

## Btf, a Novel Death-Promoting Transcriptional Repressor That Interacts with Bcl-2-Related Proteins

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**The adenovirus E1B 19,000-molecular-weight (19K) protein is a potent inhibitor of apoptosis and cooperates with E1A to transform primary rodent cells. E1B 19K shows sequence and functional homology to the mammalian antiapoptotic gene product, Bcl-2. Like Bcl-2, the biochemical mechanism of E1B 19K function includes binding to and antagonization of cellular proapoptotic proteins such as Bax, Bak, and Nbk/Bik. In addition, there is evidence that E1B 19K can affect gene expression, but whether this contributes to its antiapoptotic function has not been determined. In an effort to further understand the functions of E1B 19K, we screened for 19K-associated proteins by the yeast two-hybrid system. A novel protein, Btf (Bcl-2-associated transcription factor), that interacts with E1B 19K as well as with the antiapoptotic family members Bcl-2 and Bcl-x<sub>L</sub> but not with the proapoptotic protein Bax was identified. *btf* is a widely expressed gene that encodes a protein with homology to the basic zipper (bZip) and Myb DNA binding domains. Btf binds DNA *in vitro* and represses transcription in reporter assays. E1B 19K, Bcl-2, and Bcl-x<sub>L</sub> sequester Btf in the cytoplasm and block its transcriptional repression activity. Expression of Btf also inhibited transformation by E1A with either E1B 19K or mutant p53, suggesting a role in either promotion of apoptosis or cell cycle arrest. Indeed, the sustained overexpression of Btf in HeLa cells induced apoptosis, which was inhibited by E1B 19K. Furthermore, the chromosomal localization of *btf* (6q22-23) maps to a region that is deleted in some cancers, consistent with a role for Btf in tumor suppression. Thus, *btf* may represent a novel tumor suppressor gene residing in a unique pathway by which the Bcl-2 family can regulate apoptosis.**

Apoptosis is a genetically controlled process of cell suicide that plays a critical role in maintaining homeostasis and preventing disease (reviewed in references 35 and 90). Disruption of apoptosis leads to impaired development, cancer, neurodegenerative and autoimmune diseases, and sustained viral infection. The regulation of apoptosis is a precarious balance between factors that promote survival and those responsible for initiating and executing cell death. One major advance toward the understanding of apoptosis regulation has been the characterization of the Bcl-2 family (90). This family consists of highly conserved proteins with opposing biological functions. Antiapoptotic Bcl-2 family members, such as Bcl-2 and Bcl-x<sub>L</sub>, inhibit apoptosis triggered by many circumstances, including tumor necrosis factor alpha (TNF- $\alpha$ ), Fas, UV radiation, chemotherapeutic drugs, and growth factor or hormone withdrawal. In contrast, proapoptotic Bcl-2 family members, such as Bax, Bak, and Nbk/Bik, induce cell death in numerous model systems. The adenovirus E1B 19,000-molecular-weight (19K) protein cooperates with E1A in transformation assays and is a viral homologue of mammalian Bcl-2. Expression of E1B 19K, or Bcl-2, inhibits E1A-induced, p53-mediated apoptosis (13, 17). Like Bcl-2, E1B 19K interacts with and antagonizes several proapoptotic family members, including Bax (26), Nbk/Bik (8, 27), and Bak (19). However, it is still unclear how these proteins effect cell survival or death and whether binding to proteins unrelated to the Bcl-2 family contributes to apoptosis or other cellular processes.

While there has been some debate about the biochemical

function of E1B 19K and other Bcl-2 family members in the regulation of apoptosis, recent studies have led to several possible mechanisms. Clearly, interactions between Bcl-2 family members play an integral part in the regulation of apoptosis and transformation. A common feature of the Bcl-2 family is the occurrence of protein-protein interactions between the anti- and proapoptotic proteins, the ratio of which controls the fate of the cell (5, 58). Structural and biochemical studies of Bcl-2 family members point to the possibility that these proteins form ion channels (1, 46, 55, 71). In addition, Bcl-2 family members bind to several unrelated proteins, including R-ras (20), Nip-1, Nip-2, and Nip-3 (8), Ced-4-like proteins (12, 28, 32, 61, 79, 98, 104), Bag-1 (80), lamin A/C (65), and p28Bap31 (57). Although the functional significances of some of these interactions are not yet known, these findings suggest that Bcl-2 regulates multiple signaling pathways that influence apoptosis.

There is growing evidence from several independent studies that Bcl-2-related proteins can trigger changes in gene expression (39, 50, 66, 74, 76, 103) which may or may not be related to their role in apoptosis regulation. Initial studies of E1B 19K mutant viruses raised the possibility that 19K functions to dampen transcriptional activation by E1A (92, 95). Subsequently, it has been shown that E1B 19K and Bcl-2 alleviate the *trans*-repressive activity of E1A and p53 (66, 76, 103). This derepression of transcription has been correlated with an up-regulation of one transcriptional target of p53, Mdm-2 (82). Since Mdm-2 inhibits p53 transcriptional activity and apoptosis (53), this model provides an alternative mechanism by which E1B 19K and Bcl-2 can regulate apoptosis. Furthermore, Bcl-2 family members may actually control several additional transcription factors, including NF- $\kappa$ B (23, 34, 72), NFAT (nuclear factor of activated T cells) (39), c-Jun (74), and the glucocor-

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toid receptor (50), indicating that Bcl-2 and its related proteins can have multiple effects on gene expression that may contribute to apoptosis.

Compared to apoptosis regulation, relatively little is known about the gene-regulatory function of the Bcl-2 family. In this paper, we describe a novel transcriptional repressor, Btf, that may contribute to the modulation of transcription by Bcl-2-related proteins. Btf was identified in a yeast two-hybrid screen against E1B 19K and subsequently shown to also interact with Bcl-2 and Bcl-x<sub>L</sub>. These Bcl-2 family members inhibit the translocation of Btf to the nucleus and abrogate its transcriptional activity. We also present evidence that sustained overexpression of Btf induces apoptosis and suppresses transformation by E1A and E1B 19K or mutant p53. Thus, the interaction with Btf provides a novel pathway by which the Bcl-2 family can regulate transcription and control apoptosis.

## MATERIALS AND METHODS

**Yeast two-hybrid system.** Procedures for using the two-hybrid system to identify E1B 19K binding proteins were described previously (26). A HeLa cDNA library was constructed in the pGAD-GH vector and screened against pGBT9-E1B 19K. Plasmids were transformed into *Saccharomyces cerevisiae* YGH1 cells, and positive clones were selected based on growth in the absence of histidine and production of  $\beta$ -galactosidase. False-positive clones were eliminated by testing for interactions with an irrelevant hydrophobic protein (Apc-2) and the empty pGBT9 vector. Missense and deletion mutants of E1B 19K tested for interaction with BP-1 were described previously (26). pGBT8-Bcl-2 (20) and pGBT9-Bax (26) were also described previously. pGBT8-Bcl-x<sub>L</sub> was kindly provided by G. Nuñez (University of Michigan, Ann Arbor, Mich.).

The full-length transcript *btf*<sub>s</sub> was identified by screening a  $\lambda$  cDNA library prepared from HeLa cells (Stratagene, La Jolla, Calif.) by using conventional techniques. The cDNA sequence of *bp-1* and the subsequent full-length *btf* were analyzed with Sequenase 2.0 (U.S. Biochemical, Cleveland, Ohio) as specified by the manufacturer and later confirmed by fluorescent terminator cycle sequencing with an automated model 377 DNA sequencer (Perkin-Elmer, Applied Biosystems, Foster City, Calif.).

**Plasmid construction.** A PCR product of *btf*<sub>s</sub> from the  $\lambda$  screen was digested with *Xma*I and *Not*I (blunt) and ligated into both pGAD-GH cut with *Xma*I-*Xho*I (blunt) and pGBT9 cut with *Xma*I-*Sal*I (blunt). To prepare pcDNA3-Myc-Btf<sub>s</sub> for mammalian expression and in vitro translation, oligonucleotides encoding a Myc epitope with flanking *Kpn*I and *Xma*I sites were annealed with a PCR-cloned fragment of *btf*<sub>s</sub> from Bluescript digested with *Xma*I and *Not*I and from pcDNA3 (Invitrogen, San Diego, Calif.) digested with *Kpn*I and *Not*I. The resulting plasmid encodes a Myc epitope at the N-terminal end of Btf<sub>s</sub>. Btf<sub>s</sub> and two deletion mutants were fused in frame with the GAL4 DNA binding domain in the pm1 vector (kindly provided by C. Abate-Shen, Center for Advanced Biotechnology and Medicine, Piscataway, N.J.) for use in transcriptional reporter assays. The full-length *btf*<sub>s</sub> construct was prepared by digesting pGBT9-Btf<sub>s</sub> with *Xma*I and *Pst*I and ligating it into the same sites in the pm1 vector. The  $\Delta$ N552 and  $\Delta$ C210 mutants were generated by digesting pGBT9-Btf<sub>s</sub> with *Eco*RI-*Pst*I and *Xma*I-*Bam*HI, respectively, and ligating it into the same restriction sites within pm1. *btf*<sub>s</sub> was also cloned into pIRES-EGFP (Clontech, San Francisco, Calif.) for cell cycle analysis of Btf<sub>s</sub>-expressing cells. The construct was prepared by digesting pcDNA3-Myc-Btf<sub>s</sub> with *Sma*I and *Not*I and ligating it to pIRES-EGFP cut with *Eco*RV and *Not*I. The pcDNA3-Bcl-2 plasmid was prepared by ligating an *Eco*RI-*Xho*I fragment from pSFFV-Bcl-2 (31) (provided by S. Korsmeyer, Washington University School of Medicine, St. Louis, Mo.) into pcDNA3. The C-terminally V5/His-tagged E1B 19K was prepared by TA cloning into pcDNA3.1/V5/His-TOPO (Invitrogen) as specified by the manufacturer.

**Cell lines.** The HeLa cells were maintained in culture in Dulbecco modified Eagle medium–10% fetal bovine serum at 37°C under 5% CO<sub>2</sub>. Stable HeLa cell lines containing Bcl-x<sub>L</sub> were prepared by electroporating 1  $\mu$ g of pcDNA3-Flag-Bcl-x<sub>L</sub> (45), provided by G. Nuñez, into HeLa cells and selecting with 1.2 mg of Geneticin per ml. Expression of Bcl-x<sub>L</sub> was verified by immunofluorescence.

**Northern blotting.** Northern blot analyses were performed with commercially available blots of multiple tissues or cancer lines (Clontech). Each lane contained 2  $\mu$ g of the indicated poly(A)<sup>+</sup> RNA. The *btf*<sub>s</sub> probe was prepared by random priming with a purified *Xma*I-*Xho*I fragment from pGAD-GH-Btf<sub>s</sub>. Human  $\beta$ -actin cDNA supplied with the blots was used as a control probe to confirm equal mRNA loading. Hybridization was performed with ExpressHyb solution (Clontech) as specified by the manufacturer. Bands were visualized by autoradiography and with a PhosphorImager (Molecular Dynamics, Sunnyvale, Calif.).

**In vitro binding assay.** Binding reactions were performed by combining [<sup>35</sup>S]methionine-labeled, in vitro-translated Myc-Btf<sub>s</sub> or Myc-Bax (26) with V5/His-E1B 19K, Bcl-2, Flag-Bcl-x<sub>L</sub>, or luciferase (Promega Corp., Madison, Wis.) prepared by using the TNT T7 reticulocyte lysate system (Promega) as specified by the manufacturer. Samples were incubated with anti-Myc monoclonal anti-

body (Oncogene Research Products, Cambridge, Mass.) in 500  $\mu$ l of NETN buffer (20 mM Tris [pH 8.0], 100 mM NaCl, 1 mM EDTA, 0.2% Nonidet P-40) for 1.5 h at 4°C followed by protein A-Sepharose for 30 min. All samples were then washed three times in NETN buffer, resuspended in 2 $\times$  Laemmli buffer (12.5 mM Tris [pH 6.8], 20% glycerol, 4% sodium dodecyl sulfate [SDS], 0.5% bromophenol blue, 0.5%  $\beta$ -mercaptoethanol), boiled for 5 min, and analyzed by SDS-polyacrylamide gel electrophoresis (PAGE). The gels were fixed in 50% methanol–10% acetic acid for 1 h, dried, and then visualized by autoradiography.

**DNA binding assay.** [<sup>35</sup>S]methionine-labeled, in vitro-translated Myc-Btf<sub>s</sub> and E1B 19K were prepared from 1  $\mu$ g of pcDNA3-Myc-Btf<sub>s</sub> and pcDNA3-E1B 19K, respectively (26), using the TNT T7 reticulocyte lysate system. The proteins were incubated with 200  $\mu$ l of native DNA-cellulose (Pharmacia Biotech, Piscataway, N.J.) in 500  $\mu$ l of NETN buffer for 2 h at 4°C. The relative levels of Btf<sub>s</sub> and E1B 19K production were determined by immunoprecipitation with anti-Myc (Oncogene Research Products, Cambridge, Mass.) or anti-E1B 19K (94) in NETN buffer followed by protein A-Sepharose and then washing in NETN. Samples were resolved by SDS-PAGE and examined by autoradiography as described above.

**Indirect immunofluorescence.** HeLa cells were electroporated with 15  $\mu$ g of pcDNA3-Myc-Btf<sub>s</sub> along with 15  $\mu$ g of either pCMV-E1B 19K (94) or pcDNA3-Bcl-2. The total amount of DNA was kept constant by using empty pcDNA3 vector. Cells grown on glass coverslips were stained 24 h posttransfection as described previously (63). Briefly, the cells were fixed with 2% paraformaldehyde in phosphate-buffered saline (PBS) and then permeabilized with 0.5% Triton X-100 in PBS. The cells were double labeled with an anti-Myc mouse monoclonal antibody along with either an anti-E1B 19K rabbit polyclonal antibody (94) or an anti-Bcl-2 hamster monoclonal antibody (PharMingen, San Diego, Calif.). Antibody complexes were visualized with tetramethylrhodamine isothiocyanate-conjugated goat anti-mouse antibody along with the fluorescein isothiocyanate-conjugated goat anti-rabbit or goat anti-hamster antibodies (Jackson ImmunoResearch Laboratories, Inc., West Grove, Pa.). Immunocytochemistry was also performed with HeLa-Bcl-x<sub>L</sub> cells electroporated with 15  $\mu$ g of pcDNA3-Myc-Btf<sub>s</sub>. These cells were stained for the Myc epitope as described above. Expression of Flag-tagged Bcl-x<sub>L</sub> was confirmed by staining separate coverslips with anti-Flag M5 monoclonal antibody (Scientific Imaging Systems, New Haven, Conn.) and rhodamine-conjugated goat anti-mouse antibody. The cells were visualized by epifluorescence with an FXA microscope (Nikon Inc., Garden City, N.Y.).

**Transcription assays.** Transcriptional reporter assays were performed as described previously (11). HeLa cells were plated onto 60-mm tissue culture dishes and grown to 50 to 75% confluency. The cells were then transfected with 2.5  $\mu$ g of a GAL4 luciferase reporter construct (kindly provided by C. Abate-Shen) along with 2.5  $\mu$ g each of a pm1 vector (Btf<sub>s</sub>, Btf<sub>s</sub>- $\Delta$ N552, or Btf<sub>s</sub>- $\Delta$ C210) and a *bcl-2* family gene (pCMV-E1B 19K, pcDNA3-Bcl-2, or pcDNA3-Flag-Bcl-x<sub>L</sub>). The DNA concentrations were kept constant by using an appropriate empty vector, either pm1 or pcDNA3. The cells were transfected with SuperFect (Qiagen, Valencia, Calif.) as specified by the manufacturer and harvested 24 h posttransfection. Expression of mutant and wild-type Btf<sub>s</sub> proteins was verified by immunofluorescence with an antibody against the GAL4 DNA binding domain (Clontech). Luciferase activity was determined with the luciferase assay system (Promega) in a scintillation counter and then normalized for protein concentrations measured by the Bradford assay. Values were graphed as a percentage of the negative control (empty pm1, empty pcDNA3, and GAL-4 luciferase).

**Transformation assay.** Transformation assays of baby rat kidney (BRK) cells were performed as described previously (96). Briefly, BRK cells prepared from 6-day-old Fisher rats were electroporated with carrier DNA along with linearized test DNA (15  $\mu$ g of pCMV-E1A [91], 15  $\mu$ g of pCMV-E1B 19K or pCMV-p53DD [75], and 45  $\mu$ g of pcDNA3-Myc-Btf<sub>s</sub>). DNA concentrations were kept constant by using appropriate empty vectors. The cells were cultured for 3 to 4 weeks at 38.5°C in Dulbecco modified Eagle medium supplemented with 5% fetal bovine serum and then stained with Giemsa. Foci were counted from four dishes per condition.

**Cell cycle analysis.** HeLa cells were electroporated with 20  $\mu$ g of pIRES-EGFP-Btf<sub>s</sub> or empty pIRES-EGFP vector in combination with 10  $\mu$ g of pCMV-E1B 19K or empty pcDNA3 vector. The cells were harvested 48 and 72 h posttransfection and fixed in 2% paraformaldehyde in PBS for 30 min at 4°C. They were washed and then stained for at least 30 min at room temperature with PBS containing 1  $\mu$ g of propidium iodide per ml, 250  $\mu$ g of RNase A per ml, and 0.1% Tween 20. The fluorescence intensities for enhanced green fluorescent protein (EGFP) and propidium iodide were analyzed by fluorescence-activated cell sorting (FACS) (EPICS PROFILE-II; Coulter, Miami, Fla.). In addition, the live transfected cells were stained with Hoechst dye to visualize the DNA and photographed with an FXA microscope.

## RESULTS

**Yeast two-hybrid assay.** To identify novel cellular proteins that interact with E1B 19K, we screened a HeLa cDNA library by using the yeast two-hybrid assay. The library was constructed in the pGAD-GH plasmid in which the cDNA sequences

were fused to the GAL4 activation domain, and the bait gene, E1B 19K, was fused to the GAL4 DNA binding domain in the pGBT9 vector (26). The plasmids were transformed into the YGH1 yeast strain and screened for GAL4-inducible phenotypes, namely, growth in the absence of histidine and production of  $\beta$ -galactosidase. Three million transformants were screened, yielding seven clones (BP-1 to BP-7) that specifically interacted with 19K and not with the pGBT9 vector alone or with an irrelevant protein, Apc-2. BP-2, BP-3, and BP-4 were previously reported as lamin A/C (65), Bax (26), and Nbk/Bik (27), respectively. Here, we describe one of the novel E1B 19K-associated proteins, BP-1. A 1.5-kb cDNA containing *bp-1* was isolated seven times during the two-hybrid screen. Since Bcl-2 family members are highly homologous and frequently interact with the same cellular proteins, we tested BP-1 for interaction with Bcl-2-related apoptosis regulators. In addition to binding E1B 19K, BP-1 interacted with other related proteins, Bcl-2 and Bcl-x<sub>L</sub>, but not with the proapoptotic family member Bax (Fig. 1A).

The interaction between anti- and proapoptotic Bcl-2 family members generally occurs via their conserved domains, designated Bcl-2 homologous regions 1, 2, 3, and 4 (BH1, BH2, BH3, and BH4) (14, 15, 26, 33, 102). BH1 to BH3 are in close proximity, forming a hydrophobic cleft that is required for dimerization (55, 70). To determine the regions of E1B 19K that are required for interaction with BP-1, we tested its ability to bind a series of E1B 19K missense and deletion mutants (Fig. 1). These mutants have been characterized previously for binding to other E1B 19K binding proteins, including Bax, Nbk/Bik, lamin A/C, and Ced-4 (26–28, 65). Thus, these experiments allow us to compare the binding requirements within the E1B 19K protein with those of other 19K-associated proteins.

The E1B 19K protein may be divided into three regions: a moderately conserved N terminus which includes BH3, a highly conserved central region containing BH1, and a poorly conserved C terminus (14, 27, 96). It is important to note that most of the deletion mutants tested ( $\Delta$ N30,  $\Delta$ N64,  $\Delta$ N87,  $\Delta$ C93,  $\Delta$ C70, 30–146, 30–93, and 64–136) failed to interact with BP-1 or with Bax, Nbk/Bik, and lamin A/C and that this may be due to abnormal protein folding or masking of the binding site(s) (Fig. 1B) (26, 27, 65). Like Bax, Nbk/Bik, and lamin A/C, binding of BP-1 was retained in  $\Delta$ C146, which contains both BH1 and BH3 (26, 27, 65). The small E1B 19K fragment 19–57, containing only BH3, was also able to bind BP-1 (Fig. 1B). This fragment is also sufficient for binding to Bax as well as to Ced-4 (28). Surprisingly, we found that another small region of E1B 19K,  $\Delta$ C36, also retained binding to BP-1 (Fig. 1B). This region did not bind to other E1B 19K-associated proteins, Bax, Nbk/Bik, lamin A/C, or Ced-4 (26–28, 65). The binding of BP-1 to the E1B 19K mutants  $\Delta$ C36 and 19–57 suggests that the region of 19K immediately adjacent to BH3 (i.e., 19–36) may be sufficient for the interaction. This would represent a unique domain that contributes to protein-protein interactions and may correspond to the E1B 19K BH4, although the homology is weak.

We further addressed the binding specifications for BP-1 by using several point mutants (pm7, pm51, pm87, and pm102) that have previously been analyzed for interaction with E1B 19K-associated proteins as well as for their ability to inhibit apoptosis (14, 26–28, 96). While pm7 and pm102 retained the ability to bind to BP-1, replacement of either phenylalanine with serine at position 51 (pm51) or glycine with alanine at position 87 (pm87) resulted in a loss of binding (Fig. 1A). The lack of binding with pm51 is consistent with a role for BH3 in the interaction between E1B 19K and BP-1. Although the loss of binding with pm87 might suggest a role for BH1, it should be

noted that the glycine residue at position 87 is absolutely conserved within the Bcl-2 family and is located in an integral region adjacent to the hydrophobic cleft which serves as the BH3 binding pocket (55, 70). Therefore, the pm87 mutant may interfere with the BH3 region and/or result in a highly misfolded protein. Indeed, pm87 is generally defective in binding and at inhibiting apoptosis. Thus, taken together, the mutational analyses indicate that the BH3 and the adjacent N-terminal sequences (possibly BH4) may play a more critical role in the interaction with BP-1. This binding profile overlaps but is distinct from that for Bax and Nbk/Bik and corresponds to a region of E1B 19K that is required for inhibition of apoptosis (26–28).

**Characterization of BP-1.** To determine the size and distribution of *bp-1*, we performed a Northern blot analysis with poly(A)<sup>+</sup> RNA prepared from various tissues. Two transcripts which appeared to be ubiquitously expressed were detected at 5 and 3 kb, although only low levels were detected in the liver (Fig. 2A). The larger transcript appeared to be expressed more abundantly than the shorter form. Since the largest fragment of *bp-1* obtained in the two-hybrid screen was only 1.5 kb, we sought to recover full-length cDNAs corresponding to *bp-1*. By using conventional library screening techniques along with database searches, we were able to identify both full-length transcripts, which were named *btf* (for Bcl-2-associated transcription factor). The 3-kb transcript, *btf<sub>S</sub>*, was isolated by screening a HeLa  $\lambda$ -cDNA library. The 3' end of *btf<sub>S</sub>* was identical to *bp-1* except that 147 bp were missing within the predicted coding sequence. While the 5-kb transcript, *btf<sub>L</sub>*, could not be recovered from the  $\lambda$  screen, it was identified as a full-length expressed sequence tag (EST) within GenBank (accession no. D79986) that was isolated from the human KG-1 cell line. Unlike *btf<sub>S</sub>*, *btf<sub>L</sub>* did contain the 147-bp region present in *bp-1*. Based on comparisons of sequences from GenBank with *btf<sub>S</sub>*, we found that the remainder of the coding sequences between *btf<sub>S</sub>* and *btf<sub>L</sub>* were the same and that the large size difference between the transcripts resulted from different 3' untranslated regions.

The EST encoding Btf<sub>L</sub> was used to identify the subchromosomal location of *btf*. However, it remains to be determined whether *btf<sub>L</sub>* and *btf<sub>S</sub>* are formed from separate genes or are generated by alternative splicing of the same gene. Using the UniGene collection accessed through the National Center for Biotechnology Information, we found a 137-bp PCR fragment within the 3' untranslated region of the EST (dbSTS entry G20483) that mapped to chromosome 6 between markers D6S292 and D6S1699. These markers correspond to 6q22–23, a locus with a high frequency of deletions in tumors, particularly lymphomas and leukemias (47). Thus, the locus of *btf* correlates with a chromosomal region that may contain a tumor suppressor gene. To test whether *btf* is actually deleted in tumors, we performed a Northern blot analysis with mRNA prepared from several human cancer cell lines. While both transcripts of *btf* were observed in most cell lines tested, they appeared to be less abundant than in normal cells, and *btf* was not detected in Raji cells, which were derived from Burkitt's lymphoma (Fig. 2B). Indeed, Raji was the one cell line tested with known 6q deletions (62). The Northern blot data was therefore consistent with the chromosomal mapping of the gene. It will certainly be of interest to determine whether the loss of *btf* in Raji cells and possibly in other tumor cell lines actually contributes to tumor formation.

The primary amino acid sequences encoded by *btf<sub>L</sub>* and *btf<sub>S</sub>* are compared in Fig. 3. Btf<sub>L</sub> is 918 amino acids long and has a predicted molecular mass of 106 kDa, whereas Btf<sub>S</sub> is missing 49 amino acids near the carboxyl terminus (amino acids 797 to



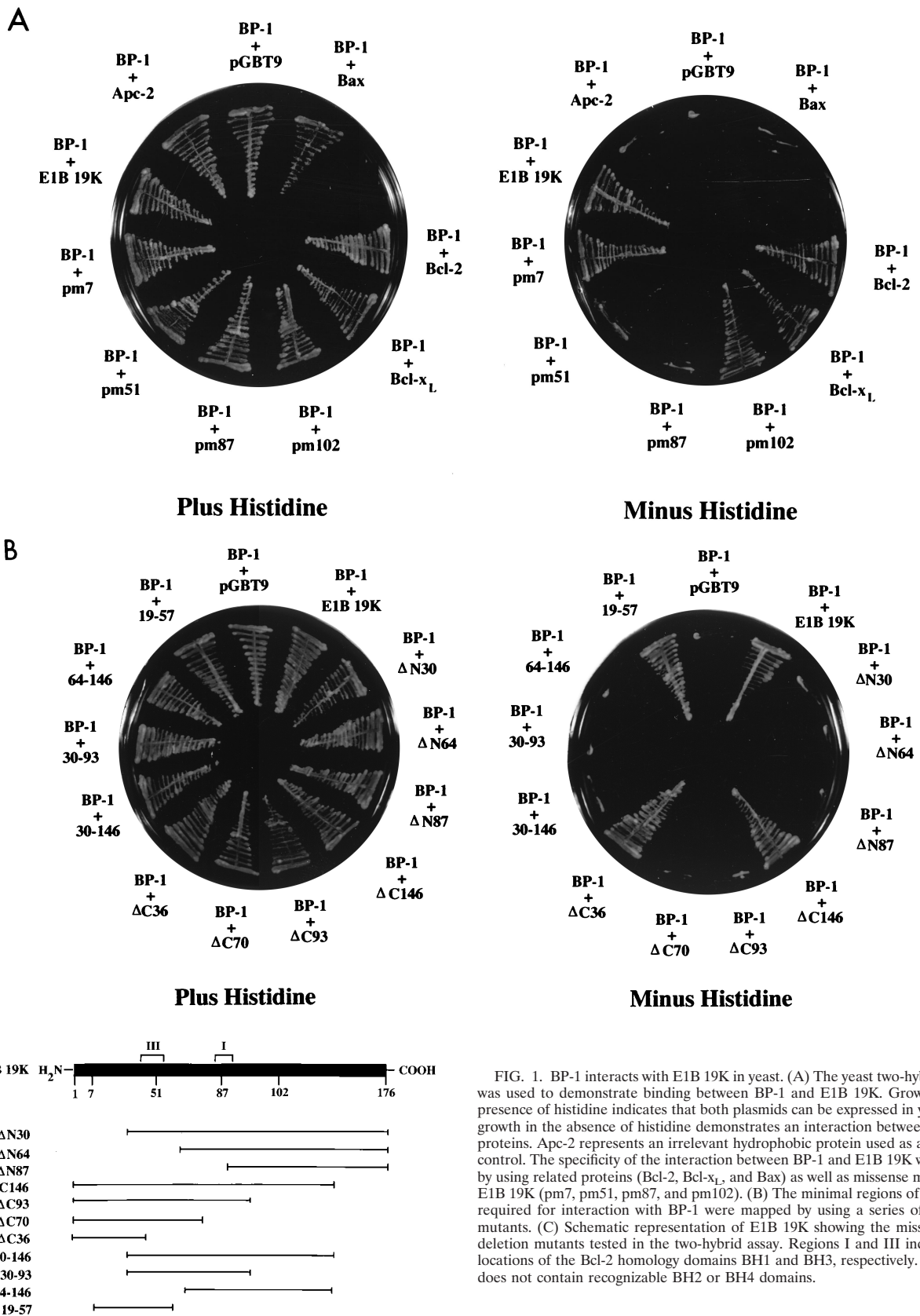


FIG. 1. BP-1 interacts with E1B 19K in yeast. (A) The yeast two-hybrid assay was used to demonstrate binding between BP-1 and E1B 19K. Growth in the presence of histidine indicates that both plasmids can be expressed in yeast, and growth in the absence of histidine demonstrates an interaction between the two proteins. Apc-2 represents an irrelevant hydrophobic protein used as a negative control. The specificity of the interaction between BP-1 and E1B 19K was tested by using related proteins (Bcl-2, Bcl-x<sub>1</sub>, and Bax) as well as missense mutants of E1B 19K (pm7, pm51, pm87, and pm102). (B) The minimal regions of E1B 19K required for interaction with BP-1 were mapped by using a series of deletion mutants. (C) Schematic representation of E1B 19K showing the missense and deletion mutants tested in the two-hybrid assay. Regions I and III indicate the locations of the Bcl-2 homology domains BH1 and BH3, respectively. E1B 19K does not contain recognizable BH2 or BH4 domains.

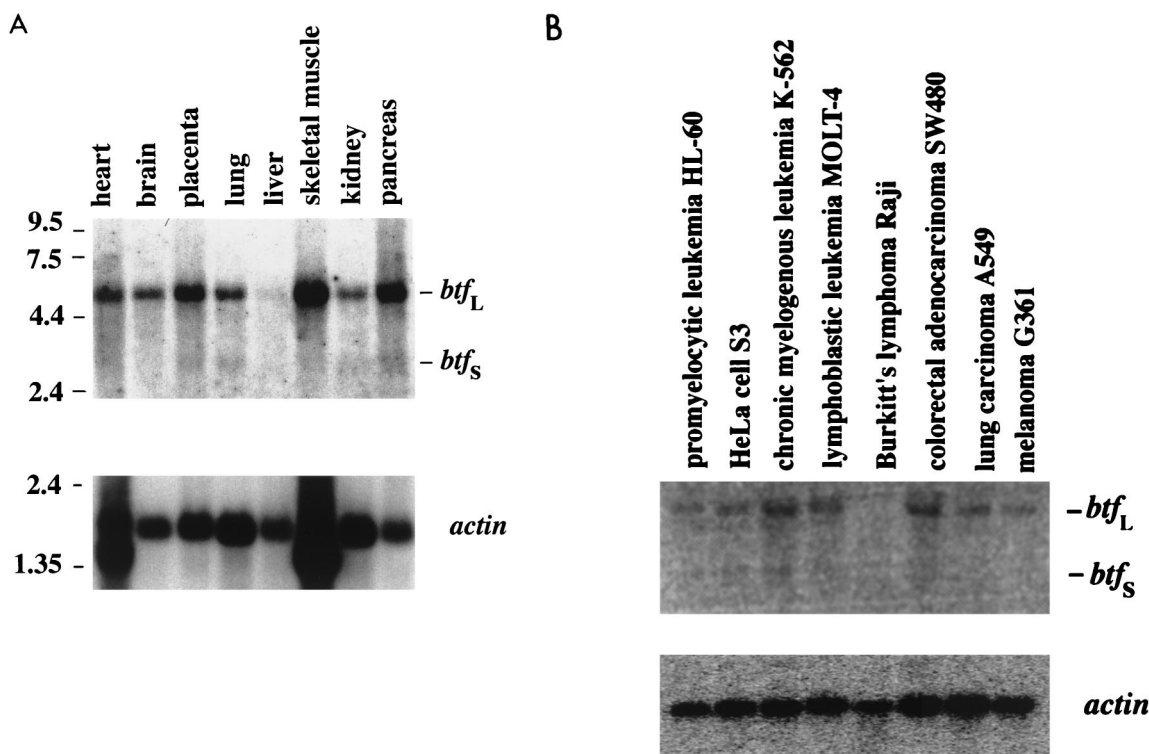


FIG. 2. Northern blotting was performed to determine *btfl* expression in various tissues (A) and cancer cell lines (B). Two transcripts were observed, at 5 kb (*btfl*) and 3 kb (*btfs*). These transcripts appeared to be ubiquitously expressed, except that *btfl* was not detected in Raji cells derived from Burkitt's lymphoma.  $\beta$ -Actin expression was examined to assess the quality of the RNA and to control for loading efficiency.

846 of Btf<sub>L</sub>) and has a predicted size of 101 kDa (Fig. 3). The original *bp-1* clone encodes amino acids 384 to 918 of Btf<sub>L</sub> (Btf  $\Delta$ N384), which is slightly more than half of the full-length protein (Fig. 3B). In general, the 49-amino-acid region specific to Btf<sub>L</sub> and  $\Delta$ N384 contains highly charged residues (48%), but the significance of this region is not known. Both Btf<sub>S</sub> and Btf<sub>L</sub> were able to bind E1B 19K in the yeast two-hybrid assay (data not shown). While the overall protein sequence of Btf is not significantly homologous to those of other known proteins, searches for conserved motifs in the PROSITE database revealed two nuclear localization signals (Fig. 3A) as well as putative DNA binding domains (Fig. 3B). There was 88% homology to the basic zipper (bZIP) DNA binding domain between amino acids 110 and 126 and 80% homology to the Myb DNA binding domain within amino acids 522 and 531 of Btf (Fig. 3B).

**Btf<sub>S</sub> is a nuclear DNA binding protein that is sequestered by the Bcl-2 family.** Since *btfs*, but not *btfl*, was isolated from the HeLa library, we concentrated our functional assays on the shorter variant. Btf<sub>S</sub> was cloned into the pcDNA3 vector with a Myc epitope for in vitro translation and mammalian expression. The protein sequence of Btf<sub>S</sub> suggested that it may bind to DNA. To test this hypothesis, [<sup>35</sup>S]methionine-labeled, in vitro-translated Myc-Btf<sub>S</sub> was incubated with native DNA-cellulose. DNA binding was detected with Btf<sub>S</sub> but not with E1B 19K, which was used as a negative control (Fig. 4). The strength of the interaction was comparable to that of a known transcription factor, Msx-1 (10), and was not competed by RNA (data not shown). In vitro-translated E1B 19K (Fig. 4) and Bcl-2 (data not shown) did not inhibit the ability of Btf<sub>S</sub> to bind to DNA, suggesting that the binding sites for DNA and for E1B 19K and Bcl-2 are in distinct domains within Btf<sub>S</sub>.

Indeed, comparison of the putative DNA binding domains and the BP-1 (Btf  $\Delta$ N384) protein which is sufficient for binding to the Bcl-2 family members is consistent with there being two separate domains.

To confirm the association between Btf<sub>S</sub> and Bcl-2 family members, we performed an in vitro binding assay. [<sup>35</sup>S]methionine-labeled, in vitro-translated Myc-Btf<sub>S</sub> or Myc-Bax, used as a positive control, was combined with E1B 19K, Bcl-2, and Bcl-x<sub>L</sub>. Immunoprecipitation with an antibody against Myc revealed that Btf<sub>S</sub> bound to all three of the antiapoptotic Bcl-2 family members but not to luciferase, used a negative control (Fig. 5). However, these associations were weaker than those with Bax (Fig. 5). These results appear to support the data from the yeast two-hybrid assay.

To examine the biological role of Btf<sub>S</sub> in vivo, we attempted to express the tagged protein in HeLa cells. While we were unable to obtain stable expression of Btf<sub>S</sub>, even with an inducible promoter, we found that transient transfection did produce low levels of Btf<sub>S</sub>. Although the levels of Btf<sub>S</sub> were not high enough to be detected by Western blot analysis of whole-cell extracts (data not shown), which would allow us to examine whether Btf<sub>S</sub> could coimmunoprecipitate with Bcl-2 family members, we were able to visualize Btf<sub>S</sub> by immunofluorescence. Its expression was confined to the nucleus, and it was present in roughly 4% of the cells (Fig. 6A). This was consistent with the DNA binding activity but not with the established localization of Bcl-2-like proteins, which are generally found associated with membrane structures, particularly the mitochondria, endoplasmic reticulum, and nuclear envelope (22, 31, 54). Unlike other Bcl-2 family members, E1B 19K is not normally present in the mitochondria but, rather, is localized predominantly to the nuclear envelope (93). However, since the Bcl-2 family can act by

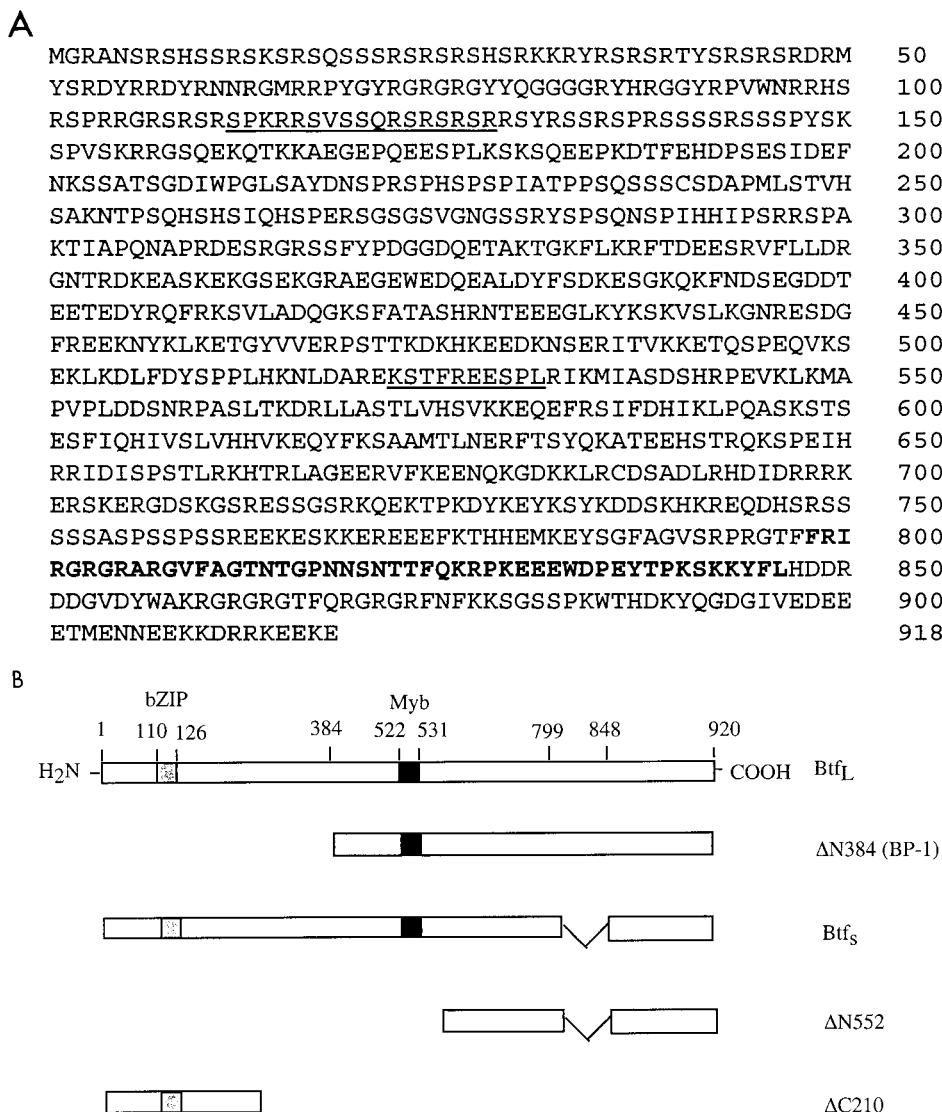


FIG. 3. (A) Amino acid sequence of Btf. Btf<sub>L</sub>, identified in the GenBank database (accession no. D79986), is predicted to encode a 918-amino-acid protein, shown here. Btf<sub>S</sub>, which was obtained in the λ screen, contains the complete Btf<sub>L</sub> sequence except that it is missing 49 amino acids between residues 797 and 846 (bold) present in Btf<sub>L</sub> and BP-1. The underlined regions represent the positions of nuclear localization sequences. (B) A schematic representation of the Btf variants and deletion mutants used to characterize Btf function. The shaded and solid boxes represent the locations of putative bZIP and Myb-DNA binding domains, respectively. BP-1 (ΔN384), identified in the yeast two-hybrid screen for E1B 19K binding proteins, contains residues 384 to 918 of Btf<sub>L</sub>. Deletion mutants of Btf<sub>S</sub>, ΔN552, and ΔC210 were used in transcriptional reporter assays to determine the regions of Btf<sub>S</sub> that contribute to transcriptional repression.

sequestering proteins (25, 28, 63, 88), we checked whether E1B 19K, Bcl-2, and Bcl-x<sub>L</sub> could alter the subcellular localization of Btf<sub>S</sub>. First, E1B 19K and Bcl-2 were cotransfected with Myc-Btf<sub>S</sub>. The cells were fixed 24 h posttransfection and then double stained for the Myc epitope present on Btf<sub>S</sub> (tetramethylrhodamine isothiocyanate) and either E1B 19K or Bcl-2 (fluorescein isothiocyanate). While the percentage of Btf<sub>S</sub>-positive cells was similar to that when it was transfected by itself, the subcellular localization was altered. Btf<sub>S</sub> colocalized with E1B 19K and Bcl-2 within the cytoplasm and nuclear periphery in almost all coexpressing cells (Fig. 6A). Subsequent experiments were performed to determine if Bcl-x<sub>L</sub> could also sequester Btf<sub>S</sub>, since these proteins also bound in the yeast two-hybrid assay and in vitro. We could not costain for Btf<sub>S</sub> and Bcl-x<sub>L</sub> with the available antibodies, so we developed a stable HeLa cell line expressing Bcl-x<sub>L</sub> and stained for Btf<sub>S</sub> with the antibody against the Myc epitope. While control HeLa

cells contained 100% nuclear Btf<sub>S</sub> expression, only 7% of the Btf<sub>S</sub>-positive cells in the HeLa-Bcl-x<sub>L</sub> cell line displayed nuclear Btf<sub>S</sub> staining and almost all of the Btf<sub>S</sub> staining was in the cytoplasm in a pattern similar to Bcl-x<sub>L</sub> (Fig. 6B). The few cells expressing nuclear Btf<sub>S</sub> may be accounted for by the variable levels of Bcl-x<sub>L</sub> observed in this cell line and/or by an incomplete ability of Bcl-x<sub>L</sub> to sequester Btf<sub>S</sub>. Nevertheless, this is in clear contrast to the parental HeLa cells, where the expression of Btf<sub>S</sub> was completely nuclear. These results suggest that E1B 19K, Bcl-2, and Bcl-x<sub>L</sub> can alter the localization, and therefore perhaps the function, of Btf<sub>S</sub>.

**Btf<sub>S</sub> represses transcription which is inhibited by the Bcl-2 family.** Since Btf<sub>S</sub> was shown to bind DNA, we checked whether it could modulate transcription. We first performed a one-hybrid assay in yeast by generating a fusion protein of Btf<sub>S</sub> with the GAL4 DNA binding domain in the pGBT9 vector. If Btf<sub>S</sub> contained a *trans*-activation domain, one would expect that the

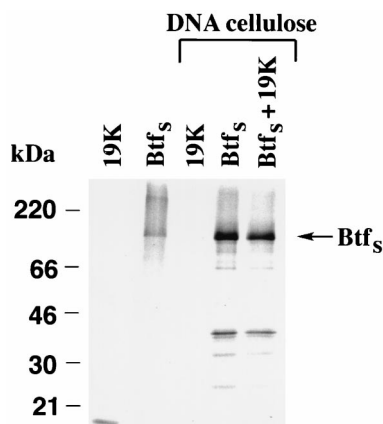


FIG. 4. Btf<sub>S</sub> binds to DNA-cellulose in vitro. [<sup>35</sup>S]methionine-labeled, in vitro-translated Myc-Btf<sub>S</sub> and E1B 19K were prepared by using the TNT T7 reticulocyte lysate system and incubated with native DNA-cellulose in NETN buffer for 2 h. Samples were washed five times in NETN, and the proteins were resolved by SDS-PAGE (17% polyacrylamide). Immunoprecipitated proteins (lanes 1 and 2) were analyzed to confirm the presence of the in vitro-translated products.

adjacent activation and DNA binding domains would lead to GAL4-inducible phenotypes in yeast. Growth was not detected in the absence of histidine, suggesting that Btf<sub>S</sub> does not contain a transcriptional activation domain (data not shown). However, it remains possible that Btf<sub>S</sub> requires mammalian cofactors to activate transcription or that Btf<sub>S</sub> represses, rather than activates, transcription.

To address these issues, Btf<sub>S</sub> was cloned into a mammalian expression vector (pm1) containing the GAL4 DNA binding domain. Transcriptional activity was monitored with a reporter construct containing GAL4 promoter sites. Transient expression of pm1-Btf<sub>S</sub> with the luciferase reporter led to nearly a 10-fold decrease in transcription compared to that for an empty pm1 vector control (Fig. 7). This effect is comparable to the *trans*-repressive activity of other proteins, such as p53 and Msx-1 (10, 66). Two deletion mutants of Btf<sub>S</sub>, ΔN522 (amino acids 522 to 918 without residues 797 to 846) and ΔC210 (amino acids 1 to 210), were also cloned into pm1 to determine the general regions that may contribute to transcriptional repression (Fig. 3B). Both deletion mutants of Btf<sub>S</sub> were detectably expressed in HeLa cells to levels comparable to wild-type

Btf<sub>S</sub> (data not shown). While Btf<sub>S</sub> ΔN522 was not able to repress transcription, the ΔC210 mutant was sufficient for repression and nearly as potent as full-length Btf<sub>S</sub> (Fig. 7). Interestingly, this fragment, which contains the bZIP homology segment, is also rich in serine and glycine residues, a feature that is present in other transcriptional repressors (11, 41, 59, 101).

Since the Bcl-2-like proteins were capable of sequestering Btf<sub>S</sub> in the cytoplasm, we hypothesized that they may also block the ability of Btf<sub>S</sub> to repress transcription. To test this possibility, we cotransfected E1B 19K, Bcl-2, and Bcl-x<sub>L</sub> with pm1-Btf<sub>S</sub> and the luciferase reporter construct. In the control samples, none of the members of the Bcl-2 family of proteins had a significant effect on transcription of the luciferase reporter (Fig. 7). However, transfection of any of the three Bcl-2 family members (E1B 19K, Bcl-2, and Bcl-x<sub>L</sub>), but not the empty pcDNA3 vector, abrogated Btf<sub>S</sub>-mediated transcriptional repression. These data suggest that E1B 19K, Bcl-2, and Bcl-x<sub>L</sub> inhibit Btf<sub>S</sub> *trans*-repression, probably by binding to and sequestering Btf<sub>S</sub> in the cytoplasm.

**Sustained expression of Btf<sub>S</sub> inhibits transformation.** Transfection of the gene for adenovirus E1A along with the gene for E1B 19K or *bcl-2* in primary BRK cells induces transformation as a consequence of dual proliferative and antiapoptotic signaling (17, 64). Expression of E1B 19K/Bcl-2 binding proteins, such as Bax and Nbk/Bik, antagonizes the antiapoptotic signal and causes a reduction in focus formation (26, 27). To determine if Btf<sub>S</sub> could also antagonize the ability of E1B 19K to transform primary cells, we transfected E1A and E1B 19K, with and without Myc-Btf<sub>S</sub>, into primary BRK cells. Btf<sub>S</sub> caused a 64% reduction in focus formation mediated by E1A and E1B 19K (Fig. 8A). This suggests that interaction between Btf<sub>S</sub> and E1B 19K inhibits 19K function in vivo and/or that Btf<sub>S</sub> is capable of suppressing transformation independently of 19K. The reduction in focus formation by Btf<sub>S</sub> and E1A compared to E1A alone suggested that the latter scenario is possible (Fig. 8A). However, we tested whether Btf<sub>S</sub> could inhibit another transforming signal, E1A with p53DD. p53DD is a p53 deletion mutant that contains the C-terminal oligomerization domain and functions in a dominant negative fashion to inhibit p53-mediated apoptosis and growth arrest (75). Since p53DD blocks both of these processes, it produces a very potent transforming signal (67). Here we show that expression of Btf<sub>S</sub> was capable of repressing focus formation by E1A and p53DD by

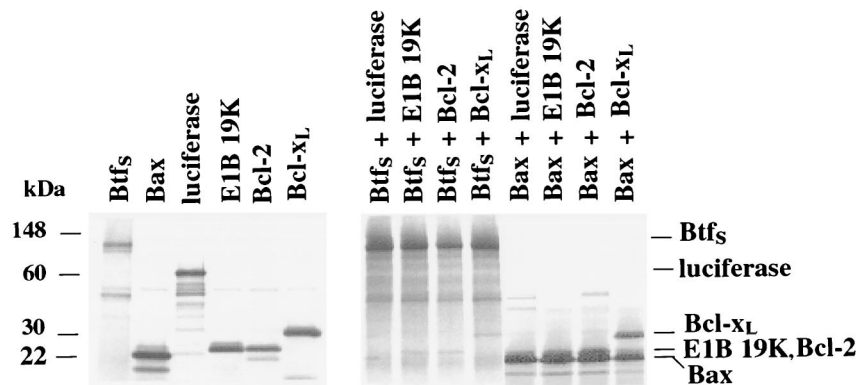


FIG. 5. In vitro interactions between Btf<sub>S</sub> and antiapoptotic Bcl-2 family members. [<sup>35</sup>S]methionine-labeled, in vitro-translated Myc-Btf<sub>S</sub> or Myc-Bax was combined with V5/His-E1B 19K, Bcl-2, Flag-Bcl-x<sub>L</sub>, or luciferase prepared by using the TNT T7 reticulocyte lysate system. The proteins were immunoprecipitated with anti-Myc monoclonal antibody in NETN buffer for 1.5 h followed by protein A-Sepharose for 0.5 h. Samples were washed in NETN buffer, resolved by SDS-PAGE (14% polyacrylamide), and visualized by autoradiography. In addition, 1 μl of each of translation reaction mixture was analyzed to verify that equal amounts of proteins were used in the binding assay.



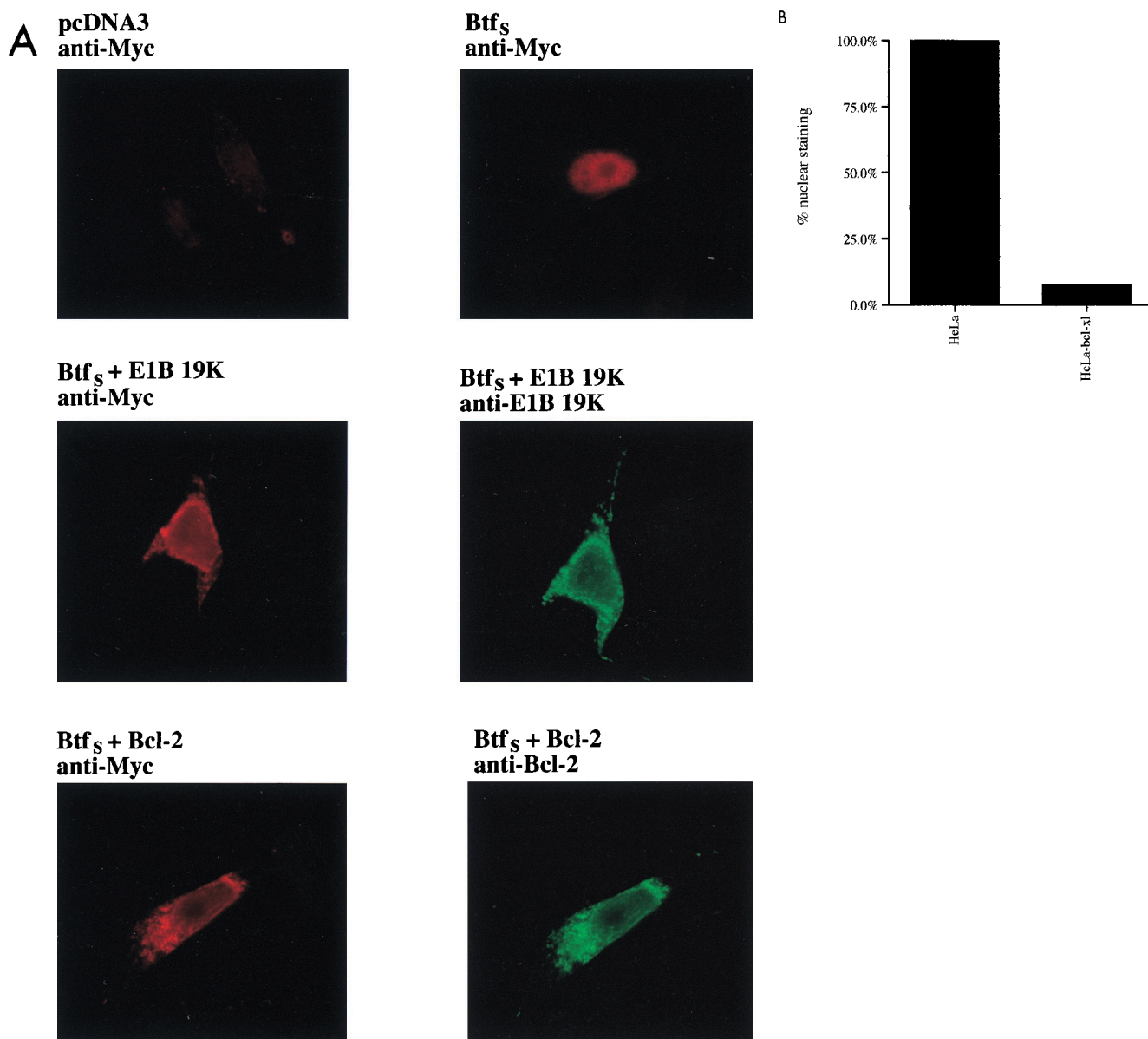


FIG. 6. Nuclear subcellular localization of Btf<sub>S</sub> is altered by E1B 19K, Bcl-2, and Btf<sub>L</sub>. (A) HeLa cells were transfected with expression plasmid pcDNA3-Myc-Btf<sub>S</sub> alone or with pCMV E1B 19K or pcDNA3-Bcl-2 as indicated. The cells were fixed 24 h posttransfection and double stained against Myc and E1B 19K or Bcl-2. Magnification,  $\times 1,000$ . (B) HeLa cells expressing Bcl-x<sub>L</sub> were transfected with pcDNA3-Myc-Btf<sub>S</sub> and stained against Myc 24 h posttransfection. Values represent the percentages of nuclear Btf<sub>S</sub> expression (Btf<sub>S</sub>/total Btf<sub>S</sub>) in these cells.

about 60%, suggesting that *btf<sub>S</sub>* may act as a general suppressor of transformation (Fig. 8B).

**Btf<sub>S</sub> functions to induce apoptosis.** To further characterize the transformation-suppressing activity of Btf<sub>S</sub>, we tested the effect of its expression on apoptosis and cell cycle progression. Attempts to generate stable BRK or HeLa cell lines expressing Btf<sub>S</sub> failed, indicating that sustained Btf<sub>S</sub> expression is incompatible with either cell proliferation or viability. To monitor Btf<sub>S</sub> expression in transient-transfection assays, the cDNA was cloned into the pIRES-EGFP vector, which provides coexpression of Btf<sub>S</sub> and the EGFP marker. HeLa cells were transiently transfected and then harvested 48 and 72 h posttransfection. The cell cycle kinetics were analyzed by FACS analysis following propidium iodide staining. Since EGFP staining with Btf<sub>S</sub> was not detectable until 48 h posttransfection, we were unable

to characterize the cell cycle characteristics at the earlier time points. The levels of EGFP expression and the cell cycle kinetics at 48 and 72 h are shown in Table 1. At 48 h posttransfection, there were only 19.5% EGFP-positive cells in the presence of Btf<sub>S</sub> whereas there were 61.2% positive cells in the control pIRES-EGFP empty vector. The FACS analysis demonstrated that Btf<sub>S</sub> led to an increase in the percentage of sub-G<sub>0/1</sub> cells from 3.4 to 15.8%, indicating an increase in cell death. No other obvious changes in cell cycle parameters were observed at this time point. To test whether E1B 19K could inhibit this cell death, we cotransfected pIRES-EGFP-Btf<sub>S</sub> with pCMV-E1B 19K. E1B 19K inhibited Btf<sub>S</sub>-mediated cell death at 48 h as indicated by an increase in the percentage of EGFP-expressing cells from 19.5 to 34.3% and a decrease in the percentage of sub-G<sub>0/1</sub> cells from 15.8 to 10.1% (Table 1).



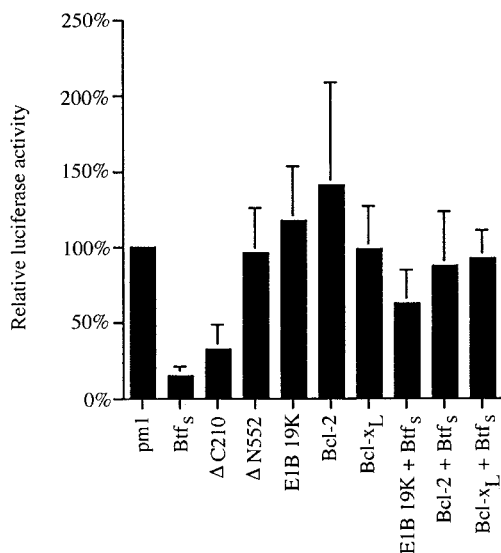


FIG. 7. Reporter assay demonstrating that Btf<sub>s</sub> is a transcription repressor and is inhibited by Bcl-2-like proteins. HeLa cells were transfected with 2.5 μg of the reporter construct (luciferase construct containing GAL4 DNA binding sites within its promoter), 2.5 μg of GAL4 DNA binding domain fusion genes (pm1-Btf<sub>s</sub>, pm1-ΔN552, and pm1-ΔC210 or empty pm1 vector control), and 2.5 μg of *bcl-2* family gene (pCMV-E1B 19K, pcDNA3-Bcl-2, pcDNA3-Flag-Bcl-x<sub>L</sub>, or empty pcDNA3 vector control). The cells were harvested 24 hours posttransfection, and the luciferase activity was measured in a scintillation counter with the luciferase substrate luciferin. Values were normalized for protein concentrations measured by the Bradford assay and graphed as a percentage of the result for the negative control (empty pm1 vector). The experiment was performed six times, and the high and low values for each sample were dropped. Bars indicate standard deviations ( $n = 4$ ).

Thus, E1B 19K expression abrogated cell death induced by Btf<sub>s</sub>. At 72 h posttransfection, the degree of cell death triggered by Btf<sub>s</sub> increased and could no longer be inhibited by E1B 19K. In the presence of Btf<sub>s</sub>, there were only 13.2% EGFP-positive cells, 29.1% of which were represented in the sub-G<sub>0/1</sub> peak (Fig. 9A). The 72-h time point also revealed a

decrease in the G<sub>2</sub>/M peak in the presence of Btf<sub>s</sub> (16.7%) compared to the control vector (40.6%). The change in the G<sub>2</sub>/M peak as a result of Btf<sub>s</sub> may be an indication that cells are exiting from these phases of the cell cycle to go into apoptosis.

Interestingly, we also observed a decrease in the G<sub>2</sub>/M peak with E1B 19K in the absence of Btf<sub>s</sub>. At 72 h posttransfection, there was a change in the number of cells in G<sub>2</sub>/M from 40.6% with the control vector to 14.8% in E1B 19K-transfected cells. Changes in cell cycle progression due to the antiapoptotic Bcl-2 family members have been observed previously; i.e., they generally produce a decrease in cell cycle progression (6, 33, 39, 83, 92). While others have shown that Bcl-2 causes an increase in the percentage of G<sub>0/1</sub> cells (33, 44, 83), we show here that E1B 19K causes a decrease in the G<sub>2</sub>/M peak. Thus, these results may reflect a difference in the mechanism of cell cycle regulation by E1B 19K and that by other, related proteins.

Cell death has been classified as either necrosis or apoptosis, where necrosis is considered a passive process of cell death associated with trauma and apoptosis is a genetically programmed active response leading to cell suicide (100). Unlike necrosis, apoptosis involves morphological changes such as chromatin condensation, DNA fragmentation, and cytoplasmic blebbing. To test whether the increase in the percentage of sub-G<sub>0/1</sub> cells as a result of Btf<sub>s</sub> was due to apoptosis, we examined the nuclei of EGFP-positive cells by staining with Hoechst dye 72 h posttransfection. Chromatin condensation observed by this approach would be one indication of apoptosis. HeLa cells transfected with the empty pIRES-EGFP vector showed EGFP-stained cells with normal nuclei. However, in cells transfected with pIRES-EGFP-Btf<sub>s</sub>, the EGFP-positive cells had condensed chromatin staining, indicating that they were dying by apoptosis (Fig. 9B). Taken together, these results suggest that Btf<sub>s</sub> can function to promote apoptosis, which may account for its ability to suppress transformation. E1B 19K can inhibit Btf<sub>s</sub>-induced apoptosis, albeit incompletely in some assays. Nonetheless, this suggests that the Bcl-2 family can affect apoptosis through modulation of transcription.

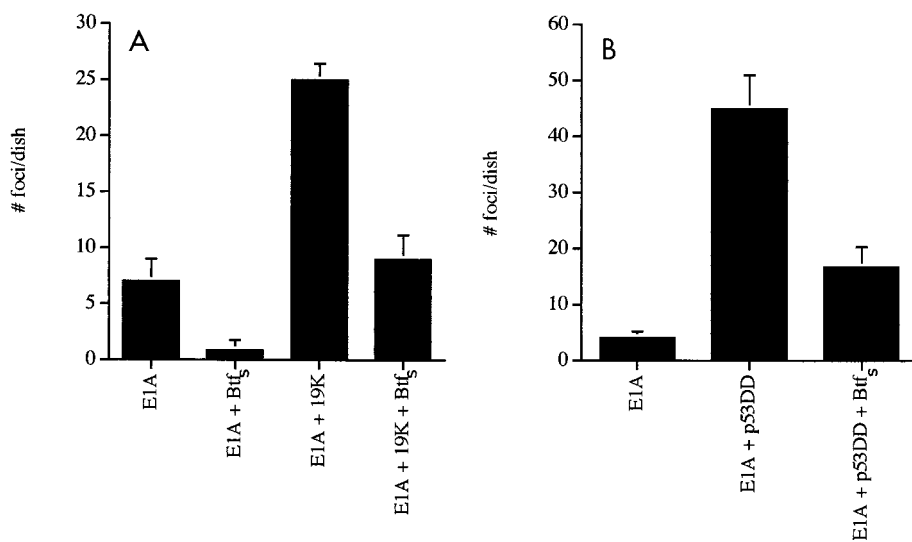


FIG. 8. Btf<sub>s</sub> inhibits transformation by E1A and E1B 19K (A) or p53DD (B). Primary BRK cells were transfected with carrier DNA along with a linearized test DNA (15 μg of pCMV-E1A, 15 μg of pCMV-E1B 19K or pCMV-p53DD, and 45 μg of pcDNA3-Myc-Btf<sub>s</sub>). DNA concentrations were kept constant by using appropriate empty vectors. The cells were cultured for 3 to 4 weeks and then stained with Giemsa. Foci were counted from four dishes per condition. Bars indicate standard deviations ( $n = 4$ ).

TABLE 1. Overexpression of Btf<sub>S</sub> induces cell death<sup>a</sup>

Vectors	48 h posttransfection					72 h posttransfection				
	% EGFP positive	% Sub-G <sub>0/1</sub>	% G <sub>0/1</sub>	% S	% G <sub>2/M</sub>	% EGFP positive	% Sub-G <sub>0/1</sub>	% G <sub>0/1</sub>	% S	% G <sub>2/M</sub>
pIRES-EGFP + pcDNA3	61.2	3.4	42.3	20.0	33.7	45.7	5.6	32.5	20.9	40.6
pIRES-EGFP + pCMV-E1B 19K	72.0	1.5	54.2	21.0	23.6	59.0	8.8	55.4	20.8	14.8
pIRES-EGFP-Btf <sub>S</sub> + pcDNA3	19.5	15.8	36.7	21.7	25.6	13.2	29.1	33.2	20.6	16.7
pIRES-EGFP-Btf <sub>S</sub> + pCMV-E1B 19K	34.3	10.1	41.8	17.8	30.0	15.4	36.9	36.0	13.3	13.6

<sup>a</sup> HeLa cells were electroporated with 20  $\mu$ g of pIRES-EGFP-Btf<sub>S</sub> or empty vector in combination with 10  $\mu$ g of pCMV-E1B 19K or empty pcDNA3 vector. The cells were harvested 48 and 72 h posttransfection, fixed in 2% paraformaldehyde, and stained with PBS containing 1  $\mu$ g of propidium iodide per ml, 250  $\mu$ g of RNase A per ml, and 0.1% Tween 20. The cells were incubated for at least 30 min at room temperature and then analyzed by FACS. The results show the percentage of EGFP-positive cells and the cell cycle kinetics for the EGFP-positive population.

## DISCUSSION

The Bcl-2 family regulates apoptosis through multiple mechanisms. For example, these proteins may function at the mitochondria by forming channels that regulate mitochondrial membrane potential and cytochrome *c* release. Alternatively, the Bcl-2 family may directly regulate caspase activation through interactions with Ced-4-like proteins. We describe a novel E1B 19K-interacting protein, Btf, isolated through a two-hybrid screen, that regulates an alternative pathway to control apoptosis. Two transcripts corresponding to *btf*, *btf<sub>S</sub>*, and *btf<sub>L</sub>*, were identified. Both appeared to be widely expressed but were deleted in some tumors. In this paper, we describe the function and biological significance of the protein product generated from the shorter form, Btf<sub>S</sub>, which differs from Btf<sub>L</sub> in just 49 amino acids in the C-terminal region.

Double-staining experiments were able to show the colocalization of Btf<sub>S</sub> with E1B 19K, Bcl-2, and Bcl-x<sub>L</sub>, supporting the interactions observed in the yeast two-hybrid assay as well as by *in vitro* coimmunoprecipitation. While transiently expressed Btf<sub>S</sub> was nuclear and could be sequestered into the cytoplasm by the antiapoptotic Bcl-2 family members, the localization of endogenous Btf<sub>S</sub> remains to be determined. It is entirely possible that in normal, nonapoptotic cells Btf<sub>S</sub> is expressed predominantly in the cytoplasm. In contrast, in dying cells, where there is a higher percentage of proapoptotic than of antiapoptotic Bcl-2 family members, Btf<sub>S</sub> may translocate to the nucleus and potentiate apoptosis.

Btf<sub>S</sub> was able to bind DNA *in vitro*, and it repressed transcription in reporter assays. The *trans*-repressive activity was inhibited by E1B 19K, Bcl-2, and Bcl-x<sub>L</sub>, correlating with their cytoplasmic sequestration potentials. While it remains possible that part of this phenomenon is a result of Btf<sub>S</sub>-induced apoptosis, the degree of cell death observed at 24 h would not account for the 10-fold reduction in transcriptional activity. The evidence that Btf<sub>S</sub> is a transcription repressor was further supported by the identification of the  $\Delta$ C210 deletion mutant that was sufficient for repressing transcription. The N-terminal fragment of Btf<sub>S</sub> is rich in glycine and serine, a feature that is common to transcriptional repressors (11, 41, 59, 101). Taken together, these data provide evidence for a novel *trans*-repressive protein that triggers apoptosis and is sequestered and inhibited by Bcl-2 family members.

One of the features of the Bcl-2 family exemplified by the Btf<sub>S</sub> interaction is their ability to sequester other cellular proteins from their normal subcellular localization. This process enables E1B 19K and Bcl-2 to inhibit apoptosis by using more than one cellular pathway. The best-characterized example concerns the association between proapoptotic and antiapoptotic Bcl-2 family members. While these interactions have been known for several years, recent studies of E1B 19K and

Bax have demonstrated that these family members can alter each other's subcellular localization (25). Bax is normally stimulated to go to the mitochondria during apoptosis and causes a loss of mitochondrial membrane potential (97). However, overexpression of E1B 19K causes Bax to be sequestered to the nuclear periphery, where E1B 19K is localized (25). This process most probably blocks the ability of Bax to disrupt mitochondrial function. The Bcl-2 family also associates with several unrelated proteins that may contribute to apoptosis regulation. For example, Bcl-2 associates with and targets the serine/threonine kinase Raf-1 to the mitochondria (88). Overexpression of Bcl-2 and that of Raf-1 cooperate to inhibit apoptosis. Another example that is now emerging is the association between the antiapoptotic Bcl-2 family members with *Caenorhabditis elegans* Ced-4 (12, 28, 79, 98) and with its mammalian homologue Apaf-1 (32, 61, 104). These interactions directly block the activation of downstream caspases. Thus far, the *C. elegans* Bcl-2 homologue, Ced-9, and Bcl-x<sub>L</sub> and E1B 19K have been shown to bind to Ced-4, and at least Ced-9 and E1B 19K can redistribute Ced-4 from the cytosol to the cytoplasmic membranes (28, 99). Bcl-x<sub>L</sub> also interacts with Apaf-1, and therefore it will be interesting to determine whether Bcl-x<sub>L</sub> can alter the localization of Apaf-1 (32, 61). In what is perhaps an analogous scenario, E1B 19K also sequesters the death-promoting protein FADD, an upstream component of the Fas- and TNF- $\alpha$ -mediated death signaling pathway (63). Overexpressed FADD becomes multimerized and produces filaments throughout the cell (63, 78). E1B 19K disrupts the FADD filaments, causing FADD to relocate in regions normally associated with 19K, and inhibits FADD-dependent apoptosis (63). Here, we show that the Bcl-2 family members also sequester a nuclear transcription factor and that this may also play a role in apoptosis. The minimal region for E1B 19K required for interaction with Btf at the amino terminus (amino acids 1 to 36) appears to be distinct from the interaction regions involved in binding to other proapoptotic proteins (Bax, Ced-4, and Nbk/Bik). This amino-terminal region of E1B 19K may correspond to BH4 of Bcl-2 and Bcl-x<sub>L</sub>, although this remains to be tested directly. Together, these studies suggest that E1B 19K and other Bcl-2 family members act as binding proteins for a number of apoptosis regulators, which could contribute to their widespread role as apoptosis inhibitors.

Transcriptional regulation often plays a critical role during apoptosis by either activating or repressing genes encoding basic apoptotic components. Indeed, inhibition of RNA and protein synthesis blocks apoptosis induced by a number of circumstances, including growth factor deprivation (42, 73) and treatment with some chemotherapeutic drugs (2, 51, 86). In contrast, others have shown that these inhibitors can actually promote cell death, suggesting that the loss of a short-lived

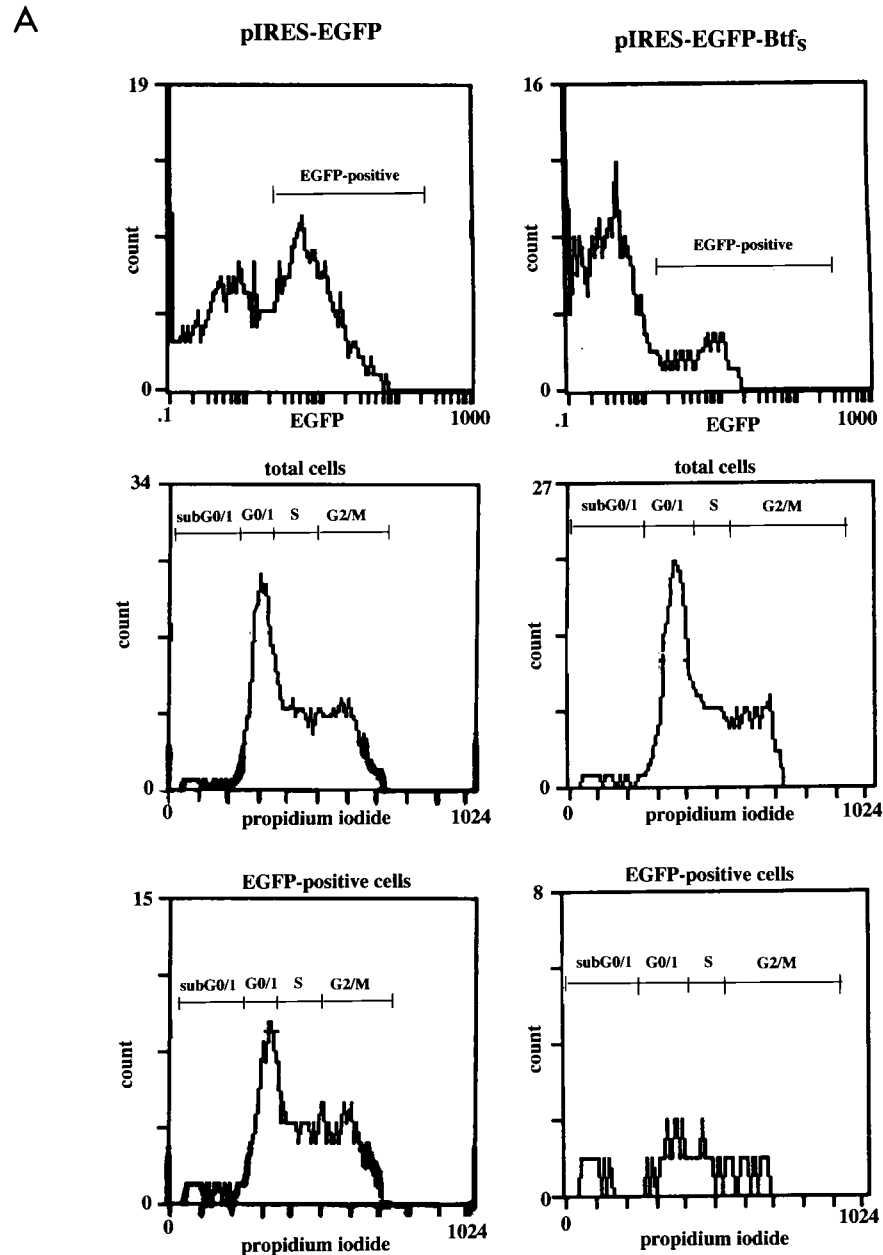


FIG. 9. Btf<sub>5</sub>-mediated cell death occurs by apoptosis. (A) Representative FACS scan from Table 1 at 72 h after transfection of pIRES-EGFP-Btf<sub>5</sub> and the empty pIRES-EGFP vector into HeLa cells. Cell cycle kinetics from propidium iodide staining are shown for the total cell population as well as the EGFP-positive cells. (B) Transfected cells were incubated with Hoechst dye 72 h posttransfection to visualize the DNA. Cells transfected with pIRES-EGFP-Btf<sub>5</sub> (right) were compared to those transfected with control pIRES-EGFP (left). Arrows for each set correspond to the same cell visualized with bright field (top), EGFP (green; middle), and Hoechst dye (blue; bottom). The presence of condensed chromatin in the presence of Btf<sub>5</sub> indicates cell death by apoptosis. Original magnification,  $\times 1,000$ .

survival factor can also lead to apoptosis (3, 43, 66, 85). A number of transcription factors that may serve as positive or negative regulators of apoptosis have been identified. For example, the NF- $\kappa$ B transcription factor plays an important role in blocking apoptosis triggered by TNF- $\alpha$  (4, 84, 87). However, in other situations, NF- $\kappa$ B activation has been associated with induction of apoptosis (23). Furthermore, Bcl-2 and E1B 19K repress NF- $\kappa$ B activity, providing another mechanism for Bcl-2 family members to control apoptosis (23, 34, 72).

Another transcription factor that may be regulated by the Bcl-2 family during apoptosis is p53 (reviewed in reference 38). The p53 tumor suppressor protein is required for apoptosis

during administration of ionizing radiation and chemotherapeutic drugs, as well as by transforming oncogenes such as *c-myc* and the E1A gene (16, 17, 30, 40). While p53 may have multiple functions, its transcriptional activity is clearly critical for the regulation of cell death in some (68) but not all (9, 29) situations. However, it is still unclear whether p53-mediated apoptosis requires its transcriptional activation or repression properties or perhaps both. On the one hand, p53 *trans*-activates both *bax* (26, 49) and *fas* (60), both of which are bona fide inducers of apoptosis. In contrast, several p53-repressible genes which may potentially contribute to apoptosis, including *bcl-2* (48), *MAP4* (56), the interleukin-6 gene (69),



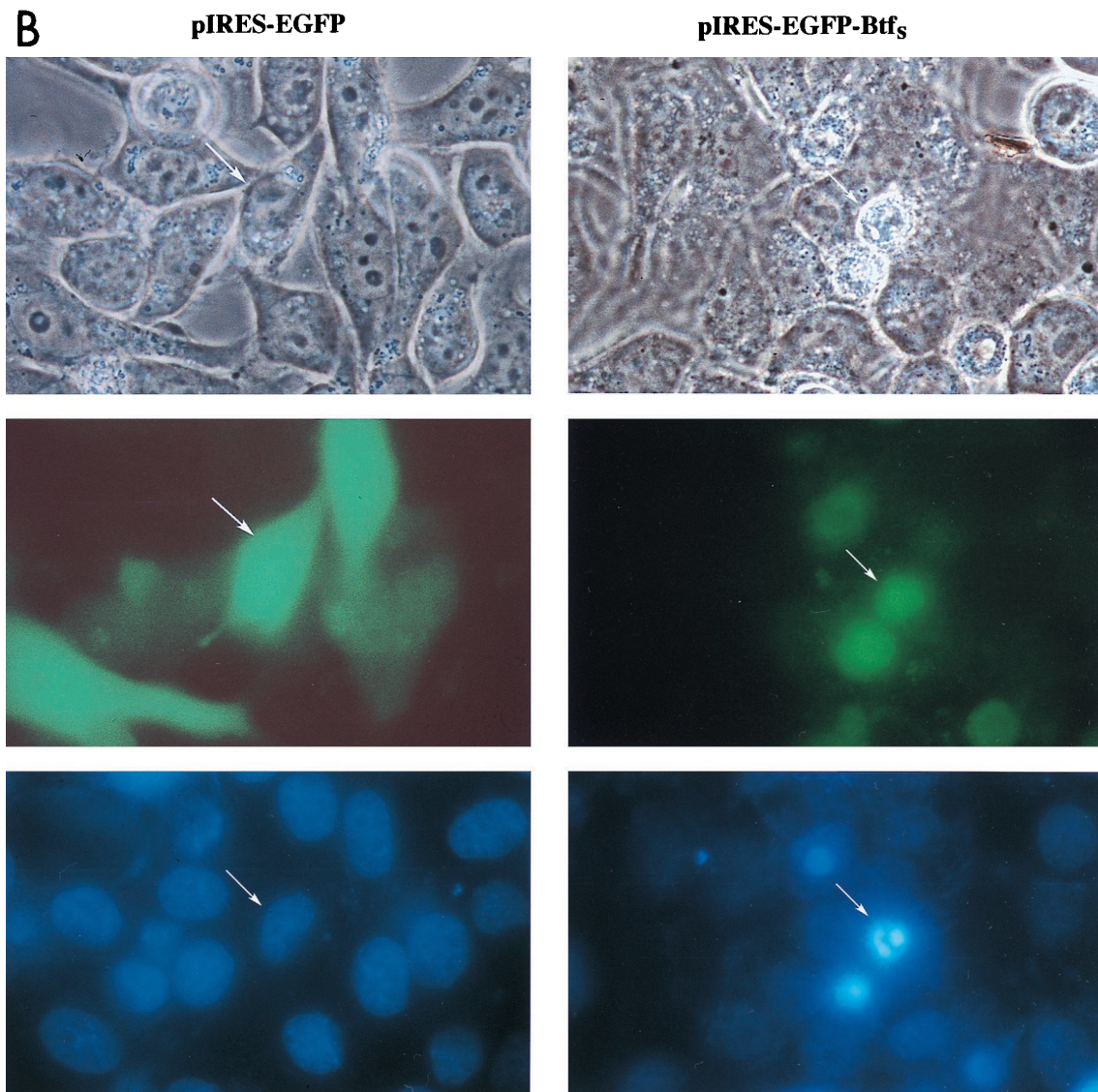


FIG. 9—Continued.

*c-fos* (37), and *c-myc* (52), have been identified. Expression of Bcl-2 and of E1B 19K alleviates the transcriptional repression activity of p53, providing a mechanism for Bcl-2 family regulation of apoptosis (66, 76). Thus, Bcl-2 family members can influence apoptosis by modulating the transcriptional activity of both NF- $\kappa$ B and p53. Inhibition of Btf<sub>S</sub>-mediated transcriptional repression and apoptosis by the Bcl-2 family establishes another connection between these apoptosis regulators and modulation of transcription. It will certainly be interesting to determine whether Btf<sub>S</sub> can influence the activities of other transcription factors related to apoptosis, such as NF- $\kappa$ B and p53.

We would predict that Btf<sub>S</sub> may repress the transcription of survival genes. Based on previous observations of regulation of gene expression by E1B 19K, one possible target may be the p53 inhibitor, Mdm-2. We have previously observed that stable expression of E1B 19K or Bcl-2 leads to an increase in Mdm-2 mRNA and protein levels (82). Such a mechanism could also account for the ability of E1B 19K to derepress the transcriptional repression mediated by p53 and E1A (56, 66, 76, 103).

While it will be worthwhile to determine whether Btf<sub>S</sub> could affect the p53-mediated modulation of these targets, the fact that Btf<sub>S</sub> was capable of inhibiting transformation by p53DD suggests that another target, which is independent of p53, also exists. We also have not been able to detect binding between Btf<sub>S</sub> and p53 (data not shown), although it is still plausible that Btf<sub>S</sub> associates with p53-dependent coactivators such as p300. Since the putative DNA binding sites within Btf<sub>S</sub> contain homology to bZIP and Myb, it is conceivable that they have similar targets. Interestingly, correlations with apoptotic regulation exist for both of these transcription factor families (reviewed in references 36 and 89). For example, application of functional blocking antibodies against the AP-1 proteins, Fos and Jun, or transfection with dominant-interfering Jun inhibits apoptosis in neuronal cells following growth factor withdrawal (18, 24). However, relevant target genes that contribute to AP-1-mediated apoptosis have yet to be identified. The Myb family of transcriptional regulators inhibits apoptosis and regulates the transcriptional activation of *bcl-2* (21, 81). This may therefore provide a feedback loop between Btf<sub>S</sub> and Bcl-2.

Future studies will be performed to determine whether Btf<sub>5</sub> regulates *bcl-2* or other genes involved in apoptosis. The link between Btf<sub>5</sub> and the Bcl-2 family may provide an alternative mechanism by which the Bcl-2 family is able to regulate cell survival.

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