and appears to be required for several forms of PCD (Lowe et al., 1993; Clarke et al., 1993). In addition, overexpression of the ced-3 homolog, interleukin-1β-converting enzyme, can directly activate PCD (Miura et al., 1993). Two related mammalian genes, bcl-2 and bcl-x, have been identified that function as suppressors of PCD. bcl-2 was discovered at the breakpoint region of a recurrent chromosomal translocation t(14;18) (q32;q21) identified in up to 85% of follicular B-cell lymphomas (Tsujimoto and Croce, 1986). The murine bcl-2 was found to be differentially regulated in human tissues (Yonish-Rouach et al., 1990). Expression of the FLAG-bcl-xL protein was determined by PCR amplification using primers corresponding to sequences in the bcl-x coding region (5′-CTAGAATTCATAAGTGCTCAGGCAACCCG-3′) and the 3′ end of the intron of bcl-x (5′-CCAGAAATTCAGCTGAAACATCG-3′). A band of appropriate size (0.8 kb) was subcloned into pBluescript and inserted sequences were sequenced. To generate a 5′ amino acid FLAG tag that contains a well-characterized epitope (Hopp et al., 1988), FLAG sequences were added to the N-terminus of the wild-type cells. These data suggest that both Bcl-2 and Bcl-xL may function by a similar mechanism to inhibit cell death. Our results show that bcl-xL is the dominant species of bcl-x mRNA expressed in murine tissues during embryonic and postnatal development. Importantly, bcl-xL is undetectable in the embryo and adult organs arguing against a role of bcl-xL in tissue development. However, a novel form of bcl-x mRNA, bcl-xS, which results from an unspliced mRNA transcript is expressed in embryonal and postnatal tissues. A truncated bcl-2 gene construct similar to bcl-xL appears capable of at least partially inhibiting apoptosis (Hockenbery et al., 1993). Together, these data suggest that in addition to bcl-2, bcl-xL will have an important role in the regulation of developmental cell death and tissue homeostasis.

**MATERIAL AND METHODS**

**Cloning and construction of plasmids**

A murine brain cDNA library cloned into the Uni-ZAP XR vector (Stratagene) was screened with a human bcl-x probe (Boise et al., 1993). Filter hybridization was performed in 50% formamide, 6× SSPE, 5× Denhardt’s solution, 0.5% SDS and 100 µg/ml denatured salmon sperm DNA at 37°C overnight. The final washing conditions were in 2× SSPE at 37°C three times. Inserts from positive clones were sequenced by a dideoxy termination method. Genomic bcl-xL clones were isolated from a murine liver library (a gift of Dr Paul Killen, University of Michigan) by hybridization with a full-length murine bcl-xL cDNA. Analysis of genomic clones was performed by restriction mapping, hybridization with oligonucleotide probes complementary to different regions of the bcl-xL cDNA and sequencing. The murine bcl-xS cDNA was isolated from a murine thymic cDNA library constructed in ZAP (Stratagene) by PCR amplification using primers corresponding to sequences in the bcl-xL coding region (5′-CTAGAATTCATAAGTGCTCAGGCAACCCG-3′) and the second intron of bcl-x (5′-CCAGAAATTCAGCTGAAACATCG-3′). A band of appropriate size (0.8 kb) was subcloned into pBluescript and inserted sequences were sequenced. To generate a 8 amino acid FLAG tag that contains a well-characterized epitope (Hopp et al., 1988), FLAG sequences were added to the N-terminus of the wild-type cells. These data suggest that both Bcl-2 and Bcl-xL may function by a similar mechanism to inhibit cell death. Our results show that bcl-xL is the dominant species of bcl-x mRNA expressed in murine tissues during embryonic and postnatal development. Importantly, bcl-xL is undetectable in the embryo and adult organs arguing against a role of bcl-xL in tissue development. However, a novel form of bcl-x mRNA, bcl-xS, which results from an unspliced mRNA transcript is expressed in embryonal and postnatal tissues. A truncated bcl-2 gene construct similar to bcl-xL appears capable of at least partially inhibiting apoptosis (Hockenbery et al., 1993). Together, these data suggest that in addition to bcl-2, bcl-xL will have an important role in the regulation of developmental cell death and tissue homeostasis.

**Cell transfection and functional analysis**

Murine FL5.12 cells were cultured as previously described (Núñez et al., 1990). Cells were transfected by electroporation (200 V, 960 µF) with 15 µg of plasmid DNA. Transfectants were selected by growth in the presence of G418 (1 mg/ml). Cell survival before and after IL-3 deprivation was assessed as previously described (Núñez et al., 1990). Expression of the FLAG-Bcl-xL protein was determined by...
flow cytometry as previously described (Boise et al., 1993). The FLAG epitope was detected using M2, a mouse anti-FLAG monoclonal antibody (International Biotechnologies). As a control, cells were stained with a murine monoclonal antibody directed to the 12CA5 influenza virus hemaglutinin protein epitope (Kolodziej and Young, 1991).

RNA preparation and northern blot analysis
Embryonal tissue was isolated from CD-1 mice (first day was taken as the day of vaginal plug appearance). Adult organs were prepared from 8- to 20-week old C57BL/6 mice (Jackson Laboratories). Total mRNA was isolated from mouse tissues by the guanidinium isothiocyanate method followed by cesium chloride gradient centrifugation (Chirgwin et al., 1987). For northern analysis, 12 µg of total mRNA was separated on agarose-formaldehyde gels, blotted onto nitrocellulose filters and hybridized overnight with 32P-labeled probes. The final washing conditions were 0.1% SSC, 0.1% SDS at 56°C for 20 minutes. Blots were stripped by boiling.

S1 nuclease protection assay
The generation of the murine bcl-2 probe has been described previously (Cuende et al., 1993). The 5’ end of the antisense strand was labeled at the BamHI site located in the coding region of bcl-2 using [32P]ATP (6,000 Ci/mmol) (Amersham) as described (Cuende et al., 1993). To construct a murine bcl-x probe, a 725 bp cDNA fragment containing the entire coding region of bcl-xL was inserted into the EcoRI polynucleotide site of pBluescript. The 3’ end of the antisense strand was labeled at the unique SalI site of bcl-xL by filling with [32P]dCTP (6,000 Ci/mmol) (Amersham) and Klenow (Promega) (see Fig. 4A). To assess mRNA expression, equal amounts of bcl-2 and bcl-xL labeled probes (10^5 cpm each) were hybridized simultaneously with total mRNA samples in the same tube for 16 hours at 55°C and then digested with 200 U of S1 nuclease for 1 hour at 37°C. Protected fragments were size separated on a 6% sequencing gel, dried, and autoradiographed. Autoradiographs were quantified by densitometry scanning with a Radioanalytic Imaging system and AMBIS Quant-Probe Software (AMBIS, Inc., San Diego, CA.)

Laserscanning microscopy
Cells were washed in PBS, adhered to polylysine-coated slides, fixed with 2% paraformaldehyde for 10 minutes and then permeabilized with 0.1% saponin (Sigma) for 15 minutes at 23°C. After washing in PBS containing 0.03% saponin, the cells were blocked with 20% normal goat serum and labeled with M2, a mouse anti-FLAG monoclonal antibody or control mouse anti-12AC5 epitope mAb (Kolodziej and Young, 1991) for 45 minutes at 23°C. The reaction was visualized with fluorescein-coupled to goat anti-mouse IgG (BRL). For double-labeling analysis, cells were first incubated with 100 nM of the dye MitoTracker (mitochondria marker) or 5 µM of CellTracker (cytosol marker) (both from Molecular Probes, Eugene, Oregon), for the dye MitoTracker (mitochondria marker) or 5 µM of CellTracker (cytosol marker) (both from Molecular Probes, Eugene, Oregon), for 15 minutes at 23°C. Cells were then permeabilized by 0.1% saponin for 10 minutes and postfixed with 1% OsO4 in rinse buffer (0.1 M sodium cacodylate, 0.12 M sucrose and 2 mM CaCl2, pH 7.4). Following washing in rinse buffer, cells were dehydrated by successive incubations in graded solutions of ethanol. Preembedding was performed in a 50% mixture of propylene oxide and Epon 812 (v/v) at 23°C for 2 hours. Cells were then embedded in Epon 812 resin at 23°C and the reaction polymerized at 60°C overnight. Ultrathin sections (70-90 nm) were mounted on mesh copper grids. Analysis was performed using a Phillips CM-10 electron microscope (Phillips Electronic Instruments).

RESULTS

Isolation and sequence analysis of the murine bcl-x cDNA
To isolate the murine bcl-x homolog, a cDNA library from adult murine brain was screened with a human bcl-x probe. Eight positive phages were isolated with inserts ranging from 1.3 to 2.7 kb. All cDNAs contained identical overlapping sequences as assessed by restriction endonuclease mapping and sequencing analysis. The longest cDNA contained an open reading frame of 233 amino acids with high level nucleotide sequence identity (93%) to the bcl-xL form of human bcl-xL (Fig. 1). None of the cDNAs isolated from the mouse brain library represented the murine counterpart of human bcl-xL (Boise et al., 1993). These results established that the cDNA isolated from brain tissue is the murine homolog of bcl-xL. Further comparative analysis revealed that the predicted coding region of murine bcl-xL displayed 97% amino acid identity to human Bcl-xL and 44% identity to mouse Bcl-2 (Fig. 2). The homology between Bcl-xL and Bcl-2 extended over the entire coding region but it was particularly striking in an internal stretch of 63 amino acids, which is absent in the predicted human Bcl-xS protein (Boise et al. 1993; Fig. 2). In addition, there were stretches of high identity between Bcl-xL and Bcl-2 at the N-terminal region (Fig. 2). Both human and murine Bcl-xL proteins contained a C-terminal domain of 19 amino acids flanked by charged residues (Boise et al., 1993; Fig. 2). This hydrophobic amino acid stretch is typical of a membrane-spanning region and has been shown to serve as an anchoring domain for Bcl-2 (Chen-Levy et al., 1989; Hockenberg et al., 1990; Nguyen et al., 1993).

Bcl-xL is a non-nuclear intracytoplasmic protein that localizes to mitochondria
The Bcl-2 protein localizes to membranes of the mitochondria, and to a lesser extent, to the perinuclear space and smooth endoplasmic reticulum and can protect cells from various forms of cell death (Hockenberg et al., 1990; Monaghan et al., 1992; Jacobson et al., 1993; Krajewski et al., 1993). Because antibodies specific for Bcl-x are not yet available, we constructed a plasmid to express an 8-amino-acid tag peptide (FLAG) fused to the N terminus of the murine Bcl-xL protein to assess its subcellular localization. Stable transfection of the FLAG-bcl-xL gene into murine FL5.12 cells resulted in high expression of the Bcl-xL protein as determined by flow cytometric analysis (Fig. 3).
Importantly, both the murine FLAG-Bcl-xL and wild-type Bcl-xL proteins inhibited the apoptotic death of FL5.12 cells deprived of IL-3 (Fig. 3B). This demonstrates that murine Bcl-xL can function to inhibit cell death induced by growth factor withdrawal and that the 8-amino acid FLAG tag does not alter its biological function. Analysis of labeled cells with anti-FLAG monoclonal antibody revealed that Bcl-xL displays an intracellular location as determined by laser scanning confocal microscopy (Fig. 3C). The labeling pattern was granular and extranuclear similar to that observed for Bcl-2 (Hockenbery et al., 1990; Jacobson et al., 1993; Krajewski et al., 1993), consistent with a localization of Bcl-xL within intracytoplasmic organelles. Dual labeling experiments showed that the staining with fluorescein-labeled FLAG-Bcl-xL is coincident with the distribution of MitoTracker, a rhodamine dye that targets to the mitochondria (Fig. 4A,B). The staining pattern of fluorescein FLAG-Bcl-xL was different from that observed with CellTracker, a dye that accumulates in the cytosolic compartment and exhibits a diffuse non-granular staining pattern (Fig. 4C,D). To further define its subcellular distribution, we performed immunoelectron microscopic studies of FL5.12 cells expressing FLAG-Bcl-xL. The analysis revealed that the bulk of the FLAG-Bcl-xL protein localizes to the periphery of the mitochondria (Fig. 5A). Within the lymphoid FL5.12 cells, which contain abundant mitochondria, the distribution pattern of FLAG-Bcl-xL complexes within individual mitochondria was non-uniform and patchy (Fig. 5A). No labeling was identified in the nucleus or plasma membrane. The staining was specific in that no immunostaining was observed when untransfected FL5.12 cells were stained with anti-FLAG antibody (Fig. 5B) or FLAG-bcl-xL transfected cells were labeled with an isotype-matched control monoclonal antibody (data not shown). Thus, the subcellular distribution of Bcl-xL is similar to that reported for Bcl-2 (Monaghan et al., 1992; Jacobson et al., 1993; Krajewski et al., 1993).

Expression of bcl-x mRNA in murine tissues
To characterize bcl-x further, its expression pattern was assessed in mouse tissues. Northern blot analysis revealed an approx. 3 kb mRNA species present in all tissues examined. Highest levels of bcl-x expression were found in the brain, thymus, bone marrow and kidney (Fig. 6). In the human, two different bcl-x mRNA species, bcl-xL and bcl-xS, have been identified by cDNA cloning and PCR analysis (Boise et al., 1993). To assess the expression of bcl-x forms in mouse tissues, a quantitative S1-nuclease assay was developed to discriminate between bcl-xL and bcl-xS forms (Fig. 7A). The assay included hybridization of mRNAs to both end-labeled bcl-x and bcl-2 S1-nuclease probes to allow assessment of the relative abundance of bcl-2 and bcl-x mRNAs. Results shown in Fig. 7B revealed that bcl-xL is the predominant mRNA expressed in postnatal tissues. bcl-xL was more abundant (2- to 6-fold) than bcl-2 in all adult tissues evaluated, except in lymph nodes. The greater abundance of bcl-xL over bcl-2 was particularly evident in adult brain, thymus, and kidney (5- to 6-fold). In the whole embryo, bcl-xL mRNA was detected at day 8.5 through 12.5 of development and was expressed at day 15.5 in several tissues examined including liver, heart, brain, and kidney (Fig. 7C). bcl-xL was particularly abundant in embryonic liver, the major site of hematopoiesis in the embryo, which is consistent with its elevated expression in adult bone marrow and thymus. Surprisingly, no bcl-xS mRNA species were detected in embryonic and adult tissues including the thymus where it was previously detected in the human by PCR analysis (Boise et al., 1993; Fig. 7B,C).
Bcl-x expression during mouse development

Detect murine bcl-x was confirmed using three additional S1-probes that were end-labeled at different sites of the 5' and 3' ends of the bcl-x coding region. Furthermore, bcl-x was also undetectable by a more sensitive PCR assay, and in tissues from two additional inbred mouse strains ruling out the possibility that the results were due to differences in genetic background (data not shown). Interestingly, a form of bcl-x mRNA, bcl-xβ, distinct from bcl-xL and bcl-xS was readily identified in mouse embryonal and postnatal tissues by S1-nuclease analysis (Fig. 7B,C). The size of the protected band identified by S1 mapping predicted that bcl-xβ and bcl-xL sequences overlap at the 5' end but differ at the 3' region of bcl-x (Fig. 7A).

The bcl-xβ form lacks a hydrophobic C-terminal domain and derives from an unspliced bcl-x mRNA transcript

To analyse in more detail bcl-xβ, genomic clones of the murine bcl-x gene were isolated and characterized by restriction endonuclease mapping and sequence analysis. These studies revealed that bcl-xβ arose from an unspliced mRNA, since it is colinear with the genomic sequences (Fig. 8A). Sequence analysis of the bcl-xβ cDNA isolated revealed an open reading frame of 209 amino acids long, of which the first 188 residues overlap with Bcl-xL including an internal region with high homology to Bcl-2 (Fig. 8B). When compared to Bcl-2, Bcl-xβ displayed the highest amino acid homology to the Bcl-2β product of bcl-2 (Negrini et al., 1987). Both Bcl-2β and Bcl-xβ proteins lack a stretch of 19 hydrophobic amino acids at the C terminus flanked by charged residues, which is present in...
both Bcl-2 and Bcl-x<sub>L</sub> proteins (Fig. 8B). Thus, both bcl-2 and bcl-x<sub>β</sub> mRNAs are generated by unspliced events of the primary mRNA transcript and their products are largely encoded by the first coding exon of bcl-2 and bcl-x.

**DISCUSSION**

In this report we have characterized the murine bcl-x gene to determine if bcl-x could be playing a role in the regulation of PCD during development and organogenesis. Our studies have shown that within murine tissues, bcl-x mRNA is expressed at various levels in most or all tissues during embryonic and postnatal development. In the adult, bcl-x was strikingly abundant in the brain, thymus, bone marrow and kidney. The highly regulated pattern of expression of bcl-x is in contrast to that observed for bcl-2, which is expressed at similar levels in all tissues (Negrini et al., 1987; Eguchi et al., 1992; Boise et al., 1993). We find that bcl-x<sub>L</sub> is the predominant bcl-x mRNA expressed in mouse organs. Importantly, the levels of bcl-x<sub>L</sub> mRNA were higher than those detected for bcl-2 in all tissues evaluated except in lymph nodes. Furthermore, murine bcl-x<sub>L</sub> can prevent cell death upon growth factor withdrawal at least as well as bcl-2, suggesting that bcl-x<sub>L</sub> may play an important role in regulating cell death during development. Although definitive assessment of the function of bcl-x<sub>L</sub> as compared with bcl-2 must await functional studies and quantitative evaluation of both genes at the protein level, the high level expression of bcl-x<sub>L</sub> in certain tissues such as brain is particularly significant. For example, recent studies have shown that the levels of Bcl-2 protein are greatly reduced or undetectable in neurons of the central nervous system (CNS) in adult mice, rhesus monkeys and humans (Merry et al., 1994). Therefore, bcl-x<sub>L</sub> expression may be critical for maintenance of neuronal survival in the adult CNS. The expression of bcl-x<sub>L</sub> in embryonic and adult tissues offers a plausible explanation to account for the normal embryonic development of mice with targeted disruption of the bcl-2 gene (Veis et al., 1993). Similarly, in bcl-2 deficient mice, there is normal maturation of lymphoid precursors in the thymus and bone marrow (Veis

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**Fig. 4.** Dual labeling of FL5.12 cells with FLAG-Bcl-x<sub>L</sub> and Mitotracker. (A,B) FL5.12 cells expressing FLAG-mbcl-x<sub>L</sub> were double-labeled with Mitotracker, a rhodamine dye that targets to the mitochondria and fluorescein-labeled FLAG-Bcl-x<sub>L</sub>. (C,D) FL5.12 cells expressing FLAG-mbcl-x<sub>L</sub> were double-labeled with CellTracker, a rhodamine dye that accumulates in the cytosol, and fluorescein-labeled FLAG-Bcl-x<sub>L</sub>. Confocal images of the cells stained with fluorescein-labeled FLAG-Bcl-x<sub>L</sub> are shown in A and C. Images of the same cells stained with Mitotracker are shown in B and those stained with CellTracker in D.
Fig. 5. Immunoelectron microscopic analysis of FLAG-Bcl-x<sub>L</sub> in FL5.12 cells. FL5.12 cells stably transfected with FLAG-bcl-x<sub>L</sub> (A) or parental untransfected FL5.12 cells (B) were fixed with 2% paraformaldehyde and immunostained in suspension using a monoclonal anti-FLAG antibody. The reaction was detected by a HRP-DAB reaction as described in Materials and Methods. In cross-section, abundant mitochondria are present in the cytosol. In A, the presence of electron-dense immune complex deposits are evident in the periphery of mitochondria (14,000×). Higher magnification (42,000×) of a random region of the cell (in box) is shown in the upper right corner. The distribution of electron-dense deposits in the periphery of mitochondria (long arrows) exhibits a patchy non-uniform pattern (in inset). Focal deposition of electron-dense complexes was also observed in perinuclear membrane (short arrow on top of inset). In B, immunostaining of parental FL5.12 cells using the monoclonal anti-FLAG is shown for comparison (magnification 14,000×). Higher magnification (42,000×) of the boxed area depicts several mitochondria indicated by arrows (in inset).
et al., 1993; Nakayama et al., 1993) which may reflect the use of bcl-xL as a survival signal by lymphocytes to complete their differentiation into mature lymphoid cells. The disappearance of lymphoid cells in adult bcl-2-deficient mice (Veis et al., 1993; Nakayama et al., 1993) is consistent with the high expression of bcl-2 in peripheral lymphoid organs such as the spleen and lymph nodes where the levels of bcl-xL are reduced relative to central lymphoid organs.

In the human, two mRNA forms, bcl-xL and bcl-xS were identified by cDNA cloning and PCR analysis (Boise et al., 1993). Expression of bcl-xS inhibited the ability of bcl-2 to promote cell survival as determined by gene transfer experiments in growth-factor dependent cells (Boise et al., 1993). Although, bcl-xS functioned as a dominant negative regulator of PCD, its expression and role in modulating developmental

![Figure 6](image.png)

**Fig. 6.** Northern blot analysis of bcl-x mRNA in tissues from adult mice. Samples of total mRNA (12 µg) were loaded in each lane. Hybridization was performed using a 725 bp cDNA fragment containing the bcl-xL coding region. In all tissues examined, an approx. 3 kb band was present. As a control to evaluate the amount of mRNA loaded in each lane, the same blot was stripped and hybridized with a murine 18S rRNA probe.

![Figure 7](image.png)

**Fig. 7.** Expression of bcl-2 and bcl-x mRNA forms in embryonic and adult mouse tissues. (A) S1-nuclease probe constructed to detect bcl-x mRNA forms. The antisense strand of the bcl-x fragment was end-labeled at the Sal site located in the coding region of the murine bcl-x cDNA. The region corresponding to the 5’ splice donor site used in the generation of bcl-xS mRNA in the human (Boise et al., 1993) is indicated by the A arrow. The exon/intron boundary in the bcl-xL cDNA is indicated by the B arrow. pBluescript sequences are indicated by a wavy line. Expected sizes of protected fragments for bcl-xL, bcl-xS and bcl-xβ mRNA forms are indicated in nucleotides. (B,C) End-labeled bcl-2 and bcl-x S1-nuclease probes were simultaneously hybridized to total mRNA samples (10 µg in adult tissues, 10 µg in whole embryo samples and 5 µg in 15.5- to 20-day embryonic tissues). Both probes were labeled at a single nucleotide to allow comparison between the relative amounts of bcl-2 and bcl-x mRNAs. Hybridization of both probes with a control tRNA sample is shown for comparison. Hybridization of thymus mRNA to the bcl-2 or bcl-x probe alone is also shown. Notice that the bcl-2 probe protected a fragment of 600 nucleotides (Cuende et al., 1993), whereas the bcl-x probe revealed protected size fragments of 683 (bcl-xL) and 545 (bcl-xS) nucleotides. An expected fragment of 356 nucleotides corresponding to the bcl-xS form was not detected (even at longer exposures) in any of the tissues evaluated. Size markers are in nucleotides.
processes remained unclear. Our analysis has revealed that in the mouse, bcl-xS is undetectable by S1-nuclease assay and PCR analysis in embryonal and adult tissues. The analysis was performed on embryonal tissues from day 8.5 to 15.5 of development, when there is extensive reshaping of tissues by PCD (Glucksman, 1951; Hinchliffe, 1981). These findings clearly argue against a role of bcl-xS in modulating physiological cell death in the mouse. However, we cannot rule out that a small population of cells expresses bcl-xS but its level in the whole organ is too low to be detectable by current methodology. In addition, bcl-xS may be expressed in cell lines or upregulated during cellular activation as reported in the human system (Boise et al., 1993). We find that bcl-xL is the dominant bcl-x form in human tissues as assessed by S1-nuclease analysis (D. Grillot and G. Núñez, unpublished data) and RNase protection (L. Boise and C. Thompson, unpublished data).

However, we have detected bcl-xβ mRNA in unstimulated human thymocytes by S1-nuclease analysis performed by PCR (Boise et al., 1993). The mechanism by which Bcl-2 and Bcl-xL block apoptosis is unclear. The bcl-xβ mRNA species is generated by alternative use of a 5′ splice site within the first coding exon of the bcl-x gene (Boise et al., 1993). Comparison between the human and murine DNA sequences revealed complete conservation of the splice donor sequences in both species. Therefore, other factors such as differences within the flanking sequences to the donor/acceptor splice sites may be involved. Initial analysis revealed that the 5′ splice site of human bcl-xβ splice to an acceptor site in mouse cells indicating that murine cells have the machinery to utilize the 5′ splice site of human bcl-x (D. Grillot and G. Núñez, unpublished data). An unexpected finding was the identification of an alternative mRNA species of bcl-xβ, bcl-xβ in mouse tissues. Our studies have shown that bcl-xβ is the result of an unspliced bcl-x mRNA transcript. The sequence of Bcl-xβ predicts that it is an intracellular soluble protein since it lacks the putative membrane-spanning region at the C terminus present in Bcl-2β and Bcl-xL proteins. Because Bcl-xβ contains the 63 amino acid domain, which is critical for Bcl-xL’s ability to block cell death, it is possible that Bcl-xβ may function in certain tissues such as the thymus where it is highly expressed. A similar Bcl-2 protein lacking the hydrophobic membrane-spanning domain appears capable of at least partially inhibiting apoptosis (Hockenbery et al., 1993). Clearly, further work is needed to assess the expression of the Bcl-xβ protein and its capacity to modulate apoptotic cell death.

The mechanism by which Bcl-2 and Bcl-xL protein function in an anti-oxidant pathway (Hockenbery et al., 1993; Kane et al., 1993). In addition, other studies have suggested that Bcl-2 may inhibit cell death by modulating Ca2+ fluxes across membranes of intracellular organelles (Baffy et al., 1993). The localization of Bcl-xL to the periphery of the mitochondria and perinuclear membrane, sites where Bcl-2 also localizes (Monaghan et al., 1992; Jacobson et al., 1993; Krajewski et al., 1993), strongly suggests that both Bcl-2 and Bcl-xL proteins function in a similar manner to prevent cell death.
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