Repression of an Interleukin-4-responsive Promoter Requires Cooperative BCL-6 Function*

Received for publication, November 9, 2004, and in revised form, January 11, 2005
Published, JBC Papers in Press, January 19, 2005, DOI 10.1074/jbc.M412649200

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BCL-6 functions as a potent transcriptional repressor that binds with specificity to DNA elements bearing marked similarity to STAT recognition sequences. Previous studies have demonstrated that BCL-6 and Stat6 can both bind and regulate the Ilp promoter that controls immunoglobulin heavy chain class switching to IgE. Examination of BCL-6−/− and BCL-6+/−Stat6−/− mice has demonstrated that BCL-6 is a repressor of IgE and that Stat6 is still required for the interleukin-4 (IL-4) induction of class switching to IgE in B cells lacking BCL-6. To define the mechanisms by which BCL-6 represses IL-4 function, we analyzed the role of BCL-6 in repressing the Ilp promoter. There are three BCL-6-binding sites within this IL-4-responsive promoter. Analysis of Ilp promoters that have mutated BCL-6-binding sites demonstrates that at least two of these sites are required for maximal BCL-6 repression of this locus. Footprinting analysis demonstrates that BCL-6 binds cooperatively to the two upstream binding sites in the Ilp promoter. This cooperative binding requires the POZ domain of BCL-6. Furthermore, activated Stat6 molecules can displace BCL-6 from one of these binding sites. These data demonstrate that cooperative interaction between BCL-6 molecules is required for repression of the Ilp promoter.

Diffuse large cell and follicular lymphomas account for the overwhelming majority of non-Hodgkin’s lymphomas seen in adult populations (1). Accordingly, much attention has been focused on determining the factors responsible for the pathogenesis of these malignancies. A surprisingly high number of diffuse large cell and follicular lymphomas have translocations involving a gene termed BCL-6/LAZ3 (2–7). BCL-6 encodes a 706-amino acid site-specific transcriptional repressor with an N-terminal POZ domain and six C-terminal zinc finger DNA-binding motifs. Much work has been directed toward elucidating the role of BCL-6 in both normal physiology and tumorigenesis. Gene targeting studies have demonstrated that disruption of BCL-6 through homologous recombination results in a striking Th2-type inflammatory disease (8–10). These reports indicate an important role for BCL-6 in the normal regulation of signaling events downstream of IL-4 and other cytokines responsible for the generation of Th2 responses. Modulation of chemokine expression provides another potential avenue of immune system regulation by BCL-6, which has been implicated in the regulation of MCP-1, MCP-3, MRP-1, MIP-1α, and IP-10 (11, 12). Further studies have implicated BCL-6 in the regulation of normal cell growth and development: mice homozygous for a deletion of BCL-6 are runted, and there is some evidence to indicate that overexpression of BCL-6 may either promote or inhibit apoptosis in various cell lines (8, 10–15). BCL-6 has also been implicated in the control of plasma cell development through the repression of Blimp-1, an important regulator of this process (16). In addition, DNA chip analysis has identified a number of genes involved in lymphocyte differentiation and cell cycle regulation as targets of repression by BCL-6 (11).

The observation that an in vitro defined binding site for BCL-6 resembles the STAT consensus sequence suggests a mechanism for BCL-6 modulation of cytokine signaling whereby BCL-6 is targeted to cytokine-regulated genes by a recognition element shared between the repressor and the STAT proteins responsible for transducing cytokine-activated transcription pathways. In a previous study, we presented evidence suggesting that BCL-6 regulates the expression of a specific subset of Stat6-dependent genes (17). In particular, BCL-6 was shown to modulate transcription of the murine germline ε promoter, but not the CD23b promoter. Lymphochip analysis of genes regulated by BCL-6 provides additional support for the argument that CD23 is not repressed by BCL-6 (11). The mechanism for the selective activity of BCL-6 is not known. However, preliminary studies have revealed two characteristics of the germline ε promoter that may contribute to its ability to act as a target of BCL-6-mediated repression. The first is the relative strength of the BCL-6-binding site at mle−111/−102 compared with its putative binding site in the CD23b promoter, as assessed by unlabeled competition experiments (17). Second, as described in this report, DNase I footprinting assays indicate the presence of two additional BCL-6-binding elements in the mle promoter. These findings suggest two non-mutually exclusive hypotheses regarding target selection by BCL-6. On the one hand, the cumulative affinities of individual BCL-6-binding elements for a promoter may determine the ability of BCL-6 to regulate transcription of a given promoter. Alternatively, the presence of multiple recognition sites may drive BCL-6 binding via cooperative interactions between BCL-6 molecules bound to a responsive promoter.

Cooperative binding requires a domain capable of associating with other proteins in a homo- or heterotypic fashion. The...
POZ domain is a 120-amino acid motif generally found at the extreme N terminus of broad-complex, traintrack and bric-a-brac/POZ proteins. Family members demonstrate extensive homology throughout the length of this hydrophobic domain, with 30–50% conservation of amino acid sequence identity (18, 19). Numerous overexpression studies have catalogued the ability of POZ domain-containing proteins to form homo- or heterodimers; furthermore, the POZ domain is suspected to mediate the formation of larger oligomeric structures in vivo (4, 18–24). The recently solved crystal structure of the POZ domain of PLZF (a zinc finger transcription factor implicated in retinoic acid-unresponsive acute promyelocytic leukemia) revealed the PLZF dimer to be considerably more stable than the monomeric form, confirming the multimeric association of the POZ domain (19, 25). The hydrophobic dimerization interface of the PLZF POZ domain is extensively distributed throughout the domain and involves ~25% of the monomer surface area in a tightly intertwined dimer, as is characteristic for an obligate homodimer (19). Thus, both biochemical and structural studies indicate a role for the POZ domain in mediating multimeric protein–protein interactions.

In this study, we investigate the cooperative and competitive associations of BCL-6 with the murine germline e promoter. Our observations suggest a mechanism for BCL-6-mediated repression that is dependent upon its ability to cooperatively bind to observations suggest a mechanism for BCL-6-mediated repression of BCL-6 by positively acting transcription factors. An activation-dependent manner through the competitive displacement of BCL-6 by Stat6 demonstrated the ability of these proteins to protect an Stat6-binding site at ~167/55 luciferase reporter construct or mutant germ line e promoter fragments (~167 to +55) were end-labeled on the noncoding strand. The probes (5000 cpm) were incubated for 20 min at room temperature with the indicated amounts of purified recombinant proteins using the Amersham Biosciences Biotech SureTrack footprinting system as described previously (17).

Cell Lines, Transient Transfection, and Reporter Gene Assays—M12.1.4 cells were maintained in RPMI 1640 medium supplemented with 10% fetal calf serum, 100 units/ml penicillin, 100 units/ml streptomycin, 2 mM L-glutamine, and 0.1 mM β-mercaptoethanol. Cells (5 × 10⁶) were transfected by electroporation as described (28). The transfection mixture contained 5 µg of either germ line e promoter ~167/55 luciferase reporter construct or mutant germ line e promoter-luciferase reporter construct (S2mut or S3mut as described above), 0.5 µg of pRL Renilla reporter plasmid (Promega), and the indicated amount of BCL-6 expression vector. Vector DNA (pMT2T) was added as necessary to achieve a constant amount of transfected DNA. Following transfection, cells were incubated in the presence or absence of 10 units/ml murine recombiant IL-4 for 24 h. After 24 h, the luciferase activity of cells transfected with the germ line e reporter was measured. Transfection efficiency was normalized relative to Renilla activity.

RESULTS

Characterization of a Novel BCL-6-binding Element within the mle Promoter—Initial DNase I footprinting studies of the murine germline e promoter using the zinc finger DNA-binding domain of BCL-6 fused to GST (GST-BCL-6ZF) and purified Stat6 demonstrated the ability of these proteins to protect an overlapping region of the promoter at ~111 to ~102 relative to the transcriptional start site (17). Additional footprinting assays of the mle promoter suggested the presence of two additional BCL-6-binding sites: one at the transcriptional start site (S1) and another (S3) located ~25 bp downstream of S1 (21). Initial footprinting experiments, wild-type and mutant germ line e promoter fragments (~167 to +55) were end-labeled on the noncoding strand. The probes (5000 cpm) were incubated for 20 min at room temperature with the indicated amounts of purified recombinant proteins using the Amersham Biosciences Biotech SureTrack footprinting system as described previously (17).

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mIdine that is thought to confer STAT binding activity to B6BS (Fig. 2A).

To assess the ability of BCL-6 and Stat6 to recognize this region of the murine germline ε promoter, an oligonucleotide probe corresponding to mIε -167/-135 was generated and used in EMSA. The probe was labeled and incubated with whole cell extracts prepared from either the Mutu I (BCL-6-positive) or Mutu III (BCL-6-negative) cell line. These cells had been cultured for 1 h prior to harvest in either the absence or presence of IL-4 (to induce the phosphorylation and DNA binding activity of Stat6). These extracts have previously been shown to have Stat6 binding activity (17). EMSA analysis of extracts prepared from the BCL-6-positive Mutu I cell line and incubated with the mIε -167/-135 probe revealed a single complex absent in EMSAs performed with extracts from the BCL-6-negative Mutu III cells (Fig. 2B). This constitutive complex was ablated upon incubation with an antiserum specific to BCL-6, but remained intact following incubation with preimmune serum, confirming that this complex contains BCL-6.

The absence of an IL-4-induced complex in extracts prepared from either the Mutu I or Mutu III cell line, as well as the lack of a complex sensitive to incubation with antiserum specific to Stat6, indicates the inability of Stat6 to bind to this region of the mIε promoter.

As we had previously demonstrated a correlation between the strength of BCL-6 interaction with its binding site within a promoter and the efficiency of BCL-6 repression of promoter activity (17), we wished to compare the affinity of BCL-6 for the S1, S2, and S3 binding sites found in the murine germline ε promoter. Unlabeled oligonucleotides derived from these three potential BCL-6 sites were assessed for their ability to compete for BCL-6 binding with a labeled probe generated from the 5′-BCL-6 recognition element of the mIε promoter (S3). Extracts prepared from cells of the murine B lymphoma line M12.4.1 were incubated with labeled S3 probe and increasing concentrations of the appropriate unlabeled competitor, and the reaction products were analyzed by EMSA (Fig. 2C). The results demonstrate that S2 and S3 competed for binding to the labeled probe with comparable efficiency, indicating that S3, like S2, is a high affinity binding site for BCL-6. However, the binding element at the transcriptional start site (S1) was unable to compete for BCL-6 binding within the range of concentrations used in this assay, and it may therefore be considered a weak binding site for BCL-6.

These experiments define a novel BCL-6 recognition element within the murine germline ε promoter. Interestingly, this sequence is the first reported BCL-6-binding element that does not also serve as a Stat6-binding site. Furthermore, unlabeled competition studies reveal a hierarchy of affinity for BCL-6 among the three potential binding sites for BCL-6 found within the mIε promoter, whereby the two most 5′ sites (S2 and S3) strongly bind BCL-6, and the 3′-element found at the transcriptional initiation site (S1) binds BCL-6 only weakly.

Repression of the mIε Promoter by BCL-6 Requires Multiple BCL-6-binding Sites—The experiments detailed above describe the presence of multiple binding sites for BCL-6 in the murine germline ε promoter. It remained uncertain, however, whether the presence of these additional binding sites is required for the efficient repression of promoter activity by BCL-6. To explore the functional significance of the BCL-6-binding sites within the IL-4-responsive region of the murine germline ε promoter −167/−55 in which the binding of BCL-6 to S2, S3, or both sites was disrupted (S2mut, S3mut, and S2S3mut, respectively). Stat6 binding to S2 remained intact in the S2 mutant as described previously (17). These promoter mutants were used to drive a luciferase reporter in transient transfection assays. The constructs were cotransfected with either a control plasmid or a BCL-6 expression vector into M12.4.1 murine B lymphoma cells. Fig. 3B demonstrates the results of experiments using a single concentration (2.5 μg) of BCL-6, whereas Fig. 3C shows a dose-response curve with increasing concentrations of BCL-6. Transfected cells were cultured either alone or in the presence of IL-4 for 24 h and then harvested and assayed for luciferase activity. Interestingly, the S3 mutants, and perhaps also the S2 mutants, were hyperactivated upon cytokine induction, perhaps due to the inability of endogenous BCL-6 to bind to the mutant promoter (Fig. 3B). As expected, cotransfection with BCL-6 resulted in repression of the IL-4-induced activation of the wild-type promoter. In contrast, the ability of BCL-6 to repress IL-4-induced transcription of the mIε promoter bearing a mutant S2 BCL-6-binding site was compromised (Fig. 3, B and C). The ability of BCL-6 to repress IL-4-induced transcription of the mIε promoter mutated at S3 was variably decreased, although never to the levels of the S2 mutant (Fig. 3, B and C). Like the S3 mutant, the S2/S3 double mutant showed markedly increased levels of induction compared with the wild-type promoter; like the S2 mutant, the double mutant was not efficiently repressed upon cotransfection of BCL-6. These results demonstrate the requirement of intact BCL-6-binding sites at both mIε −111/−102 and −156/−147 for the proper function of the repressor.

BCL-6 Binds Cooperatively to Multiple Sites in the mIε Promoter in a POZ Domain-dependent Manner—The identification of two functionally important BCL-6-binding elements within the murine germline ε promoter suggests at least two mechanisms whereby BCL-6 might regulate mIε transcription. BCL-6 might independently bind the various sites found in the germ-
line ϵ promoter; in this manner, a graded response may be effected through sequential occupancy of multiple BCL-6 sites of varying affinities. Alternatively, efficient binding of BCL-6 to the germline ϵ promoter may require the cooperative interaction of several molecules binding to multiple elements in the promoter. Cooperativity is often employed to generate a threshold response, in which small differences in protein concentration can lead to triggered responses. The sensitivity of the trigger may determined by the affinity of the stronger binding site (30–32).

To distinguish between these two models of BCL-6-mediated gene regulation, we performed in vitro DNA binding studies in which we assessed the ability of recombinant BCL-6 to bind wild-type and mutant mI ϵ promoters. Increasing concentrations of baculovirus-expressed BCL-6 were incubated with either wild-type murine germline ϵ promoter −167/−55 or promoters in which BCL-6 binding to either S2 or S3 was specifically disrupted (S2mut or S3mut, respectively). DNase I footprinting studies were performed in which increasing amounts of ΔPOZ mutant were bound to the wild-type, S2mut, or S3mut promoter prior to digestion with nuclease. These assays demonstrated little difference in the ability of the ΔPOZ mutant to protect the S2 and S3 sites of the wild-type promoter compared with either of the mutants (Fig. 4, lanes 6–9, 15–18, and 24–27). Furthermore, 20–30-fold more ΔPOZ was required to protect S2 and S3 from DNase I digestion compared with the full-length BCL-6 protein (Fig. 4, lanes 2–5 and 6–9). Interestingly, a prominent footprint at S1 was detected in assays using the ΔPOZ mutant, but not in experiments with full-length BCL-6; disruption of the S2 and S3 sites had no affect on the pattern of DNase I digestion at S1. This behavior is characteristic of the manner in which a POZ protein family member interacts with a promoter containing a single protein-binding site. Therefore, the results of these studies suggest that BCL-6 regulates transcription of the murine germline ϵ promoter by cooperatively binding to at least two sites within the promoter: S2 (−111 to −102), a site that is shared with Stat6, and S3 (−157 to −149), a newly identified site that is not recognized by Stat6. Binding of BCL-6 to the low affinity site at S1, if it occurs at all under physiologic conditions, is non-cooperative and requires very high concentrations of the repressor. Finally, the cooperative binding of BCL-6 to S2 and S3 requires an intact POZ domain and is likely mediated through the POZ-dependent dimerization of BCL-6.
Stat6 and C/EBPβ Effectively Compete for Binding to mIe S2—Stat6 and C/EBPβ are cytokine-inducible transcription factors that bind to mIe S2 and may therefore regulate germine e expression in part through competitive or cooperative association with BCL-6. In an effort to delineate the interactions of BCL-6, Stat6, and C/EBPβ on the murine germine e promoter, we performed a series of binding studies in which combinations of recombinant full-length BCL-6, recombinant phosphorylated Stat6, and GST-C/EBPβ were incubated with a labeled probe corresponding to wild-type mIe e (39). The results are given as the mean total luciferase activity of two separate experiments and are normalized to Renilla activity.

### Cooperative BCL-6 Binding Required to Repress Transcription

**Fig. 3.** Repression of germline e promoter transcription is dependent on intact BCL-6-binding sites at both S2 and S3. A, shown is a schematic diagram of the mutations used in this study. S2mut binds Stat6 (but not BCL-6) at S2. BCL-6 binding at S3 is intact. S3mut does not bind BCL-6 at S3, but has an intact S2 site. S2/S3mut binds Stat6 at S2, but does not bind BCL-6 at either S2 or S3. These mutations were generated within the context of germine e promoter −167/+55. WT, wild-type murine Ie promoter. B, 5 × 10^6 M12.4.1 cells were cotransfected with a luciferase reporter driven by the indicated variations in the germine e promoter (wild-type, S2mut, S3mut, and S2/S3mut) and either control plasmid or 2.5 μg of BCL-6 expression vector. Following electroporation, the cells were divided and cultured either alone or in the presence of IL-4 (10 units/ml) for 24 h. The results are given as the mean ± S.D. from three separate experiments and are normalized to the results of germline e promoter transcription (29). It should be noted, however, that the use of a bacterially expressed GST fusion protein confounds interpretation of these results, as C/EBPβ binding is known to be affected by posttranslational modifications (40, 41) and as the 26-kDa GST footprint was also competed out by very high levels of BCL-6 and may indicate the formation of a Stat6 tetramer at these two sites. Further study will be required to determine whether these two sites, which are separated by two helical turns, represent the association of two Stat6 dimers to form a tetramer, as has been reported for other members of the STAT family (36–38). These studies also reveal a potential role for the IL-6- and lipopolysaccharide-inducible transcription factor C/EBPβ in the regulation of BCL-6 and Stat6 binding activity. Recombinant C/EBPβ expressed as a fusion protein with GST was an extremely effective competitor of BCL-6 for binding to S2 (Fig. 5B). In contrast, C/EBPβ appeared to increase the affinity of Stat6 for S2, as was demonstrated by the strengthening of the Stat6 footprint in the presence of GST-C/EBPβ (Fig. 5C, compare lanes 4 and 5). Stat6 and C/EBPβ have similarly been reported to cooperatively interact at the corresponding site in the human germine e promoter (39). These observations correlate with the results of studies that demonstrate the requirement of an intact C/EBP-binding element for efficient IL-4- and Stat6-mediated germine e promoter transcription (29). It should be noted, however, that the use of a bacterially expressed GST fusion protein confounds interpretation of these results, as C/EBPβ binding is known to be affected by posttranslational modifications (40, 41) and as the 26-kDa GST
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FIG. 4. BCL-6 binding to the mle promoter is cooperative and depends on an intact POZ domain. Binding of increasing concentrations of purified recombinant BCL-6 or a truncated protein lacking the POZ domain (APoz) (6, 18, 54, and 162 μM) to either the wild-type murine germine e promoter (WT; lanes 1–9) or mle promoter variants in which BCL-6 binding was specifically disrupted at S2 (S2mut; lanes 10–18) and S3 (S3mut; lanes 19–27) was compared by DNase I footprinting analysis. Lanes 1, 10, and 19 (controls) were incubated with bovine serum albumin alone. The positions of S1, S2, and S3 are indicated.

moiety may itself interfere with BCL-6 binding to S2.

In conclusion, we have determined that both Stat6 and C/EBPβ are strikingly effective competitors of BCL-6 for binding to S2 in the murine germine e promoter. This is in contrast to the apparent cooperative interaction demonstrated by Stat6 and C/EBPβ at the same locus. Finally, we have evidence that suggests the presence of a novel Stat6-binding site ∼20 bp downstream of the Stat6 element at S2, which may indicate the formation of a Stat6 tetramer in the mle promoter.

DISCUSSION

In a previous study, we presented evidence indicating that BCL-6 is involved in the transcriptional regulation of only a subset of Stat6-responsive genes; we further established the murine germine e promoter as a physiologic target of BCL-6-mediated repression (17). In this study, we have described experiments designed to identify characteristics of the mle promoter that allow its regulation by BCL-6. To this end, we have identified a novel BCL-6-binding site, which we have termed S3, ∼50 bp upstream of the BCL-6/Stat6-binding site at −111 to −102 (S2) of the mle promoter. DNA binding studies using wild-type and mutant mle promoters demonstrated the highly cooperative nature of BCL-6 binding to a promoter with multiple BCL-6-binding sites. Similar studies utilizing mutant BCL-6 proteins illustrated the dependence of this cooperativity on the presence of an intact POZ domain. Consistent with a model of synergistic BCL-6 binding to S2 and S3 of the murine germine e promoter, disruption of BCL-6 binding to either one of these sites compromised the ability of BCL-6 to properly regulate the IL-4-induced activation of the promoter from the remaining BCL-6-binding site in transient transfection assays. As the mutants used in these experiments did retain some BCL-6 binding activity, the levels of BCL-6 achieved in transient transfection appeared to overwhelm the system, and the results of these transfection experiments are not as striking as might be predicted in an uncomplicated cooperative system. However, the biochemical and in vivo data together support a model in which BCL-6 modulates gene transcription by cooperatively binding to promoters that have multiple high to medium-affinity binding elements recognized by BCL-6. In addition to elucidating a mechanism of mle promoter regulation, we believe that these studies have important implications regarding the general regulation of gene expression by BCL-6.

The results of our earlier studies indicated a correlation between the affinity of BCL-6 for an individual binding site within a promoter and the efficiency of BCL-6 repression of promoter activity, leading to the speculation that BCL-6 target selection might be determined through some equation of protein concentration and binding site affinity (17). Similar gradient affinity models have been proposed for the action of other transcriptional regulators, such as was originally suggested for the Drosophila morphogen bicoid, which is responsible for determining stripe pattern in the anterior half of the fly embryo (42). This model can be extended to promoters containing multiple independent binding sites for a given transcription factor. Exquisitely graded responses might be elicited through the modulation of both protein levels and the binding affinities of the various recognition elements for that protein found within a promoter. However, the discovery of an additional, high affinity BCL-6-binding site in the murine germine e promoter that appears to cooperate with the originally defined site at −111 to −102 adds another layer of complexity to any discussion of BCL-6 target specificity.

Cooperative interactions can result in a 10–1000-fold increase in the efficiency with which a transcription factor recognizes a low affinity binding site, and are therefore less dependent on the intrinsic affinities of the involved binding sites than would be predicted by the gradient affinity model (43, 44). In a cooperative system, responsiveness is instead determined by a combination of factors related to promoter topology: the number of binding sites, the intrinsic affinities of each of those sites, the level of cooperativity among the various sites, the spacing between the sites, and the presence of binding sites for other factors with which it might also cooperatively interact. Responsiveness is also dependent upon characteristics of the protein itself, such as flexibility, potential for oligomerization, and the presence of interactive domains capable of recruiting additional factors that enhance its ability to regulate transcription. These features are therefore likely to dictate BCL-6 target specificity. However, although we have established the basic elements required to demonstrate cooperativity of BCL-6 binding and activity at the mle promoter (i.e. significantly enhanced binding and repressional synergy with the presence of an additional BCL-6-binding site), much remains to be determined regarding the structural characteristics of a promoter responsive to regulation by BCL-6.

The murine germine e promoter provides an ideal system with which to dissect the factors that determine BCL-6 target selection. The mle promoter contains three elements protected by recombinant BCL-6 in DNase I footprinting studies, yet only two of these sites (S2 and S3) appear to demonstrate a cooperative association. These two sites exhibit a relatively high intrinsic affinity for BCL-6 and are separated by 47 bp, whereas the non-interacting site (S1) binds BCL-6 only weakly and is located at the transcriptional start site, 110 and 156 bp downstream of S2 and S3, respectively. At this point, it is unclear whether the failure of S1 to participate in the repressor complex is due to its low affinity for BCL-6 (despite the documented ability of other transcription factors to drive occupancy
Control lanes (NF- also demonstrated the cooperative interaction of Stat6 and coherently interact at the same locus; previous studies have increasing concentrations of GST-C/EBP expression of the mI BCL-6 and transcriptional activators believed to regulate the promoter (54). These findings support a model of germline the presence of an intact POZ domain. 

Explanation for the dependence of transcriptional repression on the repressor complex provides a reasonable mechanistic ex-

POZ protein family members (18). It is therefore possible that BCL-6 may, in some circumstances, cooperatively associate with POZ domain-containing proteins other than BCL-6. Furthermore, protein-protein interactions mediated by the POZ domain are not limited to associations between family members. One of the more interesting developments was the discovery that the POZ domains of BCL-6, PLZF, and BACH2 are able to mediate heterophilic interactions with multiple components of the histone deacetylase repressor complex, including the SMRT/N-CoR corepressors (silencing mediator of retinoid and thyroid hormone receptor/nuclear receptor corepressor), BCoR (BCL-6-interacting corepressor, which appears to specifically interact with BCL-6 to the exclusion of other POZ proteins), mSin3A, and histone deacetylase-1 (45–52). Although apparently not a general mechanism of POZ domain-mediated repression (53), the ability of some family members to recruit the repressor complex provides a reasonable mechanistic explanation for the dependence of transcriptional repression on the presence of an intact POZ domain.

In vitro DNA binding assays performed with combinations of BCL-6 and transcriptional activators believed to regulate the expression of the mIe promoter demonstrated the ability of Stat6 and GST-C/EBPβ to cooperate very effectively with BCL-6 for binding to S2. In contrast, Stat6 and GST-C/EBPβ appear to cooperatively interact at the same locus; previous studies have also demonstrated the cooperative interaction of Stat6 and NF-κB on a fragment derived from the murine germline e promoter (54). These findings support a model of germline e transcript regulation whereby BCL-6 bound to the mIe promoter maintains the promoter in an inactive state through the activity of the associated SMRT-histone deacetylase repressor complex. Repression is relieved in an activation-dependent manner both through the competitive displacement of BCL-6 by C/EBPβ and Stat6, which is further stabilized by cooperative interactions with C/EBPβ and NF-κB, and through the recruitment of CBP/p300, which have been reported to interact with various STAT proteins (including Stat6), C/EBPβ, and NF-κB (55–59). The histone acetyltransferase activity of these coactivators can then reverse the repressive chromatin modifications catalyzed by the BCL-6-associated histone deacetylase, resulting in induction of germline e promoter transcription.

In contrast to all other known or suspected BCL-6-binding sites, the new element we have identified at S3 diverges from the Stat6 consensus motif T/CTC(N)2GA. We have demonstrated that Stat6 does not, in fact, recognize this element. The description of a physiologically relevant BCL-6-binding site that is not a target of Stat6 complements the results of studies that have demonstrated the persistence of inflammatory disease in BCL-6−/− mice (17, 60), providing further support of an additional role for BCL-6 in the regulation of non-Stat6-dependent pathways. Recent data using Lymphochips have also identified BCL-6-regulated genes that have not been identified as Stat6 target genes. Analysis of the regions of these genes required for BCL-6 repression will determine whether cooperative BCL-6 binding is a general requirement for BCL-6 regulation.

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