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The *bcl*-2 Candidate Proto-Oncogene Product Is a 24-Kilodalton Integral-Membrane Protein Highly Expressed in Lymphoid Cell Lines and Lymphomas Carrying the t(14;18) Translocation

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We have identified a 24-kilodalton protein that is the product of the human *bcl*-2 gene, implicated as an oncogene because of its presence at the site of t(14;18) translocation breakpoints. The Bcl-2 protein was detected by specific, highly sensitive rabbit antibodies and was shown to be present in a number of human lymphoid cell lines and tissues, as well as in mouse B cells transfected with a *bcl*-2 cDNA construct. Characterization of the Bcl-2 protein demonstrated that it has a lipophilic nature and is associated with membrane structures, probably by means of its hydrophobic carboxy-terminal membrane-spanning domain. In t(14;18)-carrying cell lines, the protein is predominantly localized to the perinuclear endoplasmic reticulum, with a minor fraction in the plasma membrane. These properties, together with the observations that Bcl-2 does not have a characteristic signal peptide and is not glycosylated, suggest that it is an integral-membrane protein that spans the bilayer at its C-terminal hydrophobic region but is exposed only at the cytoplasmic surface. The relative abundance of the Bcl-2 protein in various human lymphoid cell lines correlated with transcription of the *bcl*-2 gene. The protein was abundant in all t(14;18)-carrying cell lines and lymphomas and was also found at lower levels in pre-B-cell lines and nonmalignant lymphoid tissues that do not carry t(14;18) translocations. These results suggest that the Bcl-2 protein is functional in normal B lymphocytes and that a quantitative difference in its expression may play a role in the pathogenesis of lymphomas carrying the t(14;18) translocation.

The *bcl*-2 gene is the prototype for an emerging and important class of genetic elements with supposedly oncogenic character that are being discovered by virtue of their localizations at the sites of structural chromosomal aberrations in human lymphoid malignancies. *bcl*-2 is the most extensively characterized gene to be identified in this manner (1, 7, 8, 39), having been isolated and analyzed as the result of its consistent involvement by t(14;18) chromosomal translocations commonly observed in human B-cell lymphomas (16, 48). On the basis of cytogenetic observations that this translocation occurred at chromosome band 14q32, the site of the immunoglobulin heavy-chain gene (*Igh*) locus, probes for the latter were used to molecularly clone t(14;18) breakpoint DNAs (1, 7, 8, 39). These were then used to "walk" along chromosome 18 to identify a closely linked transcriptional unit for which the name *bcl*-2 was proposed (8, 39). Subsequent isolation and characterization of *bcl*-2 cDNAs showed that the gene codes for a predicted 26-kilodalton protein with a weak similarity to a predicted Epstein-Barr virus protein but no significant sequence relatedness to any previously described oncogenic proteins (9, 38).

The evidence that *bcl*-2 should be classified as a member of the proto-oncogene family is currently circumstantial yet compelling. The gene is consistently associated with the t(14;18) translocation, which itself is a constant feature of a distinctive lymphoid malignancy. Most t(14;18) translocation breakpoints have been shown either to physically disrupt the *bcl*-2 gene, tightly clustering within one of the *bcl*-2 exons, or to occur at variable distances 5′ or 3′ of the gene (1, 7–9, 39, 43). However, the *bcl*-2 coding region is never disrupted by t(14;18) translocations, implying that the gene product is important in the transformation process. After t(14;18) translocations, the *bcl*-2 gene on chromosome 18 is juxtaposed with the *Igh* gene on chromosome 14, with consequent deregulated expression of the *bcl*-2 gene (9). Only the translocated allele is transcriptionally active in cells with the t(14;18) abnormality, whereas the nontranslocated *bcl*-2 allele is transcriptionally silent (9), similar in many respects to the deregulated c-*myc* expression resulting from chromosomal translocations in B- and T-cell malignancies (14, 24). All of these findings suggest that aberrant expression of the *bcl*-2 gene product is pathogenetically important in the induction or progression of some B-cell malignancies. However, the role of *bcl*-2 in this process and its physiological role in nontransformed cells are yet unknown. Study of the biochemical properties of the protein and its cellular localization may shed light on its function.

In this report, we describe the derivation of antibodies specific for the Bcl-2 protein and the identification of this protein in human lymphoid cells. Our results demonstrate that Bcl-2 is expressed at high levels in all cells with the t(14;18) translocation. Analysis of the protein showed that it is a single subunit, nonglycosylated, membrane-spanning protein with a proposed topology distinct from those of all previously described proto-oncogenic proteins.

MATERIALS AND METHODS

Subcellular fractionation. Approximately 10^8 cells from cultures of 10^6 cells per ml were fractionated in each experimental study. The cells were washed twice with phosphate-buffered saline (PBS) and were then incubated for 10 min in 1.5 ml of cold hypotonic buffer A containing 10 mM HEPES (*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic

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acid [pH 7.8])–15 mM KCl–2mM $MgCl_2$–0.1 mM EDTA–1 mM dithiothreitol–1 mM phenylmethylsulfonyl fluoride–1 μg of antipain and 0.3 μg of leupeptin per μl. This step and all subsequent operations were performed at 4°C or on ice. The swollen cells were lysed by 15 strokes in a tight-fitting Dounce homogenizer, and the nuclear and cytoplasmic fractions were separated by a 6-min centrifugation at 1,000 × *g*. The supernatant solution from the homogenized cell extract was further centrifuged for 1 h at 150,000 × *g*. The supernatant portion of the latter centrifugation represented the soluble cytosolic fraction; the pellet, resuspended in 1.5 ml of buffer A, was the membrane and cytoplasmic organelle fraction. The nuclei obtained from the pellet of the first spin 1,000 × *g* were further purified from possible endoplasmic reticular and mitochondrial remnants by one-step sucrose centrifugation by the method of Blobel and Potter (2). The nuclear pellet was carefully suspended in 1.5 ml of buffer A containing 1.6 M sucrose, overlaid on a 2 M sucrose cushion, and centrifuged at 100,000 × *g* for 90 min. The pellet representing purified nuclei was either fixed directly for electron microscopy or was processed further for fractionation studies. For the latter studies, the purified nuclear pellet was suspended in 1.5 ml of buffer A that contained 0.5% Triton X-100, was incubated for 30 min with occasional mixing, and then was pelleted again at 1,000 × *g* for 6 min. The supernatant fraction represented the Triton-soluble nuclear fraction, which included the nuclear envelope; the pellet (suspended in 1.5 ml of sample loading buffer) represented the nuclear matrix fraction. Proteins from all four fractions were dissolved in sodium dodecyl sulfate (SDS)-sample loading buffer and were boiled for 3 min, and equal samples were loaded and run on an SDS-polyacrylamide gel.

Electron microscopy. Whole FL18 cells and isolated nuclei were prepared for electron microscopy by standard procedures (25). Briefly, pellets were fixed for 1 h at 4°C in 0.067 M sodium cacodylate buffer (pH 7.4) containing 2% glutaraldehyde–1% sucrose–2 mM calcium chloride. Fixed pellets were washed overnight in 0.1 M sodium cacodylate buffer (pH 7.4) and were then incubated for 1 h at 4°C in 2% osmium tetroxide postfixation solution. Samples were dehydrated in increasing concentrations of ethanol followed by propylene oxide. Embedding was carried out in LX-112 (Ladd Industries) which was then polymerized overnight at 60°C.

Immunofluorescence. Cultured cells (FL18 or SU-DUL-5) were placed onto glass slides by cytocentrifuging 100-μl samples of cell cultures at a density of 8×10^5 cells per ml in complete growth medium. The cell pellets were acetone fixed, air dried, and then immediately incubated for 1 h with primary antibodies diluted with PBS. Rabbit anti-Bcl-2 antiserum and preimmune serum were used at a dilution of 1:66, and monoclonal mouse anti-L26 was used at a 1:5,000 dilution. For control preparations without primary antibody, PBS alone was used. The first incubation step was followed by three PBS washes and an additional 1-h incubation with the appropriate secondary antibody (rhodamine-conjugated goat anti-rabbit immunoglobulin G [IgG] or fluorescein-conjugated goat anti-mouse IgG). Slides were rinsed in PBS and were then visualized by fluorescent microscopy.

Transfection. Cotransfection of mouse J558-L lymphocytes with p*bcl*-2/*Igh* and pSV2Neo DNA was performed by the electroporation method described by Chu et al. (6). Cells were harvested at a concentration of 10^6 cells per ml, washed with PBS, and suspended in HEPES buffer (20 mM HEPES [pH 7.05]–137 mM NaCl–5 mM KCl–7 mM Na_2HPO_4–6 mM dextrose) to a final concentration of 2.4×10^7 cells per ml. Each electroporation reaction, at a volume of 800 μl, contained 1.7×10^7 cells plus linearized plasmid DNAs at concentrations of 100 μg/ml for the p*bcl*-2/*Igh* construct and 10 μg/ml for pSV2Neo as the selective marker. The cells were exposed to a pulse of 250 V, were allowed to remain in buffer for 10 min at room temperature, and were then plated into 24-well tissue culture dishes containing RPMI 1640 growth medium supplemented with 10% fetal calf serum. After 2 days in culture, Geneticin-G418 sulfate (GIBCO Laboratories, Grand Island, N.Y.; 458 mg/ml) was added to a final concentration of 800 μg/ml. The G418 concentration used was found earlier to completely kill all parental J558-L cells in culture. Neomycin-resistant colonies started to appear after 10 days; these were expanded and examined for human *bcl*-2 construct integration by Southern blot analysis and for expression by Northern (RNA) blot analysis. One of the RNA-positive *bcl*-2 cultures, designated E.P., was further studied for Bcl-2 protein expression.

Solubilization of Bcl-2 by Triton X-114. Triton X-114 fractionation was carried out by the method of Bordier (4).

Gel electrophoresis and immunoblotting. Proteins were fractionated by discontinuous 10 to 11.5% SDS-polyacrylamide gel electrophoresis (23). Immunoblot analyses of electrophoresed proteins were done essentially as described previously (37) but by using indirect enzyme immunoassay instead of radioactive labeling. After the transfer of proteins to nitrocellulose, filters were blocked overnight at room temperature with 20% bovine serum albumin in PBS. Incubation with the primary rabbit anti-Bcl-2 antibodies or preimmune serum diluted 1:300 was carried out for 1 h at room temperature in a blotting solution (PBS–3% Carnation nonfat dry milk–0.1% Tween 20 [Sigma Chemical Co., St. Louis, Mo.]). After four brief washes with the blotting solution, the nitrocellulose was further incubated with peroxidase-labeled goat anti-IgG(H+L) rabbit antibodies (affinity purified and human serum adsorbed; KPL, Gaithersburg, Md.) diluted 1:250 in the blotting solution. Immunoreactive proteins were detected by brief soaking in 0.5 mg of diaminobenzidine per ml–0.01% H_2O_2 in PBS.

RNA isolation and Northern blots. Isolation of total cellular RNA, poly(A)$^+$ RNA selection, and Northern blot analyses were performed as previously described (9). The hybridization probe used for Northern blot analyses consisted of a fragment of *bcl*-2 cDNA containing the first 1,860 nucleotides as described previously (9).

Bacterial expression constructs and preparation of immunogens. The *trp-lac* bacterial expression vector pKK223-3 (Pharmacia, Inc., Piscataway, N.J.) was used to express the native Bcl-2 protein in *Escherichia coli*. Two constructs were made containing either the entire *bcl*-2 reading frame (p*bcl*-2) that codes for a 239-amino-acid polypeptide or a deletion construct that expressed a truncated Bcl-2 protein (p*bcl*-2Δ). The p*bcl*-2 construct consisted of the first 1,860 nucleotides of the *bcl*-2 cDNA (9) inserted into the *Eco*RI site of the pKK223-3 polylinker cloning site. The correct orientation of the cDNA insert was determined by restriction enzyme analyses. The p*bcl*-2Δ construct was derived by cutting the pbcl-2 plasmid with *Sac*I, which cut twice in the *bcl*-2 insert at nucleotides 440 and 1850. The larger *Sac*I fragment was gel purified and then recircularized to generate a smaller plasmid with a truncated *bcl*-2 open reading frame coding for a 143-amino-acid polypeptide. The C-terminal six amino acids of this polypeptide and an in-frame stop codon were coded by the polylinker of pKK223-3. Both plasmids were maintained in the protease-deficient mutant host strain

AR68 (provided by Shatzman and Rosenberg, Smith Kline & French Laboratories, Philadelphia, Pa.).

To prepare the recombinant Bcl-2 proteins for immunization, a 50-ml culture of the host strain containing the appropriate recombinant plasmid was grown at 30 to 32°C in rich medium. At an optical density at 600 nm of 1.0, the *lac* inducer IPTG (isopropyl-β-D-thiogalactopyranoside) was added to a concentration of 2 mM, and incubation was continued with vigorous aeration for 3 to 8 h at 42°C. The bacteria were harvested by centrifugation, washed twice with 10 mM Tris hydrochloride–1 mM EDTA (pH 7.5), and frozen at −20°C. Bacterial pellets were suspended in 5 ml of 25% sucrose–10 mM Tris hydrochloride (pH 7.5) and were sonicated to lyse the bacterial cells. The insoluble material was sedimented by centrifugation at 12,000 × *g* for 5 min and was suspended in 0.5 ml of 10% sarcosyl. The recombinant Bcl-2 proteins were detected and purified from this sarcosyl extract.

Preparation of antiserum against Bcl-2. Large quantities of recombinant Bcl-2 or Bcl-2Δ proteins, recovered from the sarcosyl extracts of transfected *E. coli* cells, were purified by preparative SDS-polyacrylamide gel electrophoresis and used as antigens to immunize rabbits. Two adult New Zealand White rabbits were injected subcutaneously with 100 to 200 μg of purified Bcl-2 protein and then with two intramuscular booster injections of 100 to 200 μg each of purified Bcl-2Δ protein. Sera obtained 14 days after the second booster injection had high titers of antibodies against recombinant Bcl-2 proteins as detected by immunoblotting.

Cell lines. The derivation and characterization of t(14;18)-carrying cell lines SU-DHL-4 and FL18 have been described previously (12, 13). Cell lines FL218 and FL318 were established by methods similar to those described for FL18 and were kindly provided by Shirou Fukuhara, Kyoto, Japan. The establishment and characterization of human cell lines SU-DHL-9, SU-DHL-10, SUP-B2, SU-DUL-5, and 697 have been described previously (13, 15, 33, 34). The mouse myeloma cell line J558L is an *Igh* mutant variant of J558, as described previously (17). All cell lines were maintained in RPMI medium containing 10% fetal calf serum (GIBCO).

***bcl-2/Igh* expression construct.** The structure of the human *bcl-2* gene construct that was expressed in mouse myeloma cells is illustrated (see Fig. 2A). The *bcl-2* gene containing a complete *bcl-2* open reading frame was placed upstream of and in the same transcriptional orientation as a human immunoglobulin $C_{\gamma 2}$ heavy-chain gene so that a hybrid *bcl-2/Igh* mRNA would be expressed, initiating at the *bcl-2* promoter under the control of the *Igh* enhancer. This construct precisely recapitulated the genomic configuration of the *bcl-2* gene resulting from many t(14;18) translocations, except that it lacked the large *bcl-2* intervening sequence. The *bcl-2* portion consisted of a 4.5-kilobase *Bam*HI fragment containing most of the *bcl-2* 5′ exon and several kilobases of 5′-flanking sequence containing the *bcl-2* promoter. This fragment was ligated to a 1,260-base-pair *Bam*HI-*Eco*RI segment of the *bcl-2* cDNA (nucleotides 595 to 1860) containing the remaining coding region and a portion of the 3′ untranslated region (9). Directly downstream of the *bcl-2* portion was placed a 20-kilobase *Eco*RI fragment (fragment B) (8), a genomic DNA that contained an *Igh* $C_{\gamma 2}$ gene, the *Igh* enhancer, and J_H segments 4, 5, and 6. The *bcl-2/Igh* construct was propagated in the bluescript cloning vector.

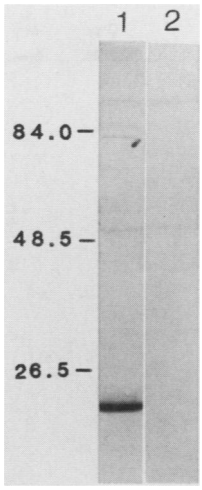

FIG. 1. Detection of human Bcl-2 protein with a specific rabbit antiserum. Western blot analysis was performed on SDS extracts from human lymphoid cell line FL18 carrying the t(14;18) translocation with immune (lane 1) and preimmune (lane 2) sera, both diluted 1:300. Molecular mass standards (in kilodaltons) are shown.

RESULTS

Detection of a *bcl-2* gene product by specific antibodies. To examine the immunoreactivity of anti-Bcl-2 antibodies with the human Bcl-2 protein, we initially examined protein extracts from the lymphoid cell line FL18, which was established from a human follicular lymphoma, carries the t(14;18) translocation, and expresses high levels of *bcl-2* mRNA (see Fig. 3B, Northern blot). Western (immuno-) blot analysis showed that a specific, Bcl-2-reactive protein was detected in FL18 cells. The antiserum detected a single 24-kilodalton polypeptide in a total FL18 cell extract, whereas preimmune rabbit serum showed no immunoreactive protein bands in the same cell extract (Fig. 1). The molecular mass of the Bcl-2-immunoreactive band (24 kilodaltons) was very close to the 26-kilodalton molecular mass predicted for the *bcl-2* gene product on the basis of the *bcl-2* cDNA sequence (9). These results suggested that the rabbit antiserum specifically recognized the Bcl-2 protein present in FL18 human lymphoma cells.

Identification of the human Bcl-2 polypeptide in transfected cells. To confirm that the immunoreactive protein detected by our antibodies corresponded to the polypeptide encoded by the *bcl-2* open reading frame, the mouse lymphoid cell line J558-L, which does not express detectable levels of *bcl-2* mRNA (not shown), was transfected with a *bcl-2/Igh* DNA construct by the electroporation method. This construct (Fig. 2A) was designed to mimic the configuration of the *bcl-2* gene after t(14;18) translocations in lymphoid malignancies (9). The construct contains the immunoglobulin enhancer site that enables expression of the *bcl-2* gene in a tissue-specific manner in lymphocytes. Western blot analysis with the anti-Bcl-2 antibodies (Fig. 2B) demonstrated that the transfected J558L mouse cells (E.P. cells) have a specific 24-kilodalton immunoreactive band that is not present in the parental or mock-transfected J558-L cells. This band comigrated with the Bcl-2 polypeptide band observed in the t(14;18)-carrying FL18 human lymphoid cell line (Fig. 2B). As expected, the transfected cells also expressed human *bcl-2* mRNA as determined by Northern blot analysis (not shown).

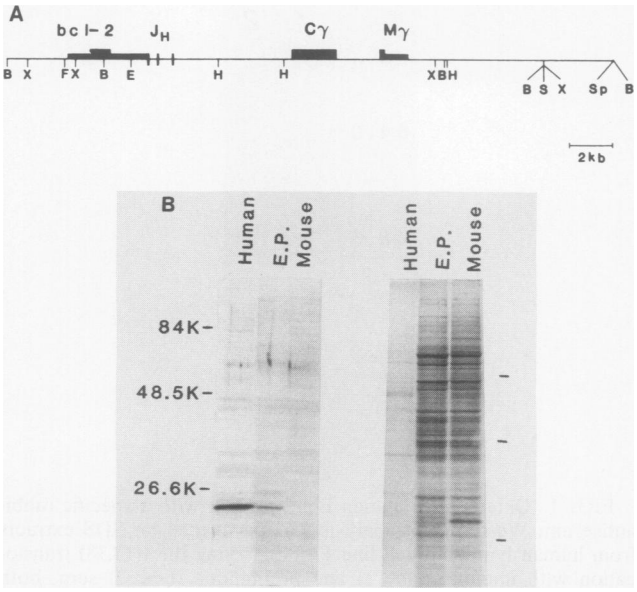

FIG. 2. Expression of human Bcl-2 protein in transfected murine lymphoid cells. (A) Physical map of the *bcl-2/Igh* fusion construct used for mouse-cell transfections is shown oriented in a 5′-to-3′ direction. Thick lines denote coding regions, and intermediate lines denote 5′ or 3′ untranslated segments of *bcl-2* and *Igh* genes. Restriction enzyme cleavage sites: B, *Bam*HI; X, *Xho*I; F, *Fsp*I; E, *Eco*RI; H, *Hin*dIII; S, *Sal*I; Sp, *Spe*I. (B) Total cell extracts fractionated through an SDS-polyacrylamide gel and subjected to Western blot analysis with the same anti-Bcl-2 rabbit antibodies as for Fig. 1. Samples consisted of human lymphoid cell line FL18 (Human), electroporated mouse cell line J558L (E.P.), and the parental J558L mouse cell line (Mouse). Additional faint bands in all lanes correspond to nonspecific background seen in whole-cell extracts. Immunoblot (left) and Coommassie blue-stained gel of the same samples (right) are shown. Molecular masses of protein standards (in kilodaltons) are shown or are denoted by dashes.

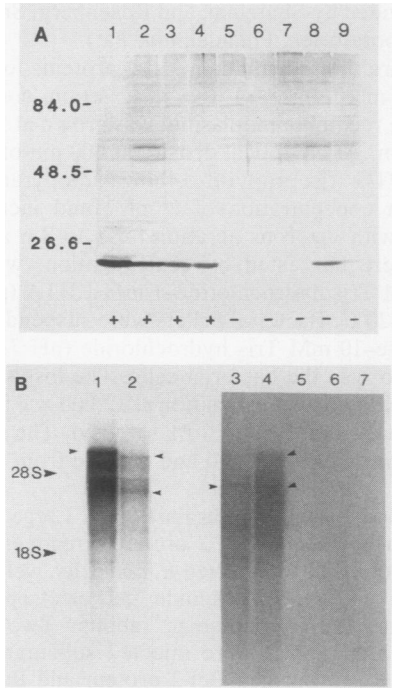

FIG. 3. Expression of Bcl-2 protein and mRNA in various human lymphoid cell lines. (A) Total cellular proteins from 2×10^6 cells were fractionated through an SDS-polyacrylamide gel and analyzed for the presence of Bcl-2 protein by the Western blot procedure with anti-Bcl-2 rabbit antibodies. The cell lines examined were as follows: lane 1, FL18; lane 2, DHL-4, lane 3, FL218, lane 4, FL318; lane 5, DUL-5; lane 6, DHL-9; lane 7, DHL-10; lane 8, SUP-B2; lane 9, 697. The presence (+) or absence (−) of a t(14;18) cytogenetic abnormality in each cell line is indicated. Samples were standardized by loading equal numbers of cells in each lane. Migrations and molecular masses of protein standards (in kilodaltons) are shown. (B) Equivalent amounts of polyadenylated RNAs were fractionated in formaldehyde-agarose gels and analyzed by the Northern blot procedure. The hybridization probe consisted of a 1.8-kilobase fragment of *bcl-2* cDNA as described previously (9). The seven cell lines examined were the same as described for panel A. Size markers consisted of 28S and 18S rRNAs. Arrows indicate positions of hybrid *bcl-2/Igh* transcripts which vary in size because of breakpoint heterogeneity as described elsewhere (9). Lanes 1 and 2 represent a sixfold less exposure than lanes 3 to 7.

The appearance in the transfected cells of a new 24-kilodalton immunoreactive polypeptide and its comigration with the band detected in the cell line FL18 indicate that a full-length Bcl-2 protein can be expressed in mouse lymphoid cells by using the *bcl-2/Igh* construct. These results also confirmed that the rabbit antibodies specifically recognized the human *bcl-2* gene product. We decided, therefore, to use these anti-Bcl-2 antibodies for further characterization of the Bcl-2 protein.

Expression of Bcl-2 protein in human lymphoid cell lines correlates with transcription of the *bcl-2* gene. Nine lymphoid cell lines were examined for Bcl-2 expression. Four of them carry the t(14;18) translocation (FL18, SU-DHL-4, FL218, and FL318), whereas the other five do not carry cytogenetic abnormalities of chromosome 18. In Western blot analysis (Fig. 3A), the Bcl-2 protein was readily detected in total SDS crude extracts of all four cell lines that carried the t(14;18) translocation. Three lymphoma cell lines (DUL-5, DHL-9, and DHL-10; lanes 5 to 7) that lack a t(14;18) translocation did not express detectable Bcl-2 protein in Western blot analysis. In two cell lines (SUP-B2 and 697) without cytogenetic abnormalities of chromosome 18, lower levels of Bcl-2 protein, comigrating with the Bcl-2 bands of the t(14;18) translocation-carrying cell lines, were observed (lanes 8 and 9). Both the SUP-B2 and 697 cell lines were derived from pre-B-cell leukemias and probably express the physiological, untranslocated *bcl-2* gene.

The relative abundance of Bcl-2 protein in the various cell lines correlated with transcription of the *bcl-2* gene in the same cells (Fig. 3B; compare with Fig. 3A). All of the t(14;18) translocation-carrying cell lines had an abundant Bcl-2 immunoreactive polypeptide band as well as detectable *bcl-2* transcripts, which correspond to hybrid *bcl-2/Igh* transcripts of heterogeneous size resulting from t(14;18), as described elsewhere (9). The levels of *bcl-2* mRNA and protein roughly correlated with each other: the FL18 cell line had the highest levels of both *bcl-2* RNA and protein, and FL218 had the lowest. In the lymphoma cell lines that lacked a t(14;18) translocation (DUL-5, DHL-9, and DHL-10), neither a *bcl-2* transcript (Fig. 3B, lanes 5 to 7) nor Bcl-2 protein expression (Fig. 3A, lanes 5 to 7) could be detected. The parallel abundance of *bcl-2* RNA and protein in the various cell lines further established the identification of the immunoreactive 24-kilodalton band as the *bcl-2* gene product. This study also demonstrated the high sensitivity of our antibodies to Bcl-2 in Western blot analysis; even a low level of Bcl-2, such as that observed in the 697 cell line (Fig. 3A, lane 9), could be easily detected in a crude total cell extract.

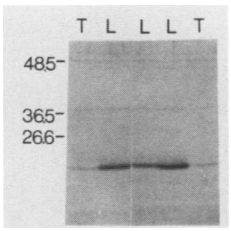

FIG. 4. Expression of Bcl-2 protein in human lymphoid tissues as demonstrated by Western blot analysis of Triton X-100 total extracts of malignant and normal lymphoid tissues. Samples were standardized by loading comparable total protein amounts of the tissue extracts as examined by Coomassie blue staining of the electrophoresed samples (not shown). T, Reactive tonsillar tissue; L, follicular lymphoma tissues. Molecular masses of protein standards (in kilodaltons) are shown.

Detection of Bcl-2 protein in human lymphoid tissues. Expression of Bcl-2 was also observed in Triton X-100 extracts of several human lymphoid tissues. In lymph nodes involved by t(14;18)-carrying lymphomas, abundant Bcl-2 expression was detected at levels comparable to those observed in the t(14;18)-carrying cell lines (Fig. 4), indicating that significant amounts of Bcl-2 are present in these tissues. Bcl-2 was also present, although at much lower levels, in reactive tonsillar tissues not involved by malignancy (Fig. 4). These determinations of Bcl-2 abundance in the lymphoid tissues were standardized by loading comparable amounts of total protein for each tissue extract. Our results clearly demonstrate abundant Bcl-2 in neoplastic t(14;18)-carrying tissues and a low but detectable level of Bcl-2 in reactive lymphoid tissues. The latter observation suggests that Bcl-2 expression may play a role in the normal immune response; however, the specific cells responsible for the expression of Bcl-2 and the relative levels expressed in each type of cell cannot be identified by our methods.

Membrane localization of Bcl-2 protein. The subcellular localization of the Bcl-2 protein was determined by both biochemical fractionation and immunofluorescence analyses of lymphoid cell lines SU-DHL-4 and FL18. Figure 5 represents typical results of a fractionation study. FL18 cells were lysed by hypotonic shock and then fractionated, as described in Materials and Methods. The nuclear first pellet was spun through a 2 M sucrose cushion to shear away any cytoplasmic remnants (2). Electron microscopy of this second nuclear fraction revealed intact nuclei with apparently undamaged nuclear envelopes and no obvious attached rough endoplasmic reticular (RER) membranes (data not shown). Although contamination of this fraction by other subcellular components is not totally excluded by this analysis, the amount of possible contamination is obviously very low. Bcl-2 was present in the Triton extract of the nuclear fraction as well as in the cytoplasmic membrane fraction (Fig. 5). No Bcl-2 was detected in the cytosol, and only residual amounts of Bcl-2 remained in the nuclear matrix after one Triton extraction of the nuclei. This profile of Bcl-2 localization suggested an association of the protein with cellular membranes, namely, surface or cytoplasmic membranes and possibly the nuclear envelope. A very similar pattern of Bcl-2 fractionation was found in SU-DHL-4, another human lymphoid cell line that carries a t(14;18) translocation.

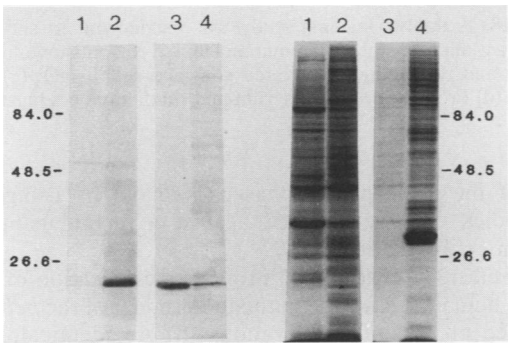

FIG. 5. Biochemical fractionation of lymphoid cell line FL18 to determine subcellular localization of Bcl-2 protein. Subcellular fractions, standardized to represent equal numbers of cells in each (all fractions brought up to 1.5 ml), were electrophoresed in SDS-polyacrylamide gel and analyzed by Western blot technique with anti-Bcl-2 antibodies (left) and by Coomassie blue staining (right). Lanes: 1, soluble cytosolic fraction; 2, membrane and cytoplasmic-organelle fraction; 3, Triton extract of nuclear fraction; 4, nuclear matrix fraction. Sizes of standards (in kilodaltons) are shown.

Immunofluorescence studies confirmed and extended the fractionation results. Figure 6 demonstrates a fluorescent visualization of FL18 cells. The intensity of anti-Bcl-2 staining in FL18 cells (Fig. 6b) was significantly above the levels observed with preimmune serum (Fig. 6d) or that observed with anti-Bcl-2 in SU-DUL-5 cells, which do not express the Bcl-2 protein (data not shown). Most of the Bcl-2 immunofluorescent staining of FL18 cells was localized around the nucleus and in cytoplasmic organelles with a perinuclear distribution (Fig. 6b). This pattern of staining, in conjunction with the fractionation results, is consistent with localization to the RER and its direct continuation, the nuclear envelope (26). In addition, a significant although low level of Bcl-2 staining was clearly visible at the plasma membrane. The nucleus, other than its envelope, was not stained by anti-Bcl-2 antibodies. A control anti-L26 antibody, specific for a B-cell surface marker (20), stained FL18 cells predominantly at the plasma membrane (Fig. 6c).

Transmembrane character of the Bcl-2 protein. The cellular localization of the Bcl-2 protein clearly indicated that it is a membrane-associated protein. To analyze the hydropathic character of the Bcl-2 protein, we performed a Triton X-114 extraction and phase separation, a procedure that separates hydrophilic soluble proteins from amphiphilic integral-membrane proteins (4). Nuclei of the lymphoid cell line FL18 were extracted by one step with 0.5% Triton X-114, and the buffer and detergent phases were separated at 30°C. Figure 7A shows the SDS-polyacrylamide gel electrophoresis profile of the total protein content of the fractions and the partitioning of the Bcl-2 protein detected by anti-Bcl-2 antibodies, in each phase. Although most of the proteins in the sample were found in the buffer phase, Bcl-2 was recovered almost exclusively in the detergent-rich phase. The small amount of Bcl-2 (less than a few percent) observed in the buffer phase is due to a low level of detergent micelles present in this phase, as described elsewhere (4). Such fractionation of Bcl-2 is indicative of its lipophilic nature and suggests that Bcl-2 is an integral-membrane protein.

We also analyzed the predicted amino acid sequence of Bcl-2 and found one very hydrophobic stretch of 23 residues at its C terminus (Fig. 7B). The calculated hydropathy average of this sequence, by the method of Kyte and Doolittle (22), is 1.6, which is typical of a transmembrane-spanning domain. Following this lipophilic region, the Bcl-2 protein sequence shows two basic C-terminal amino acids (His and Lys) that probably permit its membrane anchorage.

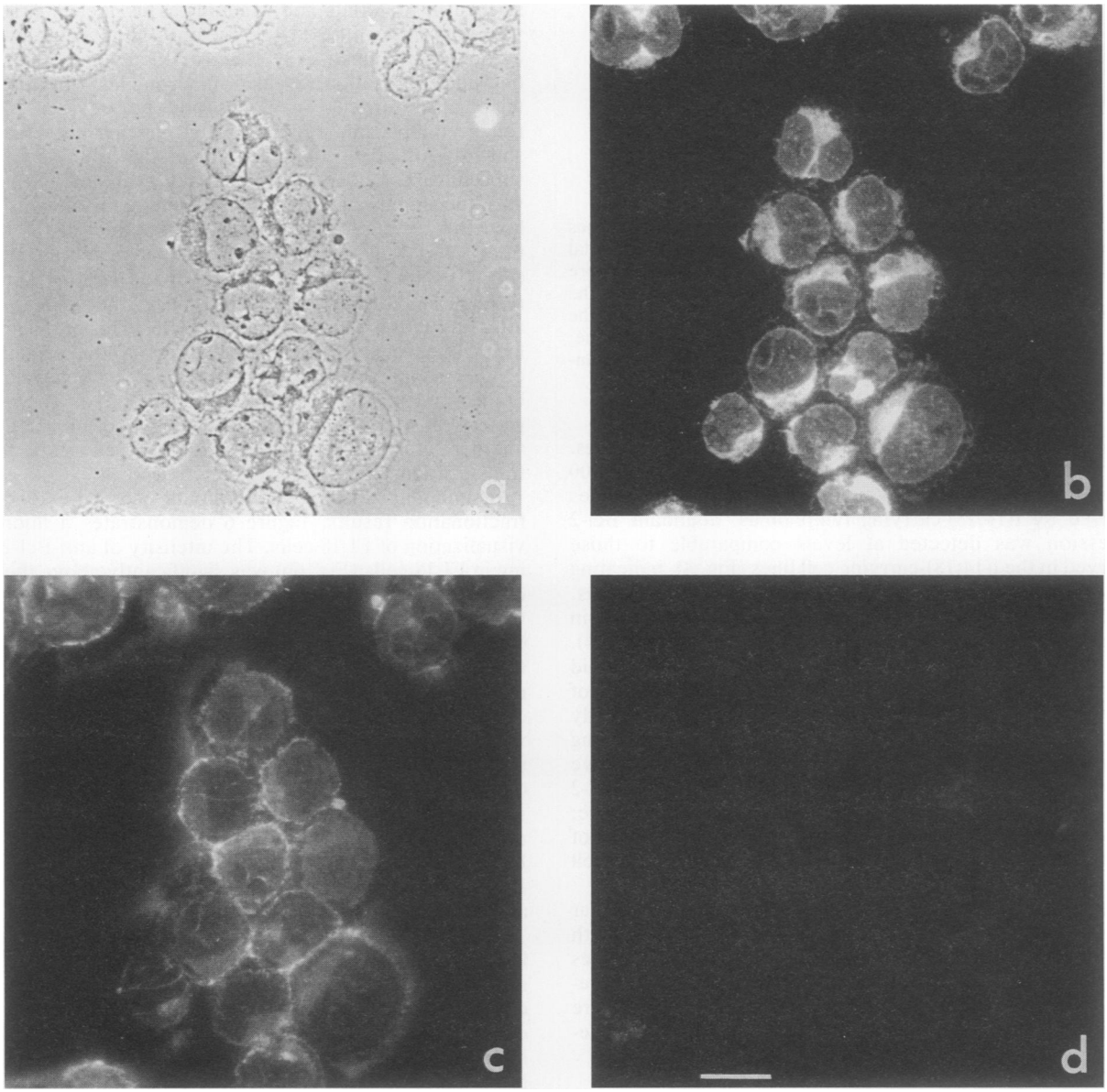

FIG. 6. Immunofluorescent localization of Bcl-2 in lymphoid cell line FL18. A double staining study was carried out to show Bcl-2 localization compared with that of a B-cell surface marker, L26. (a) Phase-contrast microscopy of the immunostained region shown in panels b and c. (b) Immunofluorescent microscopy using rabbit anti-Bcl-2 antiserum and rhodamine-conjugated goat anti-rabbit-IgG. (c) Mouse anti-L26 monoclonal antibody and fluorescein-conjugated goat anti-mouse IgG. (d) Preimmune rabbit serum and rhodamine-conjugated goat anti-rabbit IgG as a control staining of a different slide. Bar, 10 μm.

This analysis and the Triton X-114 extraction results confirm the membrane association of Bcl-2 and suggest that it is probably an integral-membrane protein that spans the lipid bilayer.

DISCUSSION

The *bcl*-2 gene has been previously described as a candidate proto-oncogene because of its involvement by t(14;18) translocations in many human lymphomas in which it is juxtaposed with the *Igh* locus. In the present work, we have identified a protein in human lymphoid cells that is the product of the *bcl*-2 gene. We have also carried out a preliminary characterization of this protein and studied its abundance in several lymphoid cell lines and tissues. Detection of the Bcl-2 protein was carried out by using rabbit antibodies that were raised against a recombinant Bcl-2 protein expressed in *E. coli*.

Identification of the Bcl-2 protein. Identification of a protein in human cells as the authentic product of the *bcl*-2 gene is based on several observations. (i) An identical, single, immunoreactive band was detected in all the positive cell lines and tissues by using a highly specific antiserum. (ii) The 24-kilodalton molecular mass of the immunoreactive protein in human lymphoid cell extracts corresponded to that predicted by translation of the *bcl*-2 cDNA sequence. (iii) After transfection with a human *bcl*-2 cDNA construct, mouse myeloma cells assumed high-level expression of an immunoreactive protein with a molecular mass identical to that of

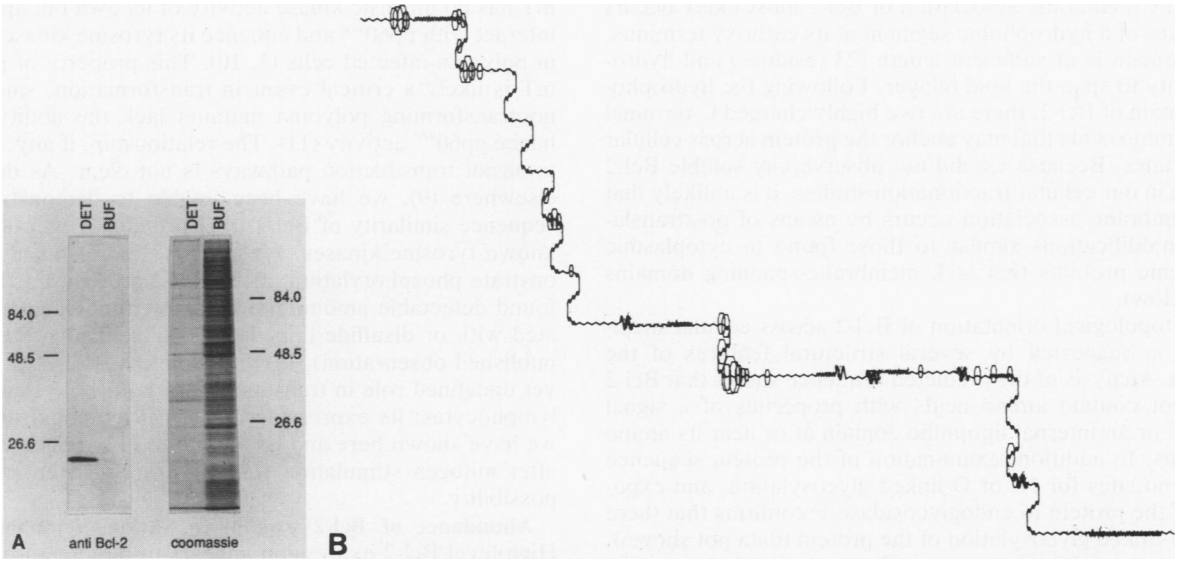

FIG. 7. (A) Triton X-114 extraction of the Bcl-2 protein from the FL18 lymphoid cell line. Protein fractions standardized to represent equal numbers of cells from the Triton extraction were fractionated in an SDS-polyacrylamide gel and subjected to Western blot analysis with anti-Bcl-2 antibodies (left). A Coomassie blue-stained gel (right) is shown for comparison of the protein content of each fraction. DET, Proteins that partitioned to the detergent-rich phase; BUF, proteins remaining in the buffer (detergent-poor) phase. Migrations of molecular weight standards (in kilodaltons) are shown. (B) Hydropathy profile of the Bcl-2 protein portrayed by squiggle plot analysis. The hydropathy characteristics of the Bcl-2 protein based on the predictions of Chou and Fasman (5) are presented. The Bcl-2 protein sequence is portrayed as a squiggly line where prolines are denoted by 180° turns. Hydrophilic and hydrophobic residues with scores greater than 1.3 are denoted by octangles and diamonds, respectively.

the human Bcl-2 protein. (iv) The presence of the protein in various lymphoid cell extracts correlated with *bcl*-2 mRNA expression as detected by Northern blot analysis of the same cells. We thus concluded that our anti-Bcl-2 serum specifically detected the product of the human *bcl*-2 gene. The protein detected by our antibodies is likely the same protein as that called Bcl-2α, recently detected by other investigators of the 697 pre-B-cell line using a different rabbit antiserum (40).

Identical migrations of the Bcl-2 protein were observed in several t(14;18)-carrying lymphoid cell lines and lymphomas, in human pre-B-cell lines without the translocation, in some normal reactive lymphoid tissues, and in mouse myeloma cells transfected with a normal human *bcl*-2 cDNA construct. These findings indicate that there are no major structural alterations or modifications that would distinguish the Bcl-2 protein in cells with the t(14;18) translocation versus those without the translocation. This conclusion is consistent with molecular data characterizing a number of t(14;18) breakpoints that occur in or flanking the *bcl*-2 gene but never in the protein-coding portions. Our results, however, cannot rule out the possibility that point mutations resulting in single amino acid substitutions, not significantly affecting gel migration of the protein, may be associated with oncogenic activation of the *bcl*-2 gene in some lymphomas. The mobility of the identified Bcl-2 polypeptide on SDS gels corresponds to 24 kilodaltons, which is very close, but not identical, to the 26-kilodalton molecular mass predicted for the protein. It is not clear whether this difference is due to posttranslational modifications or some structural features that affect gel mobility. Glycosylation is apparently not a cause for the mass differences, since we have shown that Bcl-2 is not glycosylated (unpublished observations).

Our studies of several Bcl-2-expressing cell lines and lymphoid tissues suggest that there is only one form of the Bcl-2 protein, which corresponds to a single polypeptide with an apparent molecular mass of 24 kilodaltons. We found no indications for the existence of an additional form of the protein, designated Bcl-2β, proposed by others on the basis of analysis of cDNA clones from a single cell line (38). However, if such a protein is expressed at very low levels, it might be below the threshold of our detection system, although our antibodies were demonstrated to be highly sensitive in Western blot analyses.

Localization of the Bcl-2 protein. Our results show that the Bcl-2 protein has a lipophilic nature and is intimately associated with cellular membranes. Most of the protein, as shown by both biochemical fractionation and immunofluorescence studies, localized to cellular membranes in a perinuclear cytoplasmic distribution. Presumably this perinuclear membrane association corresponds to the RER and its continuum, the nuclear envelope (26). The latter appears to contain up to 50% of the total Bcl-2 protein found in the lymphoid cells we studied, although potential contamination of the nuclear fraction by cytoplasmic organelles might have some effect on the estimated percentage. Because the cell lines used to localize Bcl-2 protein were shown to express very high levels of the protein, it is possible that the predominant RER localization simply reflects the site of its biosynthesis and processing. A minor fraction of the immunodetectable protein was also observed in the plasma membrane, but it is not clear whether this, in fact, represents the physiological site of function for Bcl-2. Further studies are necessary to determine the functionally important site for the role of Bcl-2 in normal and neoplastic lymphocyte physiology and to determine whether there is any requirement for at least a fraction of total cellular Bcl-2 to localize to the plasma membrane. A similar localization (21, 29, 36) has been described for the oncogenic proteins v-*erb*-B and polyoma middle T (mT) antigen (see below).

Bcl-2 appears to be an integral-membrane protein since it partitions to the detergent phase in a Triton X-114 extrac-

tion. The membrane association of Bcl-2 most likely occurs by means of a hydrophobic segment at its carboxy terminus. This domain is of sufficient length (23 residues) and hydrophobicity to span the lipid bilayer. Following the hydrophobic domain of Bcl-2, there are two highly charged C-terminal basic amino acids that may anchor the protein across cellular membranes. Because we did not observe any soluble Bcl-2 protein in our cellular fractionation studies, it is unlikely that the membrane association occurs by means of posttranslational modifications similar to those found in cytoplasmic oncogenic proteins that lack membrane-spanning domains (see below).

The topological orientation of Bcl-2 across cellular membranes is suggested by several structural features of the protein. Analysis of the predicted sequence shows that Bcl-2 does not contain amino acids with properties of a signal peptide or an internal lipophilic domain at or near its amino terminus. In addition, examination of the protein sequence shows no sites for N- or O-linked glycosylation, and exposure of the protein to endoglycosidase-F confirms that there is no N-linked glycosylation of the protein (data not shown). These structural features are similar to those of cytochrome b_5 (35), a microsomal protein that is involved in electron transport. Cytochrome b_5, like Bcl-2, has no signal peptide, is not glycosylated, and has a C-terminal hydrophobic domain that is inserted into the RER membrane after synthesis of the protein on free cytosolic ribosomes (44). On the basis of the structural similarities of Bcl-2 and cytochrome b_5, we suggest that the Bcl-2 protein is similarly synthesized and inserted into the membrane by means of its C-terminal hydrophobic domain and, like cytochrome b_5, is oriented into the cytoplasm and not into the extracellular space. The topological orientation and lack of glycosylation could account for the observed low level of Bcl-2 at the plasma membrane which may simply result from bulk membrane flow out of the RER (28), although we cannot rule out a specific modification that would direct some Bcl-2 to the cell surface.

Membrane associations of oncogenic proteins are not unique, since all of the characterized cytoplasmic oncogene products have been found to associate with membrane structures. The *src*, *ras*, and *abl* proteins are all cytoplasmic proteins that also localize to the inner side of the plasma membrane surfaces (32). However, they are distinguished from Bcl-2 by not having segments that span the bilayer, by being partly in a soluble state, and by undergoing posttranslational modifications that facilitate their association with membranes (31, 45, 46). Although Bcl-2 shows no sequence relatedness to any of these or other previously described oncogenic proteins, it can provisionally be placed into the category of cytoplasmic oncogenic proteins and, similar to the known members of this class, it is intimately associated with the cytoplasmic surface of cellular membranes. It has recently been shown that Bcl-2 can collaborate with *myc* to promote the immortalization of pre-B cells in culture (42).

The oncogenic proteins most similar to Bcl-2 in distribution and topology are v-*erb*-B and polyoma mT antigen (21, 29, 36). Bcl-2, v-*erb*-B, and polyoma mT antigen are predominantly localized to the RER, and a fraction of each protein also makes its way to the plasma membrane (21, 29, 36). Most of the v-*erb*-B and polyoma mT polypeptides, like Bcl-2, presumably face the cytoplasm and have membrane-spanning domains with small extracellular segments. However, v-*erb*-B is clearly a glycoprotein and is known to be a truncated form of the epidermal growth factor receptor with features of a tyrosine-specific protein kinase (41). Polyoma mT has no intrinsic kinase activity of its own but appears to interact with $pp60^{src}$ and enhance its tyrosine kinase activity in polyoma-infected cells (3, 10). This property of polyoma mT is likely a critical event in transformation, since some nontransforming polyoma mutants lack the ability to enhance $pp60^{src}$ activity (11). The relationship, if any, of Bcl-2 to signal transduction pathways is not clear. As described elsewhere (9), we have been unable to demonstrate any sequence similarity of Bcl-2 to G proteins, *ras* proteins, or known tyrosine kinases. Preliminary studies failed to demonstrate phosphorylation of the Bcl-2 protein, nor have we found detectable amounts of the protein covalently associated with or disulfide linked to other cellular proteins (unpublished observation). It is possible that Bcl-2 plays an as yet undefined role in transduction of mitogenic signals in B lymphocytes; its expression in normal lymphoid tissues as we have shown here and its induction in lymphocytes soon after mitogen stimulation (18, 30) are consistent with this possibility.

Abundance of Bcl-2 protein in various lymphoid cells. High-level Bcl-2 expression appears to be a feature of cells that have a t(14;18) chromosomal translocation. For a number of B-lymphoma cell lines that we studied, expression of the protein correlated with the presence of the translocation, and several lymphoma cell lines without t(14;18) translocation were completely Bcl-2 negative. In a preliminary examination of lymphoid tissues analyzed by the anti-Bcl-2 antiserum on Western blots, the Bcl-2 protein was easily detected in Triton X-100 total extracts of malignant follicular lymphomas, whereas relatively lower levels were observed in normal lymphoid tissues. Although there are limitations to quantitative protein determinations made on whole tissues, these results and our previous immunocytochemical studies (27) suggest that t(14;18)-carrying lymphoma cells express Bcl-2 protein at relatively high levels, compared with reactive lymphoid cells.

The levels of Bcl-2 in t(14;18)-carrying cells appear to be equal to or greater than those seen in pre-B-cell lines. Previous studies of human and mouse lymphoid cell lines have shown that *bcl*-2 mRNA is expressed at relatively high levels in early-B-lineage cells and decreases progressively with B-cell differentiation to undetectable levels in plasma cells (18, 19). Our detection of Bcl-2 protein in pre-B cells is consistent with these earlier observations and likely reflects the physiological expression of the protein at this stage of B-cell differentiation. The t(14;18) chromosomal translocation, which occurs during the earliest stages of *Igh* rearrangement, may prevent the *bcl*-2 gene from progressing through its normal differentiation-linked down-regulation. The resultant high-level, constitutive, or inappropriate expression of the Bcl-2 protein in more-mature B cells might contribute to the subsequent development of lymphoid tumors carrying this translocation. A role for simple overexpression of membrane-associated oncogenic proteins in neoplastic transformation is not unprecedented. Several studies have shown that increased expression of a number of different receptor tyrosine kinases is associated with certain human malignancies and in vitro transformation of appropriate target cells (for a review, see reference 47). Furthermore, the structural and topological similarities of Bcl-2 protein and polyoma mT antigen provide a working model for how overexpression of an integral-membrane protein that does not seem to fall into any of the known oncogene categories may contribute to the neoplastic transformation of appropriate target cells.

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