

BCL-6, a POZ/zinc-finger protein, is a sequence-specific transcriptional repressor

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ABSTRACT Approximately 40% of diffuse large cell lymphoma are associated with chromosomal translocations that deregulate the expression of the *BCL6* gene by juxtaposing heterologous promoters to the *BCL6* coding domain. The *BCL6* gene encodes a 95-kDa protein containing six C-terminal zinc-finger motifs and an N-terminal POZ domain, suggesting that it may function as a transcription factor. By using a DNA sequence selected for its ability to bind recombinant BCL-6 *in vitro*, we show here that BCL-6 is present in DNA-binding complexes in nuclear extracts from various B-cell lines. In transient transfection experiments, *BCL6* can repress transcription from promoters linked to its DNA target sequence and this activity is dependent upon specific DNA-binding and the presence of an intact N-terminal half of the protein. We demonstrate that this part of the *BCL6* molecule contains an autonomous transrepressor domain and that two noncontiguous regions, including the POZ motif, mediate maximum transrepressive activity. These results indicate that the BCL-6 protein can function as a sequence-specific transcriptional repressor and have implications for the role of *BCL6* in normal lymphoid development and lymphomagenesis.

The *BCL6* gene was identified by virtue of its involvement in chromosomal translocations affecting band 3q27 in non-Hodgkin lymphoma (1–5). Subsequent studies have demonstrated that rearrangements of the *BCL6* gene can be found in 30–40% of diffuse large cell lymphoma and 6–11% follicular lymphoma (6–8). These alterations cause the deregulated expression of the *BCL6* gene by a mechanism called promoter substitution, that is the juxtaposition of heterologous promoters, derived from other chromosomes, to the *BCL6* coding domain (9). Recent evidence also indicates that the 5' non-coding region of the *BCL6* gene is altered by somatic point mutations that are found, independent of rearrangements, in ≈70% diffuse large cell lymphoma and 45% follicular lymphoma (10). Thus, most cases of diffuse large cell lymphoma and a significant fraction of follicular lymphoma carry structural alterations of the regulatory region of the *BCL6* gene, suggesting that deregulated *BCL6* expression may be important for lymphomagenesis (11).

The BCL-6 protein is a 95-kDa nuclear phosphoprotein detectable at low abundance in multiple tissues and expressed at high levels exclusively in mature B cells (12–14). Within the B-cell lineage, the expression of the BCL-6 protein is specifically regulated during differentiation since it is only detectable in B cells within germinal centers (GCs) but not in pre-GC cells or in differentiated progenies such as plasma cells (12–14). The structure of the BCL-6 protein includes six Krüppel-type C-terminal zinc-finger (ZF) motifs (2, 4, 5) which have been shown to recognize specific DNA sequences *in vitro* (15, 16), and a N-terminal POZ (also called ZIN or BTB; refs. 17–19) motif shared by various ZF molecules including the *Drosophila* develop-

mental regulators *Tramtrack* and *Broad-complex* (20, 21), the human KUP (22), ZID (19), and PLZF (23) proteins as well as by POX viruses (24) and the actin-binding *Drosophila* oocyte protein *Kelch* (25). These structural features and the expression pattern of the BCL-6 protein suggest that it may function as a transcription factor involved in the control of B-cell development.

This study was aimed at elucidating the role of *BCL6* in transcriptional regulation. We demonstrate that *BCL6* functions as a potent transcriptional repressor of promoters linked to its DNA target sequence and we map this transrepression activity to two noncontiguous N-terminal regions of the protein, one of them including the POZ domain.

MATERIALS AND METHODS

Plasmids. The eukaryotic expression vector pMT2-BCL-6 has been described (12). To construct the plasmids expressing the Gal4-BCL-6 fusion proteins, various segments of *BCL6* cDNA were first obtained by polymerase chain reaction (PCR) using pairs of oligonucleotide primers in which *BCL6*-specific sequences are linked to a *Bam*HI restriction site and then subcloned into the *Bam*HI restriction site of the Gal4 plasmid (26). To construct Gal4-BCL-6 fusion protein plasmids with internal *BCL6* deletions (constructs J, K, and L; see Fig. 5A), *BCL6* cDNAs were first obtained by PCR using *BCL6* primers linked to a 5' *Eco*RI site and a 3' *Sma*I site and then cloned into the Gal4 plasmid to form an intermediate fusion protein construct. A second *BCL6* cDNA obtained using primers linked to a 5' *Sma*I site and 3' *Bam*HI site was then cloned into the *Sma*I/*Bam*HI sites of the intermediate fusion protein construct to create the final construct. All the fusion constructs express Gal4 (amino acids 1–147) fused to variable domains of *BCL6* via five residues (PEFPG) encoded by the multiple cloning site of the Gal4 plasmid. Translation termination is provided by three-frame termination codons in the Gal4 plasmid. All PCR-derived sequences as well as subcloning junctions were verified by DNA sequencing. B6BS-TK LUC was constructed by insertion of a single copy of the *BCL6* binding site (B6BS: GAAAATTCCTA-GAAAGCATA; B.H.Y. *et al.*, unpublished data) into the *Bam*HI site of TK-LUC. Luciferase reporter constructs (G5-TK-LUC and TK-LUC) were obtained from K. Calame (Columbia University). TK-CAT reporter constructs [G5(+1000)-TK-CAT and G5(-750)-TK-CAT] and SV-CAT reporter constructs [G5-SV-CAT, G5-SV'-CAT, and G5(110)-SV-CAT] were obtained from L. Lania (27) and F. Rauscher (28), respectively (TK, thymidine kinase; CAT, chloramphenicol acetyltransferase; LUC, luciferase; SV, simian virus).

Antisera and Immunoprecipitation. The generation and characterization of polyclonal anti-BCL-6 antisera (N70–6; C73–6) have been described (12). Polyclonal anti-yeast Gal4 antiserum was obtained from Upstate Biotechnology (Lake Placid, NY).

Cell Lines, Transient Transfection, and Reporter Gene Assays. The Mutu I and Mutu III cell lines were obtained from A.

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Abbreviations: ZF, zinc-finger; GC, germinal center; CAT, chloramphenicol acetyltransferase; SV, simian virus; LUC, luciferase, EMSA, electrophoretic mobility shift assay; B6BS, BCL-6 binding site.

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Rickinson (29). Transfections of Mutu III cells (1.5×10^7) were performed by electroporation using Gene Pulser (Bio-Rad) apparatus. Transfections in NTera-2 cells (from U. Siebenlist, National Institutes of Health), 293T cells (from J. Krolewski, Columbia University), and NIH 3T3 cells were performed by calcium-phosphate precipitation procedure (30). Assays for LUC and CAT activities were performed as described (30).

Electrophoretic Mobility Shift Assay (EMSA). Preparation of nuclear extracts, EMSA, and antibody-mediated supershift assays were performed as described (30) except that the EMSA reaction buffer was 20 mM Hepes (pH 7.5), 50 mM KCl, 5 mM $MgCl_2$, 10 μ M $ZnCl_2$, 4% Glycerol, and 100 μ g of BSA per ml.

RESULTS

Specific DNA-Binding by BCL-6 in B-Cell Nuclear Extracts.

Using the cyclic amplification and selection of targets (CAST) method (31), we have identified a 20-bp DNA B6BS (GAA-AATTCCTAGAAAGCATA) capable of binding *in vitro* translated or bacterially produced BCL6 (B.H.Y. *et al.*, unpublished data). Compared with the sites previously reported (15, 16), B6BS contains additional flanking residues that increase affinity and contains unequivocal residues at various previously undefined positions. To determine whether B6BS could serve as a target for naturally synthesized BCL-6 in nuclear extracts, we used it as a probe in an EMSA by using nuclear extracts prepared from B-cell lines expressing (Mutu I) or lacking (Mutu III) BCL6 RNA and protein (12). Fig. 1 shows that a DNA-binding protein complex was detectable only in nuclear extracts from cells expressing BCL6 (Mutu I), but not in control cells (Mutu III). Cold cognate DNA competition showed that the detected complex bound B6BS specifically; antibody-mediated supershift analysis using anti-

sera against the N-terminus (Fig. 1) or the C-terminus (not shown) of BCL6 showed that this complex contained BCL-6 and that the full-length protein was present in the complex. Analogous EMSA performed on nuclear extracts from a panel of B cells representative of discrete stages of B-cell differentiation indicated complete concordance between formation of BCL-6 containing protein-DNA complexes and protein expression (data not shown). As a further control for specificity, we compared the B6BS-binding complexes in cells (NTera-2; lacking BCL6 expression) transfected with a BCL6 expressing vector or with a control vector (30). The result (Fig. 1) shows that a DNA-binding complex, specific for B6BS (see cold cognate competition) and containing BCL-6 (see supershift analysis), is also detectable in BCL6 transfected cells, but not in control-transfected cells. The complex detected in NTera-2-transfected cells appeared to be similar in migration and abundance to the one detected in B-cell lines, suggesting either that the BCL-6 protein is the only component of the complex or that, if a cofactor is present, it is expressed in cell types that do not express BCL6. Taken together, these results demonstrate that the B6BS sequence can serve as a DNA target site for *in vivo* synthesized full-length BCL-6 protein.

BCL6 Represses Transcription from Promoter Linked to Its DNA Target Site. To investigate the role of BCL6 in the regulation of transcription, we analyzed the ability of a BCL6 expression vector to affect the transcription of reporter genes linked to a promoter and the B6BS site in transient transfection assays in Mutu III cells (Fig. 2A). Cotransfection of the BCL6 expression vector with a reporter gene (LUC) driven by the constitutively active TK promoter linked downstream to a single-copy B6BS led to a strong (90% using 1 pmol effector plasmid) and dose-dependent repression (Fig. 2B). The transrepression activity was dependent upon the presence of the

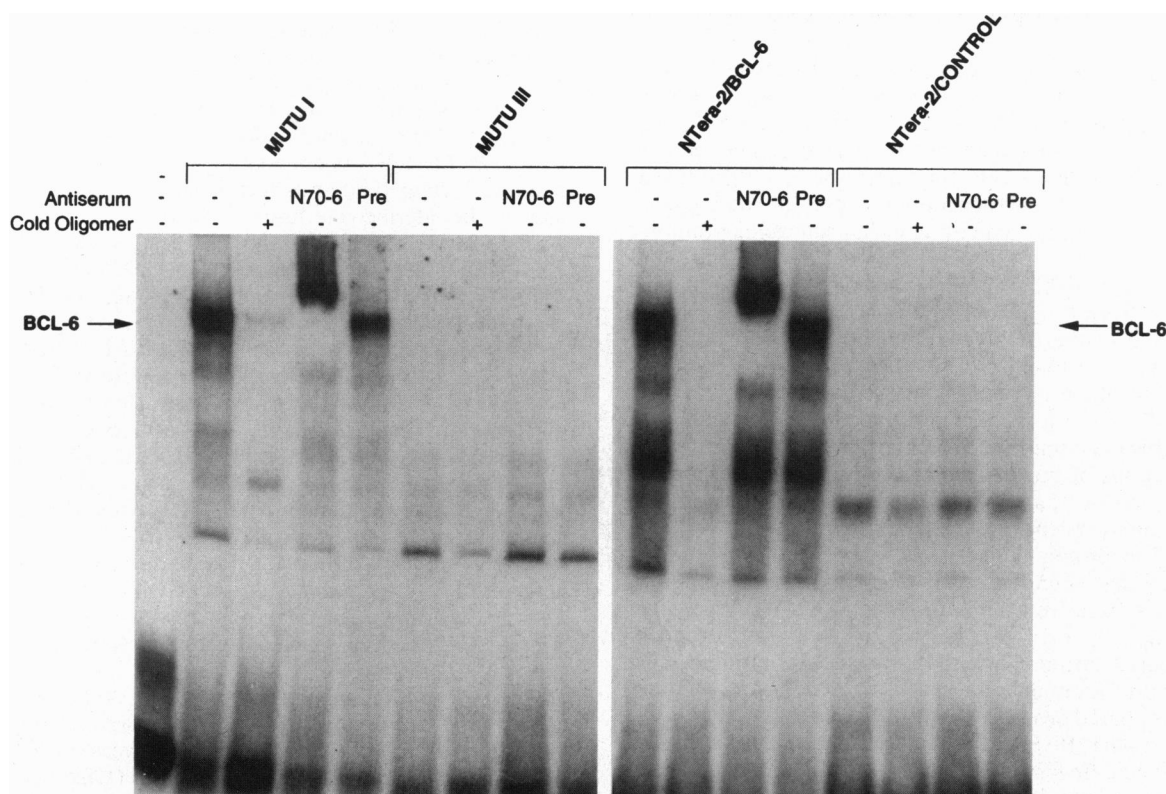


Fig. 1. Identification of DNA-binding complexes containing BCL-6 in nuclear extracts from B-cell lines. Nuclear extracts from Mutu I (subclone of lymphoma line, Mutu BL, positive for BCL6 RNA and protein), Mutu III (a subclone of Mutu BL, negative for BCL6 RNA and protein), NTera-2 transfected with a BCL6 expression vector (NTera-2/BCL-6), or a control vector (NTera-2/control) were assayed for binding to the B6BS probe by EMSA. Arrows point to BCL-6-containing complexes. Cold cognate oligomer competition was performed using a 10-fold excess of the B6BS probe. Supershift analysis was performed by using an anti-BCL-6 (N70-6) antiserum recognizing the N-terminus of BCL6 or a pre-immune (Pre) serum as a control for specificity.

BCL6 DNA-binding site since a reporter plasmid (TK-LUC) lacking B6BS was only marginally affected by *BCL6*. This activity is most likely due to nonspecific "squenching" since it is detectable independently of the presence of the B6BS site on the reporter gene (not shown). The transrepression activity of *BCL6* was also dependent upon the presence of an intact N-terminal half of the protein since a vector expressing only the *BCL6* ZF domain retained the ability to bind DNA specifically (not shown), but lost the ability to repress transcription. Analogous results were obtained in additional non-B cell lines that do not express *BCL6* (293T cells), while transfection assays performed in B-cell lines expressing high levels of endogenous *BCL6* led to only minimal levels of repression (data not shown). These results indicate that *BCL6* is a site-specific transcriptional repressor and suggest that its transrepressive activity is mediated by its N-terminal region.

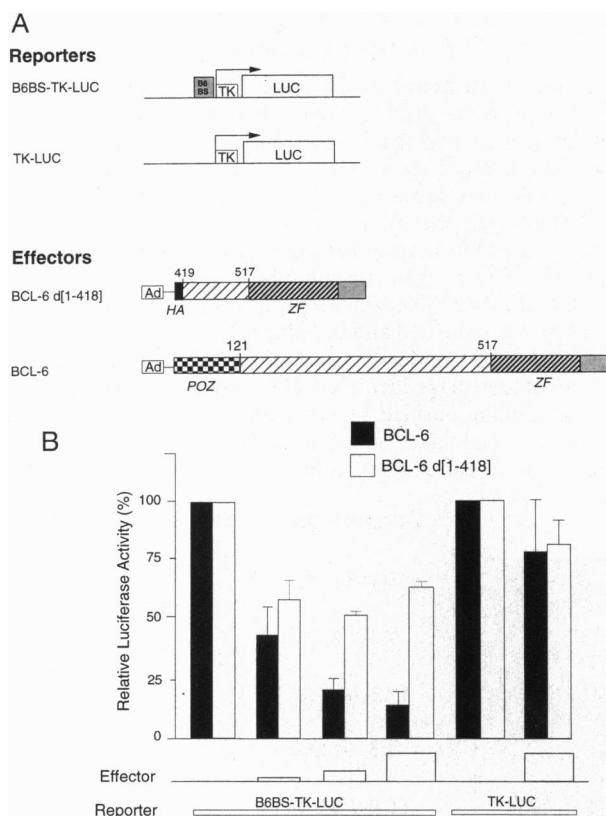


FIG. 2. *BCL6* can repress transcription from a promoter linked to the B6BS site. (A) Schematic representation of the reporter and effector vectors used. B6BS, 1 copy of the B6BS 20 bp site; TK, promoter region of the thymidine kinase gene; LUC, coding region of the luciferase gene; Ad, adenovirus major late promoter region; HA, influenza-hemagglutinin epitope tag. (B) Increasing amounts (0, 0.1, 0.3, 1.0 pmol) of the pMT2-BCL-6 or pMT2-BCL-6 d[1-418] effector vectors were cotransfected into Mutu III cells with 0.4 pmol of target plasmid (pB6BS-TK-LUC or TK-LUC) by a modified calcium phosphate precipitation method. The total amount of transfected DNA and vector sequences was kept constant in each experiment by adding pMT2 DNA to reach 30 μ g. Two micrograms of a plasmid expressing the bacterial β -galactosidase gene were also cotransfected in each experiment to serve as an internal control for transfection efficiency. Forty-eight hours after transfection, cells were harvested and luciferase activity was measured in a luminometer. Values are expressed as percent of those obtained using the reporter vector alone after normalization for β -galactosidase values. No significant difference was detectable between the basal levels of expression of B6BS-TK-LUC and TK-LUC (not shown). Experiments were performed in triplicate and error bars are shown. The results indicate that *BCL6* expression leads to a dose-dependent repression of the reporter gene activity and that *BCL6*-mediated repression is dependent upon the presence of the N-terminal half of the BCL-6 protein and the B6BS site on the reporter plasmid.

The N-Terminal Half of the BCL-6 Protein Is Capable of Autonomous Transrepression Activity. To conclusively determine whether the N-terminal region of *BCL6* contains a transrepression domain and whether this domain was capable of acting autonomously, we tested whether the *BCL6* transrepression activity was transferable to an heterologous DNA-binding domain such as the one from the yeast Gal4 transcription factor. We constructed a vector [Gal4-BCL-6(2-517)] expressing a fusion protein in which the non-ZF region (amino acids 2-517) of *BCL6* was linked to the Gal4 DNA binding domain (amino acids 1-147) and cotransfected it (293T cells) with a reporter gene construct (G5-TK-LUC) driven by the TK promoter region linked to Gal4 binding sites (G5) (Fig. 3A). The results (Fig. 3) indicate that the Gal4-BCL-6[2-517] fusion protein is capable of strong (90% repression obtained with 0.1 pmol Gal4-BCL-6 vector) and dose-dependent repression of reporter gene expression. The observed activity is specific since it is dependent upon the presence of the *BCL6* domain in the effector vector (see modest activity of Gal4 vector) and the Gal4 DNA binding site in the reporter vector (see modest repression of the TK-LUC reporter gene). Analogous experiments performed using reporter genes driven by minimal promoters (E1B, SV40) lead to some degree of repression (not shown), the specificity of which, however, was difficult to establish due to the low basal activity of these promoters. Taken together, these results indicate that the N-terminal domain of *BCL6* (amino acids 2-517) contains a potent autonomous transrepression domain.

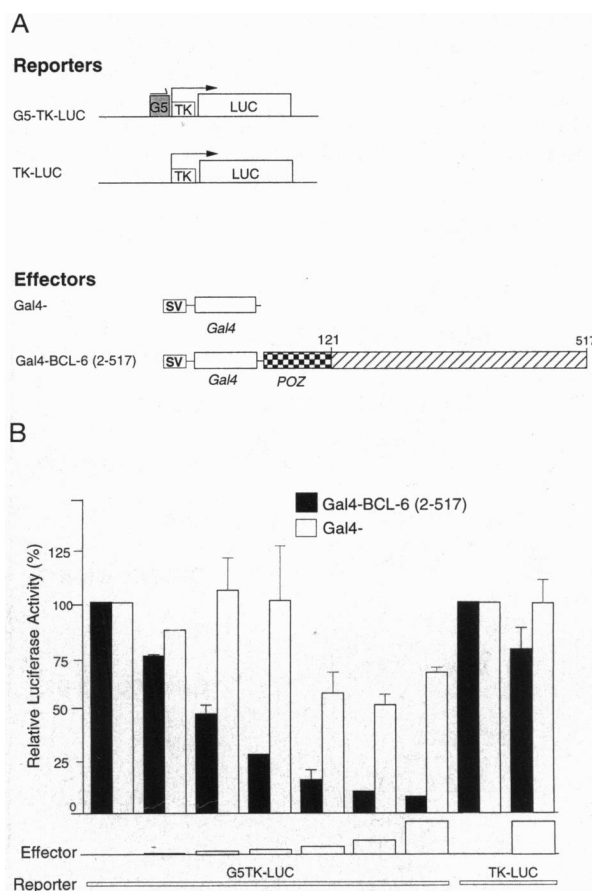


FIG. 3. The N-terminal half of *BCL6* contains an autonomous transrepression domain. (A) Schematic representation of reporter and effector vectors used. G5, five copies of the Gal4 binding site (32); SV, SV-40 promoter-enhancer region; other abbreviations are as in Fig. 2A. (B) Increasing amounts (0, 0.001, 0.003, 0.01, 0.03, 0.1, and 0.3 pmol) of the Gal4-BCL-6(2-517) or Gal4 effector vector were cotransfected with 2 μ g of the G5-TK-LUC or TK-LUC and 2 μ g SV- β -galactosidase (see Fig. 2B) into 293T cells. Values are expressed and normalized as described in Fig. 2.

The Transrepression Activity of *BCL6* is Position-, Orientation-, and Distance-Independent. To characterize further the transrepression activity of *BCL6*, we investigated the effects of varying the distance, 5'-3' position and orientation between the DNA binding site and the promoter of the reporter gene. Toward this end, the Gal4-BCL-6[2-517] vector was cotransfected into 293T cells with various constructs in which the Gal4 binding site was positioned at variable distance 5' [750 bp: G5(-750)-TK CAT; 110 bp: G5(-110)-TK CAT], or 3' [G5(+1000)SV-CAT], or at opposite orientations (G5-SVCAT versus G5-SV'CAT) with respect to the promoters (TK or SV40) driving the CAT reporter gene (Fig. 4). The results show that the transrepression activity was maintained on all reporter constructs tested. Thus, *BCL6*-mediated transrepression can act on DNA binding sites independently of their position (3' versus 5'), orientation and distance from the promoter of the target gene.

Mapping of the *BCL6* Transrepression Domain. To determine which region within the N-terminal half (amino acids 2-517) of *BCL6* was responsible for the transcriptional repression activity, we constructed various deletion mutants of the *BCL6* portion of the Gal4-BCL-6[2-517] vector (Fig. 5A) and tested their ability to repress the expression of the G5-TK-LUC reporter gene in cotransfection experiments in 293T and in NIH 3T3 cells. In both cell lines, significant loss of activity was associated with mutants lacking a relatively central domain of the *BCL6* molecule (amino acids 267-395) (see mutant C versus D), part of the POZ domain (F, G, H, and L), or both (I). In both cell lines, maximal levels of transrepression activity (>95%), comparable to those obtained with Gal4-BCL-6[2-517], were obtained only in those mutants that retained these two domains, while deletion of variable portions between them was functionally irrelevant (J and K). Accordingly, the presence of only one of the two domains (POZ in E; amino acids

267-395 in F) was insufficient to confer maximal transrepression activity.

The observed reduction in reporter gene activity was not the result of differences in transfection or expression efficiencies among the various effector constructs since immunoprecipitation analysis of transfected cells indicated comparable levels of expression for all Gal4-BCL-6 fusion proteins (representative results in Fig. 5B). The observed results were not due to differences in subcellular localization or DNA binding among the various proteins since comparable levels of specific DNA binding were observed in nuclear extracts from transfected cells by EMSA using as a probe a synthetic oligonucleotide corresponding to the Gal4 binding site (representative results are shown in Fig. 5C). Thus, these results indicate that two noncontiguous domains, corresponding to POZ (amino acids 11-121) and to amino acids 267-395, are necessary for maximal transrepression by *BCL6*.

DISCUSSION

Based on the presence of ZF motifs and a N-terminal POZ domain, previous studies have suggested that the BCL-6 protein may function as a DNA-binding transcription factor (2, 4, 5). The present study demonstrates that nuclear extracts from B-cell lines contain protein complexes in which BCL-6 binds DNA at its specific target sequence and that *BCL6* can repress transcription from promoters linked to the same DNA target site. These results have implications for the function of BCL-6 and other POZ/zinc finger proteins as well as for the role of *BCL6* in normal and neoplastic lymphoid development.

DNA-Binding Complexes Containing BCL-6 in B Cells. Two previous studies have identified DNA consensus sequences capable of binding purified recombinant BCL-6 *in vitro* (15, 16). One of this candidate sites was also shown to bind protein complex in nuclear extracts from cells expressing *BCL6*, but the

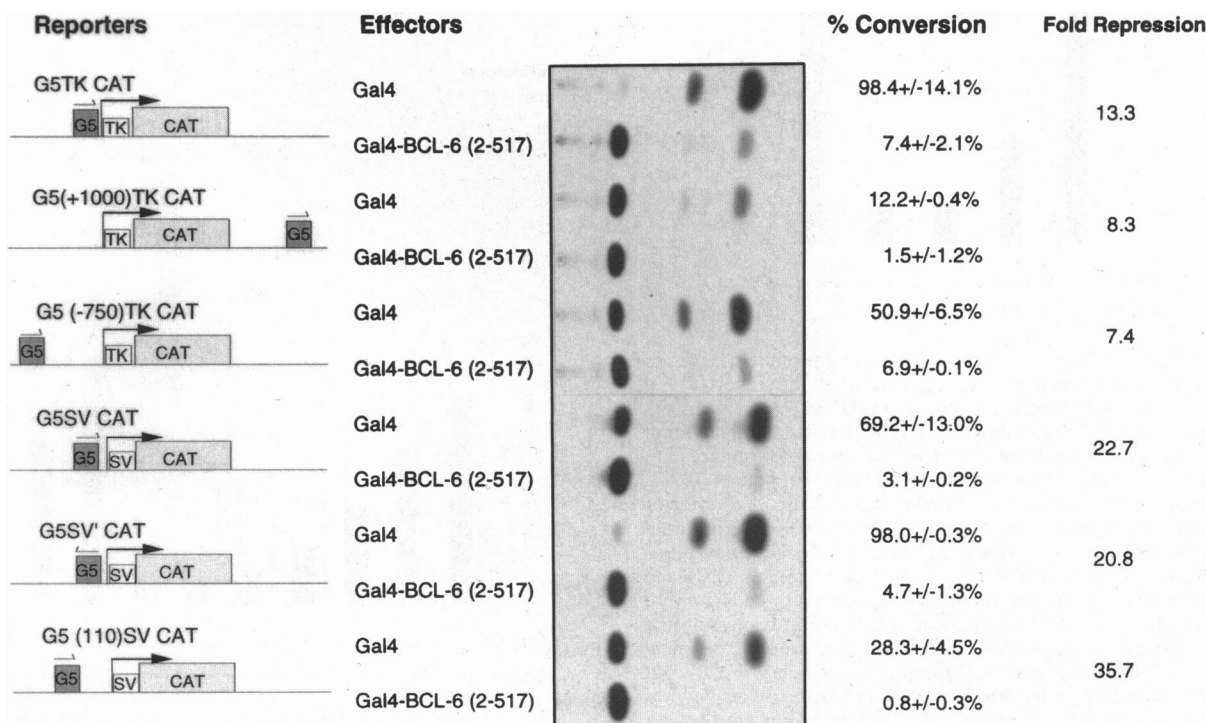


FIG. 4. Position-, distance-, and orientation-independent transcriptional repression by Gal4-BCL-6 fusion proteins. 293T cells were transfected with 0.1 pmol Gal4-BCL-6 (2-517), 1 μ g SV- β -galactosidase plasmid, and 2 μ g of various reporter plasmids (CAT and SV-40 promoter region) as described in Figs. 2 and 3. CAT activity was measured 48 hr after transfection by thin layer chromatography. A representative chromatogram showing the results obtained with each vector combination is shown. Values are expressed as percent conversion of chloramphenicol and as fold-repression obtained by Gal4-BCL-6 versus Gal4 vector with each reporter vector. The results indicate that comparable levels of repression are induced by *BCL6* on the TK (7.4-13.3 fold) or SV (20.8-35.7) promoters regardless of the position (3' or 5'), orientation and distance of the Gal4 DNA-binding site from the promoter.

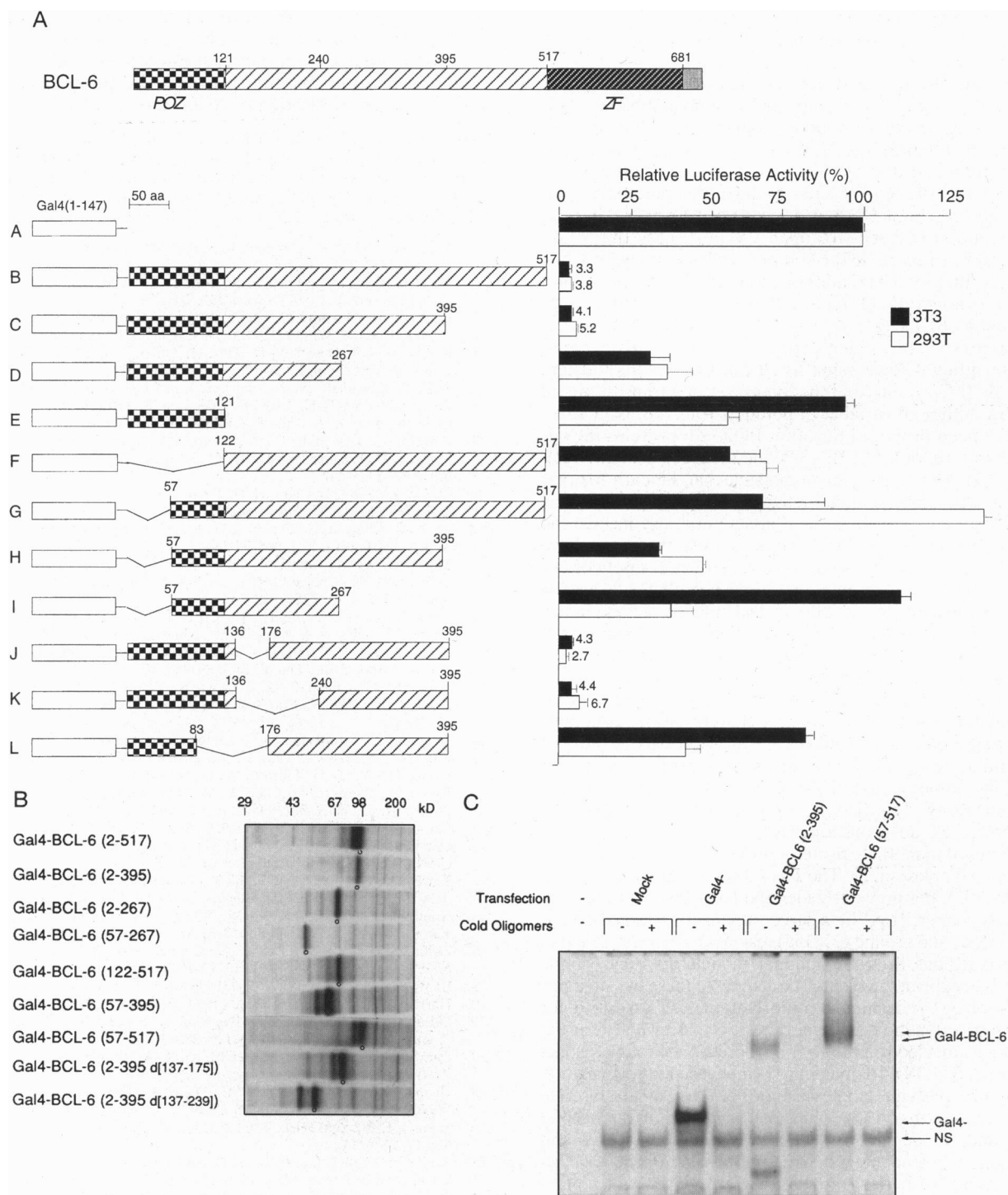


FIG. 5. Mapping of the *BCL6* transrepression domain to two noncontiguous regions in the N-terminal half of *BCL6*. (*A*) Schematic representation of Gal4-BCL-6 deletion mutant vectors (*Right*; compare with schematic representation of the BCL-6 protein on top) and their activity in repressing transcription from a cotransfected G5-TK LUC reporter vector (assayed as described in Fig. 3) in 293 and 3T3 cells (*Left*). (*B*) Immunoprecipitation analysis of Gal4- and Gal4-BCL-6 fusion proteins expressed in transfected 293T cells. Anti-Gal4 antiserum was used to immunoprecipitate [³⁵S]methionine-labeled proteins that were resolved in 10% SDS/PAGE. Bands corresponding to Gal4-BCL-6 fusion proteins are indicated by open circles. Proteins with molecular mass smaller than that predicted for the corresponding fusion protein represent partial degradation products. Only representative data are shown since different fusion proteins required different gels for analysis. The results indicate that the transfected constructs express the Gal4-BCL-6 proteins at comparable levels. (*C*) Gal4-BCL-6 deletion mutants retain the ability to bind DNA. Nuclear extracts of 293T cells transfected with the Gal4-BCL-6(2-395), Gal4-BCL-6(57-517), Gal4 vectors or mock-transfected (Mock) were analyzed by EMSA using as a probe a 27-bp oligomer containing the Gal4 DNA-binding site (CGGAAGACTCTCCTCCG; ref. 32). Fifty-fold cold cognate oligomer was used in the assay to control for specificity. NS, nonspecific band. The results indicate that Gal4-BCL-6(2-395) (active in transrepression; see *A*) and Gal4-BCL-6(57-517) (inactive) display comparable DNA-binding activities.

presence of BCL-6 within *in vivo* formed DNA-binding complexes could not be demonstrated (16). Using a target DNA sequence optimized for *in vitro* binding to BCL-6 as well as

specific immunodetection of BCL-6 in DNA binding assays, we provide direct evidence for the presence of BCL-6 within naturally occurring complexes in various B-cell types (Fig. 1). This

result validates the B6BS site as the best target for *BCL6* *in vitro* and suggest that this site may represent the target for *BCL6* binding *in vivo*.

Our results also demonstrate that BCL-6 contacts DNA as a full-length protein in *in vivo* formed protein complexes. This finding is relevant in view of the observation that another POZ/zinc finger protein, ZID, cannot bind DNA as a full-length protein and that binding-inhibition is mediated by the POZ domain (19). Our results exclude the possibility that BCL-6 can only bind DNA in a processed form lacking the POZ domain (19), but leave open the possibility that other proteins may be present in the complex and necessary for DNA binding by BCL-6. The finding of apparently identical complexes in a variety of cell types either expressing endogenous or exogenous *BCL6* (Fig. 1) would suggest that, if a partner protein is present, it is widely expressed in various tissue types.

Transcriptional Repression by *BCL6*. Our results indicate that the BCL-6 protein can function as a strong transcriptional repressor. Although variable in potency, transrepressor functions have been proposed for other POZ/ZF proteins including the B isoform of the γ FBP (33), and the ZF5 (17) and PLZF proteins (23). Although it remains possible that, similarly to other ZF proteins, the transcriptional activity of POZ/ZF proteins may be dependent on the cellular environment and may include the ability of transactivate, all POZ/ZF proteins studied so far have displayed a consistent transrepressive activity in a variety of cell types and on various promoters, suggesting that site-specific transrepression may represent the critical function of these family of nuclear factors. During the preparation of this manuscript, analogous results on the transrepressive activity of *BCL6* have also been reported by others (34).

The mechanism by which transrepression occurs remains to be elucidated. Our results suggest that two noncontiguous domains are necessary for maximal transrepressive activity of *BCL6* and a similar conclusion has been recently reached for the highly homologous PLZF protein (J. Licht, personal communication). The first domain virtually coincides with POZ sequences, an evolutionarily conserved motif that has been proposed to mediate protein-protein interactions in large multimeric complexes (35). The second set of sequences (amino acids 240–395), not previously identified as a distinct functional domain, is characterized by a large number of charged amino acids (35/155) and proline (21/155) residues as typically found in regulatory regions of transcriptional modulators. One simple hypothesis to explain the synergistic activity of these two domains is that they may determine the conformation of *BCL6* critical for transrepressive function. Alternatively, it is conceivable that the two domains may contribute to transrepression through distinct mechanisms. The POZ domain may be involved in the formation of multimeric protein complexes and their targeting to discrete nuclear substructures (35), while the structure of the second domain and the ability of *BCL6* to act in a distance- and orientation-independent manner are more reminiscent of silencers interfering with the formation of an active transcription complex (36). The identification of molecules interacting with these two domains should provide insights into their role in *BCL6*-mediated transrepression.

Implications for the Role of *BCL6* in Normal Lymphoid Development and Lymphomagenesis. The BCL-6 protein is expressed in both proliferating and nonproliferating B cells within GCs, but not in pre-GC cells or in differentiated progenies such as plasma cells (12). This pattern suggests that *BCL6* expression may be needed for GC development, while its downregulation may be necessary for differentiation of B cells into plasma cells. Based on these observations, the finding that *BCL6* functions as a site-specific transcriptional repressor suggests that *BCL6* may regulate the GC phenotype by silencing the genes determining plasma cell differentiation or those preventing the death of B cells within the GC. This implies that

the same genes, and thus differentiation and death, may be constitutively repressed in lymphomas in which *BCL6* expression is deregulated by promoter substitution or mutation. The results reported in this study provide the experimental framework for the identification of *BCL6* target genes.

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