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Functional Long-range Interactions of the IgH 3' Enhancers with the *bcl*-2 Promoter Region in t(14;18) Lymphoma Cells

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Abstract

To better understand the mechanisms underlying the role of the immunoglobulin heavy chain gene (IgH) 3' enhancers on *bcl*-2 transcriptional deregulation in t(14;18) lymphoma, we characterized the physical interactions of the IgH 3' enhancer region with the *bcl*-2 promoters. Using the chromosome conformation capture (3C) technique, we found that the IgH 3' enhancers physically interact with the *bcl*-2 promoter region over a 350 kb genomic region in t(14;18) lymphoma cells. No interactions of the *bcl*-2 promoter region with sequences distant to the IgH enhancers were observed. The physical interactions of the IgH enhancers with the *bcl*-2 5' region are functionally involved in the transcriptional control of *bcl*-2. The histone deacetylase inhibitor, trichostatin A (TSA), repressed *bcl*-2 transcription and decreased the IgH enhancer-*bcl*-2 promoter region interactions. We showed by chromatin immunoprecipitation assay (ChIP) and siRNA transfection studies that the POU2 family transcription factor Oct-2 and its cofactor Bob-1 play a critical role in mediating the IgH enhancer-*bcl*-2 promoter region interactions. This study reveals a new aspect of the regulatory role of the IgH 3' enhancers on *bcl*-2 transcription in t(14;18) lymphomas.

Keywords

t(14;18) lymphoma; *bcl*-2; IgH enhancer; spatial interactions

INTRODUCTION

The t(14;18)(q32;q21) is the most common chromosomal translocation in human low grade lymphomas. More than 85% of follicular lymphomas and 25% of diffuse B-cell lymphomas possess this translocation. As a result of the translocation, one allele of the anti-apoptotic *bcl*-2 gene from chromosome 18q21 is juxtaposed to the immunoglobulin heavy chain (IgH) locus on chromosome 14q32. In t(14;18) lymphoma, the untranslocated *bcl*-2 allele is silent, and the translocated *bcl*-2 allele is aberrantly transactivated (Graninger *et al.*, 1987). The prolonged cell survival due to increased *bcl*-2 expression from the translocated allele has been shown to contribute to the development of lymphomas and confer resistance to a variety of anticancer therapies (Desoize, 1994; Hockenberry *et al.*, 1990; Reed *et al.*, 1994; Schmitt and Lowe, 2001).

Two promoters mediate transcriptional control of the *bcl-2* gene (Seto *et al.*, 1988). The 5' promoter (P1) is located 1,386 to 1,423 bp upstream of the *bcl-2* translational start site, and it

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is GC-rich with multiple Sp1 sites. The start sites of the 3' promoter (P2) are located 1.3 kb downstream of the P1 promoter. The P2 promoter has a classic TATA and CAAT box. The major positive cis-elements for P1 activity are a cAMP response element (CRE) and several Sp1 sites (Ji *et al.*, 1996; Wilson *et al.*, 1996). NF-κB family members bind to these sites and are essential for *bcl*-2 deregulation in t(14;18) cells (Heckman *et al.*, 2002). The major positive regulatory site of P2 is a binding site for the homeodomain protein Cdx. A-Myb, a strong activator of P2 promoter activity, acts through the Cdx site (Heckman *et al.*, 2000), as does C/EBPα. (Heckman *et al.*, 2003b). We also showed that Oct transcription factors mediate t (14;18) lymphoma cell survival by directly regulating *bcl*-2 expression (Heckman *et al.*, 2006).

The 3' IgH enhancers consist of four DNase I-hypersensitive sites, HS1, 2, 3, and 4, which have been shown to function as a locus control region in B cells (Khamlichi *et al.*, 2000; Madisen and Groudine, 1994; Mills *et al.*, 1997). (HS sites 1 and 2 are located within 0.5 kb of each other and are usually assayed together as HS1, 2.) Several transcription factor-binding sites have been identified in the IgH 3' enhancer regions, including sites for NF-κB, Oct, Cdx, Pax5/Bsap (B-cell lineage-specific activator protein), Bach2/Maf, AP1, and Ets proteins. The conservation of the Oct, NF-κB, Cdx, AP1 and Ets sites across different species suggests that these sites may be critical for the function of the enhancers (Khamlichi *et al.*, 2000; Mills *et al.*, 1997).

Using in vitro and in vivo models, we have characterized the role that the IgH 3' enhancers play in the deregulation of *bcl*-2 transcription in t(14;18) lymphomas. Our initial transient transfection study showed that the murine IgH 3' enhancers HS1-4 increased *bcl*-2 transcription when they were linked to the *bcl*-2 promoter (Heckman *et al.*, 2003a). To further characterize the role of the IgH 3' enhancers in *bcl*-2 transcriptional deregulation, we established an episomal reporter gene model system in which the *bcl*-2 promoter was linked to regions of the IgH 3' enhancers. This model system reproduces many aspects of *bcl*-2 deregulation in vivo, including the promoter shift and the increased histone H3 acetylation at the promoters (Duan *et al.*, 2007). Our study of endogenous *bcl*-2 mRNA transcription in t(14;18) lymphoma cells using real-time reverse-transcriptase PCR revealed that the increased *bcl*-2 transcripts were derived from both the P1 and P2 promoters (Duan *et al.*, 2007). Interestingly, the activation of the P2 promoter was more dramatic than that of the P1 promoter.

It is thus becoming increasingly clear that the IgH 3' enhancers are critically involved in aberrant transactivation of *bcl*-2 in t(14;18) lymphoma cells, but how the IgH enhancers mediate *bcl*-2 transactivation is not understood. Using the recently developed chromosome conformation capture (3C) technique to detect long-range interactions (Dekker *et al.*, 2002), we demonstrate here that the IgH 3' enhancer regions are physically associated with the *bcl*-2 promoter region over a 350-kb genomic distance. These spatial interactions are functionally involved in the transcriptional deregulation of *bcl*-2 in t(14;18) lymphoma cells. Moreover, Oct-2 and its cofactor Bob-1 are important factors mediating these physical interactions.

RESULTS

Experimental design of the chromosome conformation capture assay for the study of the physical interactions between the IgH enhancers and the*bcl*-2 promoter region

To determine whether the IgH 3' enhancers interact with the *bcl*-2 promoter region, 3C analysis was performed with primers near the *bcl*-2 transcriptional start sites on chromosome 18 and primers on chromosome 14 (105,051,517-105,351,516). The region of chromosome 14 is centered approximately between the two IgH 3' enhancer regions downstream of Cα1 and Cα2 (Figure 1a, b).

The 3C assay measures the proximity and frequency of interaction between two points in chromatin. For the 3C assay, cells are treated with formaldehyde to cross-link proteins to other nearby proteins and DNA. After digestion with an appropriate restriction enzyme, ligation is performed under conditions of low DNA concentration to promote intramolecular ligation of the interacting cross-linked DNA fragments. The cross-links are reversed, and the ligation products are quantified by PCR. 3C analysis using BAC DNA was performed as a control for the real-time PCR efficiency of each primer/probe set (Hagege *et al.*, 2007). To compare the 3C analysis between DHL-4 and DHL-9 cells, the interactions of two separate BamHI sites that are 11.5 kb away from each other in the GAPDH locus on chromosome 12 were determined. Similar interactions were observed in 3C samples at the GAPDH locus from DHL-4 and DHL-9 cells. (DHL-4 cells have the t(14;18) translocation, and DHL-9 cells lack this translocation.)

A higher resolution of the linear arrangement of the *bcl*-2 promoter region (BamHI sites 1-3) and the IgH enhancers (BamHI sites 4-8) in the t(14;18) translocation is shown in Figure 2a. Due to the conserved sequence in the enhancer regions 3' of Cα1 and Cα2, the primers for the two sets of IgH enhancers are the same. Examples of the PCR products with *bcl*-2 promoter region BamHI sites 1, 2, and 3 primers and primers at BamHI sites 4 though 8 of the IgH enhancer region are shown in Figure 2b. The PCR products with the primers at the GAPDH locus in DHL-4 and DHL-9 cells are shown in Figure 2c. As shown in Figure 2d (lanes 1-3), no PCR product was observed with DNA that was not cross-linked and ligated. When PCR was performed with cross-linked and ligated 3C DNA using *bcl*-2 promoter region BamHI site 1 primer and a primer at -90 (BamHI site 10) relative to the IgH enhancer site 4, a very low level of product was observed (Figure 2c, lane 4). In contrast, the yield of the PCR product was higher when *bcl*-2 promoter region BamHI site 1 primer was used with the primer at IgH enhancer BamHI site 4 (Figure 2c, lane 5).

Interactions of the core regulatory regions of the IgH 3' enhancers and the*bcl*-2 promoter region in t(14;18) DHL-4 cells but not in DHL-9 cells

Specific interactions of the *bcl*-2 promoter region BamHI site 1 were observed at the five BamHI sites in the IgH enhancer region, but no significant interactions of BamHI site 1 were observed with regions 5' or 3' to the IgH enhancers (Figure 3a). The results with sequences 5' and 3' to the Cα1 enhancers are shown, and numbering of the IgH region is relative to BamHI site 4, which was set at 0. Similar results were obtained with sequences flanking the Cα2 enhancers (data not shown). *Bcl*-2 promoter region BamHI site 2 also interacted with the five BamHI sites at the IgH enhancers but not with sequences 5' or 3' of the IgH enhancers (Figure 3b). The 3C results demonstrate that interactions of the *bcl*-2 promoter region with the IgH locus peak at the core enhancer regions and dramatically decrease with increasing genomic distance from the enhancers. These studies suggest that the interactions of the *bcl*-2 promoter region with the IgH enhancers are specific and most likely functionally related.

We focused on the interactions of the *bcl*-2 promoter region BamHI sites 1, 2, and 3 with the five BamHI sites at the IgH enhancers as shown in Figure 3c. The BamHI sites 1 and 2 at the *bcl*-2 promoter region have stronger interactions with the IgH enhancer regions than site 3, suggesting that BamHI sites 1 and 2 at the *bcl*-2 promoter region are in closer proximity than site 3 with the IgH enhancer regions. Moreover, the interactions of the *bcl*-2 promoter region with BamHI site 8 are significantly higher than with the other sites in the IgH enhancer region, suggesting a closer proximity of HS4 with the *bcl*-2 promoter regions compared to HS3 and HS1, 2. No interactions of the IgH enhancers with the *bcl*-2 promoter region were detected in DHL-9 cells which lack the t(14;18) translocation (Figure 3d).

The physical association of the IgH enhancers with the*bcl*-2 promoter region is functionally involved in the transcriptional regulation of*bcl*-2

To demonstrate the functional involvement of the physical interactions of the IgH enhancer regions with the *bcl*-2 promoter region, we examined the effect of trichostatin A (TSA) on the physical association of the IgH enhancers with the *bcl*-2 promoter region. We have previously shown that TSA decreases the transcription of the translocated *bcl*-2 gene in t(14;18) lymphoma cells (Duan *et al.*, 2005). DHL-4 cells were treated with 500 ng/ml TSA for 18 hours, and the relative association of the IgH enhancers and the *bcl*-2 promoter region was compared in cells treated with TSA or with solvent only. After normalization for PCR efficiency of the different primer/probe sets, the relative associations of the *bcl*-2 promoter region BamHI sites 1 and 2 and the IgH enhancers are shown in Figure 4a and b, respectively. TSA treatment dramatically decreased the IgH enhancer and *bcl*-2 promoter region interactions at both BamHI sites 1 and 2. We showed previously that treatment with 500 ng/ml TSA for 18 hours decreased *bcl*-2 transcription by 90% (Duan *et al.*, 2005). The markedly decreased interaction of the IgH enhancers with the *bcl*-2 promoter region by treatment of the cells with TSA suggests that the association of the IgH enhancers with the *bcl*-2 promoter region correlates well with transcription.

Oct-2 and its co-activator Bob-1 are involved in mediating the physical association of the IgH enhancers and the*bcl*-2 promoter region

Previously, we determined whether the level of expression of several transcription factors involved in the regulation of *bcl*-2 (CREB, CBP, Sp1, C/EBP, p53) and/or their binding to the *bcl*-2 promoter changed following treatment of DHL-4 cells with TSA (Duan *et al.*, 2005). To extend these studies, we examined the level of expression of Oct family members in DHL-4 cells with and without TSA treatment. As seen in Figure 5a and b, TSA decreased both Oct-1 and Oct-2 protein levels in whole cell lysates and in nuclear extracts. A dose-dependent decrease of Oct-2 mRNA was also observed by real-time RT-PCR analysis (data not shown). TSA did not have a major effect on Bob-1 expression.

To further characterize the influence of TSA on Oct family members in the regulation of *bcl*-2 transcription, quantitative ChIP assays were performed to measure the binding of Oct-1, Oct-2, and Bob-1 to the consensus octamer sites in the IgH enhancer HS1, 2 and HS4 regions. As shown in Figure 6a and b, Oct-2 and Bob-1 bound to the IgH enhancer HS1, 2 and HS4 regions in DHL-4 cells. The binding of Oct-1 to HS1, 2 and HS4 was not above the background level. No significant binding of these factors to the HS3 region was observed (data not shown), which is consistent with the lack of an octamer site in HS3. Treatment with 500 ng/ml of TSA for 18 h dramatically decreased the binding of Oct-2 and Bob-1 to HS1, 2 and HS4 and had no significant effect on Oct-1 binding (Figure 6a and b).

There are no octamer sites in either *bcl*-2 promoter region, but if Oct-2 and Bob-1 are involved in mediating the interactions of the *bcl*-2 promoter region with the IgH enhancers HS1, 2 and HS4, it may be possible to detect Oct-2 and Bob-1 binding at the *bcl*-2 promoter region. To determine whether Oct-2 and Bob-1 bound to the major active sites of the *bcl*-2 P1 and P2 promoters, ChIP assays were performed over these regions. Oct-2 bound to the *bcl*-2 P1 promoter region, and TSA decreased its binding to P1 by more than 50% (Figure 6c). Oct-1, Oct-2, and Bob-1 all bound to the *bcl*-2 P2 promoter, and TSA treatment decreased the binding of all three proteins to the P2 promoter by 40 to 55% (Figure 6d). As there are no consensus octamer motifs at the *bcl*-2 promoter regions, their binding to the promoter regions may derive from the IgH enhancers, which are physically associated with the *bcl*-2 promoter regions in t (14;18) cells. As controls, we examined the binding of Oct-1, Oct-2 and Bob-1 to a region in exon 3 of the *bcl*-2 gene. No octamer binding sites exist in this region. As shown in Figure 6e, no binding above background was detected in exon 3 and there was no change with TSA

treatment. We wished to determine whether the binding of Oct-2 to the *bcl*-2 promoter was dependent on the presence of the IgH enhancers. We performed ChIP assays at the P1 and P2 promoters in the DHL-9 cell line which lacks the t(14;18) translocation. As shown in Figure 6f and 6g, there was no binding of Oct-1, Oct-2 or Bob-1 to either the P1 or P2 *bcl*-2 promoter in DHL-9 cells.

To further investigate the role of Oct-2 in mediating the interaction of the *bcl*-2 promoter region with the IgH enhancers, we used siRNA to decrease Oct-2 expression and then performed 3C analysis. Oct-2 siRNA transfection resulted in an 80% reduction of Oct-2 protein expression in the transfected DHL-4 cells, similar to our previous report (Heckman *et al.*, 2006). Decreased interactions of the IgH enhancers with *bcl*-2 promoter region BamHI site 2 were observed by 3C analysis in cells transfected with siRNA to Oct-2 (Figure 6h). The interactions of IgH enhancer BamHI sites 4, 5, 6, 7, 8 with *bcl*-2 promoter region BamHI site 2 were decreased by 63%, 58%, 42%, 53% and 54%, respectively, relative to the results with the non-targeting siRNA. We also observed a decrease in the interactions of the IgH enhancers with *bcl*-2 promoter region BamHI site 1 in the cells transfected with siRNA to Oct-2 (data not shown). These results demonstrate that Oct-2 is involved in the physical association of the IgH enhancers with the *bcl*-2 promoter region. The effect of the Oct-2 siRNA on disruption of the interactions of the IgH enhancer with the *bcl*-2 promoter region was less than the effect of TSA, suggesting that additional transcription factors are also involved in mediating this association.

DISCUSSION

Gene expression is regulated in part by chromatin accessibility for transcription factors. It is also clear that the spatial structure of the chromatin is critical in the regulation of gene transcription. The β-globin genes and the locus control region, which is located over 50 kb away, form a loop structure during transcription, and the spatial interactions correlate with transcription of the β-globin genes (Palstra *et al.*, 2003; Tolhuis *et al.*, 2002). 3C analysis in murine plasmacytomas cells revealed mutual interactions over 22 kb at the transcriptionally active Igκ locus among the Vκ promoters and three Igκ enhancers (Liu and Garrard, 2005). Interactions between the IgH variable region and the IgH enhancers have also been reported recently (Ju *et al.*, 2007).

It is widely believed that the IgH enhancers are responsible for the increased expression of *bcl*-2 in lymphomas with the t(14;18) translocation, but it is not known how this effect is mediated over a distance of 350 kb. Using chromosome conformation capture, we showed that the IgH enhancers physically associated with the *bcl*-2 promoter region. When we used the two BamHI sites flanking the *bcl*-2 promoter region as anchors, we found that the peak interactions of the *bcl*-2 promoter region were centered at the IgH enhancer HS sites, and they decreased rapidly with increasing genomic distance both 5'and 3' to the enhancers. It is likely that the intervening sequences between the *bcl*-2 promoter region and the IgH enhancers and the sequences between the two enhancer regions loop out, thus allowing the association of the *bcl*-2 promoter region with the IgH enhancers. Further evidence provides support for the hypothesis that these physical interactions are functionally involved in the transcriptional regulation of *bcl*-2 by the IgH enhancers.

We found that TSA, a transcriptional repressor of *bcl*-2, decreased the physical association of the IgH enhancers with the *bcl*-2 promoter region. Our previous study showed that histone H3 localized at the *bcl*-2 promoter region is hyperacetylated in t(14;18) DHL-4 cells compared to non-t(14;18) DHL-9 cells (Duan *et al.*, 2007). TSA treatment decreased *bcl*-2 transcription and decreased histone H3 acetylation at the *bcl*-2 promoters (Duan *et al.*, 2005). These results suggested that the IgH enhancers increased *bcl*-2 transcription through increasing *bcl*-2

promoter histone acetylation in t(14;18) cells. In the current study, we showed that TSA nearly abrogated the physical association of the IgH enhancers with the *bcl*-2 promoter region. The physical interactions of the IgH enhancers and the *bcl*-2 promoter region likely serve as the structural basis for the IgH enhancer-mediated *bcl*-2 promoter histone acetylation and increased transcription.

It is reasonable to assume that several transcription factors are involved in the physical association of the IgH enhancers with the *bcl*-2 promoter region. We investigated whether the expression levels of any of the transcription factors that are known to interact with the IgH enhancers were decreased by TSA treatment. TSA decreased the expression of both Oct-1 and Oct-2 at the protein level. We reasoned that decreased levels of Octamer proteins might be involved in the dramatic reduction in the interactions between the IgH enhancers and the *bcl*-2 promoter region with TSA treatment. Quantitative ChIP assays revealed that the binding of Oct-2 and Bob-1 at HS1, 2 and HS4 was decreased to baseline levels with TSA treatment.

We showed previously that levels of Oct-2 are increased in lymphoma cells with the t(14;18) translocation, and the Oct transcription factors mediate t(14;18) lymphoma cell survival by directly upregulating *bcl*-2 expression (Heckman *et al.*, 2006). We previously observed binding of Oct-2 to the *bcl*-2 P2 promoter region despite the fact that there are no octamer sequences in this region. We have now shown that Oct-2 also binds to the *bcl*-2 P1 promoter. TSA decreased the binding of Oct family members, particularly Oct-2, to the *bcl*-2 promoters. Since no canonical octamer motifs exist at the *bcl*-2 promoters, the Oct family members at the *bcl*-2 promoter region are likely bound at the octamer sites in the IgH enhancers, which are then physically associated with the *bcl*-2 promoters. Decreased expression of Oct-2 by siRNA transfection results in decreased association of the IgH enhancers with the *bcl*-2 promoter region, providing further support for the critical involvement of Oct-2 in mediating the IgH enhancer and *bcl*-2 promoter region interactions.

In summary, our study provides solid evidence for the functional interactions of the IgH enhancers with the *bcl*-2 locus in t(14;18) lymphomas. Oct-2 is one of the transcription factors that bridges the IgH enhancers and the *bcl*-2 promoter region, and the Oct factors play a role in mediating the transcriptional deregulatory effect of the IgH enhancers on the *bcl*-2 promoters. The study of *bcl*-2 deregulation in t(14;18) lymphoma not only provides insight into the pathogenesis of the disease with the potential for identification of new targeted therapies, but additionally, the translocation itself serves as an ideal and unique model system in the study of the transcriptional regulation of a gene by remote enhancers. Furthermore, these results may be relevant to the molecular mechanisms involved in the deregulation of other oncogenes in translocations involving the IgH locus.

MATERIALS AND METHODS

Cell lines, drug treatment and siRNA transfection

The human t(14;18) lymphoma cell line DHL-4 and the DHL-9 lymphoma cell line that lacks a t(14;18) have been described previously (Ji *et al.*, 1996). They were maintained in RPMI medium supplemented with 10% fetal calf serum, 100 U of penicillin/ml, 100 μg of streptomycin/ml, and 2 mM L-glutamine.

Where indicated, cells were treated with trichostatin A (TSA) (Sigma) for 18 h before further analysis. SiRNA targeting Oct-2 and control non-targeting siRNA were obtained from Dharmacon. Transfection of DHL-4 cells with siRNA molecules using Amaxa's Nucleofector device (Amaxa Biosystems) was performed as previously described (Heckman *et al.*, 2006). The transfected cells were purifed by Ficoll separation to separate the dead cells before further analysis.

Chromatin conformation capture (3C) assay

Generation of 3C templates was performed following the standard protocol with minor modifications (Hagege *et al.*, 2007; Miele *et al.*, 2006). Briefly, approximately 2×10^7 cells were centrifuged and resuspended in 45 ml of fresh RPMI1640 medium. The suspended cells were cross-linked by 1% formaldehyde for 10 min at room temperature and then quenched by the addition of glycine to 0.125 M with incubation for 5 min at room temperature. After sitting on ice for 15 min, the cells were harvested and lysed in 2 ml ice-cold lysis buffer (10 mM Tris-Cl, pH 8.0, 10 mM NaCl, 0.2% NP-40) containing protease inhibitors (1 mM phenylmethylsulfonyl fluoride, 1 μg/ml leupeptin, and 1 μg/ml pepstain A) for 15 min. Nuclei were harvested and washed once using ice-cold 1x BamHI buffer and then resuspended in 1x BamHI buffer containing 0.3% sodium dodecyl sulfate (SDS) and incubated at 65°C for 10 min. Triton X-100 was added to 1.8%. 5×10^6 cross-linked nuclei were digested with 400 units BamHI (New England Biolabs) at 37°C overnight with constant rotating to achieve >80% digestion. (BamHI was chosen for the 3C analysis because it cuts regularly several kilobases around the *bcl*-2 promoters and the IgH 3' enhancers. Several other BamHI sites located at a distance from the IgH enhancers were also chosen for the comparison of the relative associations of the core regulatory regions and the flanking regions with the *bcl*-2 promoters.) The restriction enzyme was inactivated by the addition of SDS to 1.6% with incubation at 65° C for 30 min. Triton X-100 was added to 1%, and 5×10^6 digested nuclei were subjected to ligation with 4000 Weiss units of T4 DNA ligase (New England Biolabs) in 7.5 ml of 1x ligation buffer and 1% BSA for 5 h at 16°C. 50 μl of 10 mg/ml proteinase K was added to the ligation complex followed by incubation at 65°C overnight to reverse the cross-links. The DNA was purified twice by standard phenol-chloroform extraction and ethanol precipitation. Purified DNA was dissolved in TE and digested with 10 μg/ml DNase free RNase A at 37°C for 15 min. 100 ng of DNA was used for each PCR analysis. The PCR primers were designed to hybridize as close as possible to the restriction sites.

A control template was utilized to optimize the PCR conditions and determine the minimal amount of ligation product that can be reliably quantified and to control for differences in amplification efficiency between primer sets. Generation of the control 3C template using BAC clone DNA was essentially the same as described above. BAC clone RP11-495C15 or CTD2270-P21 on chromosome 18 was utilized with RP11-815P20 or RP11-731F5 covering 105,051,517 to 105,351,516 on chromosome 14 (Invitrogen). 20 μg of one BAC clone or equal molar amounts of two BAC clones were used to generate 3C control templates which were then mixed with 100 ng of genomic DNA prior to PCR. To control for differences in the 3C efficiency in different samples, an internal standard was utilized, the association of two BamHI sites in the GAPDH locus (Hagege *et al.*, 2007; Splinter *et al.*, 2004). PCR was performed at 95°C for 15 min, cycles 2-45 of 95°C for 10 s, 60°C for 60 s. To confirm that the correct *bcl*-2/IgH hybrid molecules were present in the 3C analysis, the PCR products were electrophoresed on 2% agarose gels, and the gel-purified DNA was sequenced.

The following primers and probes were used for the analysis of*bcl*-2 and IgH enhancer interactions by TaqMan primer/probe based real-time PCR (probes are double-dye 5'FAM and 3'TAMRA).

bcl-2 BamHI site primers and probes:

#1 CAAGATGCCACATAAGGAATCAGTC,
CCTTTGCTCCCACAGAGCCTCACTCTATG

#2 TCATGTGTGTGGAGAGCGTCAAC,
ATGACTGAGTACCTGAACCGGCACCTGCAC

#3 CTGGAAGAATTTGCTAAAGGGTGAAAAG,

TTGGGAATCTGGAAGTCCCAACCCCTTTAG

IgH enhancer BamHI site primers:

#4 CAAAGAAGCCTGCTCCTAAGAACTAC; #5 CACTGAGCCCTGGACCAGAC;

#6 GATGACTCTGAGCATCACGCTGTC; #7 GCTGCCCTCACCACCTGCTG;

#8 TCCAGGGAGGACTCAAGTTTGAG

IgH flanking BamHI site primers:

#9 CACTGAGGAGCTGAGGTTCTGGAGAG; #10 TAGAGTTGAGTGCCTGTGGCTTTTCC

#11 AAATGTATGCCCACCTGGAACCTCAG; #12 ATGGATGCAGTTTCTCCTCCTGCTG

#13 CACGCCTTGAGCTAGTCCATGTGC; #14 CTGTTCGCGGATGCCACAGCCATCTC

#15 ATGCTATCCTCCCACCTCAGTCTTCG;

#16 CTGTGGCTGGTATGTGAGCAGCCAGGT

All quantitative 3C assay results are presented as the average and standard deviation from at least three independent preparations of 3C DNA followed by duplicate real-time PCR analysis.

Quantitative ChIP assay

The ChIP assay was performed as outlined previously (Duan *et al.*, 2005). Antibodies for Oct-1, Oct-2, Bob-1, and IgG were from Santa Cruz Biotechnology. Real-time PCR was performed to quantify the amount of immunoprecipitated DNA. The primer and probe sequences for the ChIP assay of the *bcl*-2 promoter P1 and P2 regions and exon 3 were described previously (Duan *et al.*, 2005; Duan *et al.*, 2007). The following primer and probe sequences were used for the analysis of IgH enhancers HS1, 2 and HS4 regions:

HS1, 2 forward primer: CATGCAAATGGTTGTTTGTTCCAC

HS1, 2 reverse primer: GTGGAGAATCGTGCAAGCTATTT

HS1, 2 probe: Probe: [6-FAM]TTTCTTGCCCTCTGAGGCTGTTTCCA[Tamra-Q]

HS4 forward primer: AGATGGCGATTTGCATTGG

HS4 reverse primer: GAATAGTCAGGAATCCTGCAAACC

HS4 probe: [6-FAM]AAGGCTGGCACCCAGGCAGCT[Tamra-Q]

All quantitative ChIP assay results are presented as the average and standard deviation from at least three independent immunoprecipitations followed by duplicate real-time PCR analysis.

Western blot analysis

Generation of the whole cell lysates and nuclear extracts has been described previously (Duan *et al.*, 2005). Cells were washed with ice-cold PBS and lysed in Triton X-100 extract buffer. Samples (50 μg) were electrophoresed in a 10% SDS-polyacrylamide gel. All antibodies were

from Santa Cruz Biotechnology. Detection of β-actin expression was performed to ensure equivalent protein loading.

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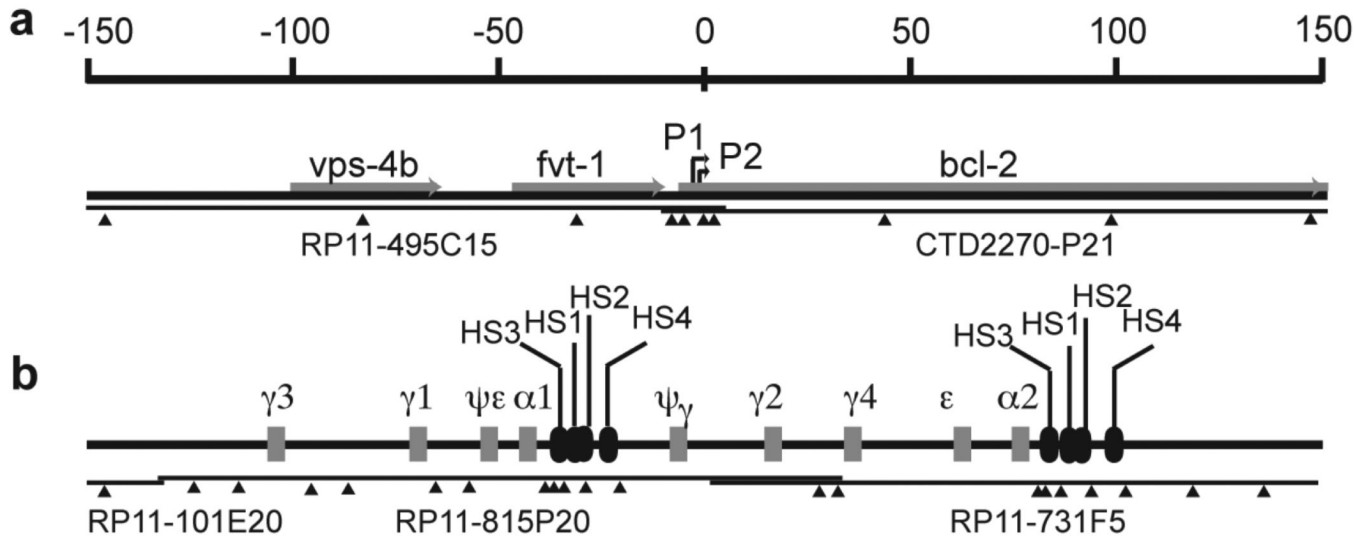

Figure 1. Diagram of the regions of human chromosomes 18 and 14 utilized in the 3C analysis of interactions between the*bcl*-2 promoter region and the IgH enhancers

(**a**) 300 kb genomic region on chromosome 18 from 58,986,879 to 59,286,878 in the *bcl*-2 transcriptional orientation. The thick black line represents the genomic region. Gray lines above this line show the genes in the region with the arrows indicating the transcriptional orientation of each gene. Two vertical arrows represent the major transcriptional start sites of *bcl*-2 (P1 and P2). The lower lines indicate the span of BAC DNAs used to control for PCR efficiency in the 3C analysis. BamHI restriction sites are shown as small triangles under the BAC DNA. (**b**) 300 kb genomic region on chromosome 14 from 105,051,517 to 105,351,516 in the IgH gene transcriptional orientation. The thick black line represents the genomic region, and the IgH genes are shown as gray rectangles. The black ovals following Cα1 and Cα2 represent the hypersensitive sites (HS3, HS1, HS2, and HS4). BAC DNAs and BamHI restriction sites are also shown.

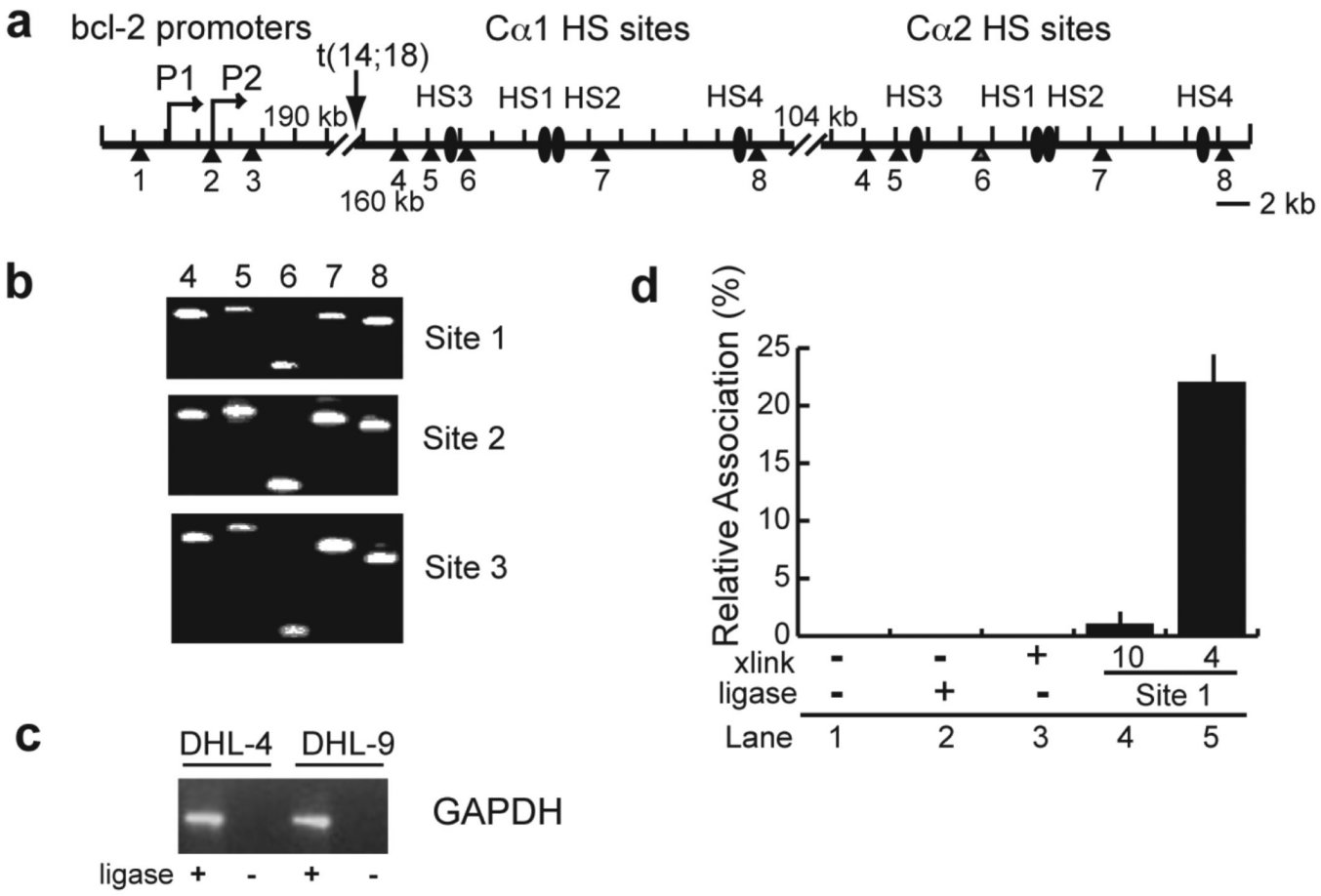

Figure 2. Schematic of the BamHI sites at the*bcl*-2 promoter region and IgH enhancers, and controls for 3C analysis

(**a**) Diagram of the *bcl*-2 promoters and the IgH 3' enhancers in t(14;18) lymphoma cells. The transcription start sites of *bcl*-2 (P1 and P2) and the IgH hypersensitive sites (HS3, HS1, HS2, HS4) following Cα1 and Cα2 are labeled. Numbers under each triangle show the BamHI sites in this region. Approximate distances in kilobases are shown at each of the gaps in the diagram.

(**b**) Agarose gels (2%) showing the PCR products of the 3C analysis of interactions of *bcl*-2 promoter region BamHI sites 1, 2, and 3 with IgH BamHI enhancer sites 4 through 8 in DHL-4 cells.

(**c**) Agarose gel showing the PCR products of the 3C analysis of interactions of two BamHI sites in the GAPDH locus in DHL-4 and DHL-9 cells.

(**d**) Negative controls for 3C analysis in DHL-4 cells. Real-time PCR was performed with *bcl*-2 promoter region BamHI site 1 primer and probe and IgH enhancer BamHI site 4 primer on non-cross-linked DNA (lanes 1 and 2) and cross-linked DNA before and after ligation (lanes 3 and 5). The primer/probe at *bcl*-2 promoter region BamHI site 1 was also used in real-time PCR with the IgH locus BamHI site 10 primer (lane 4). (Lanes 4 and 5 utilized cross-linked and ligated DNA.)

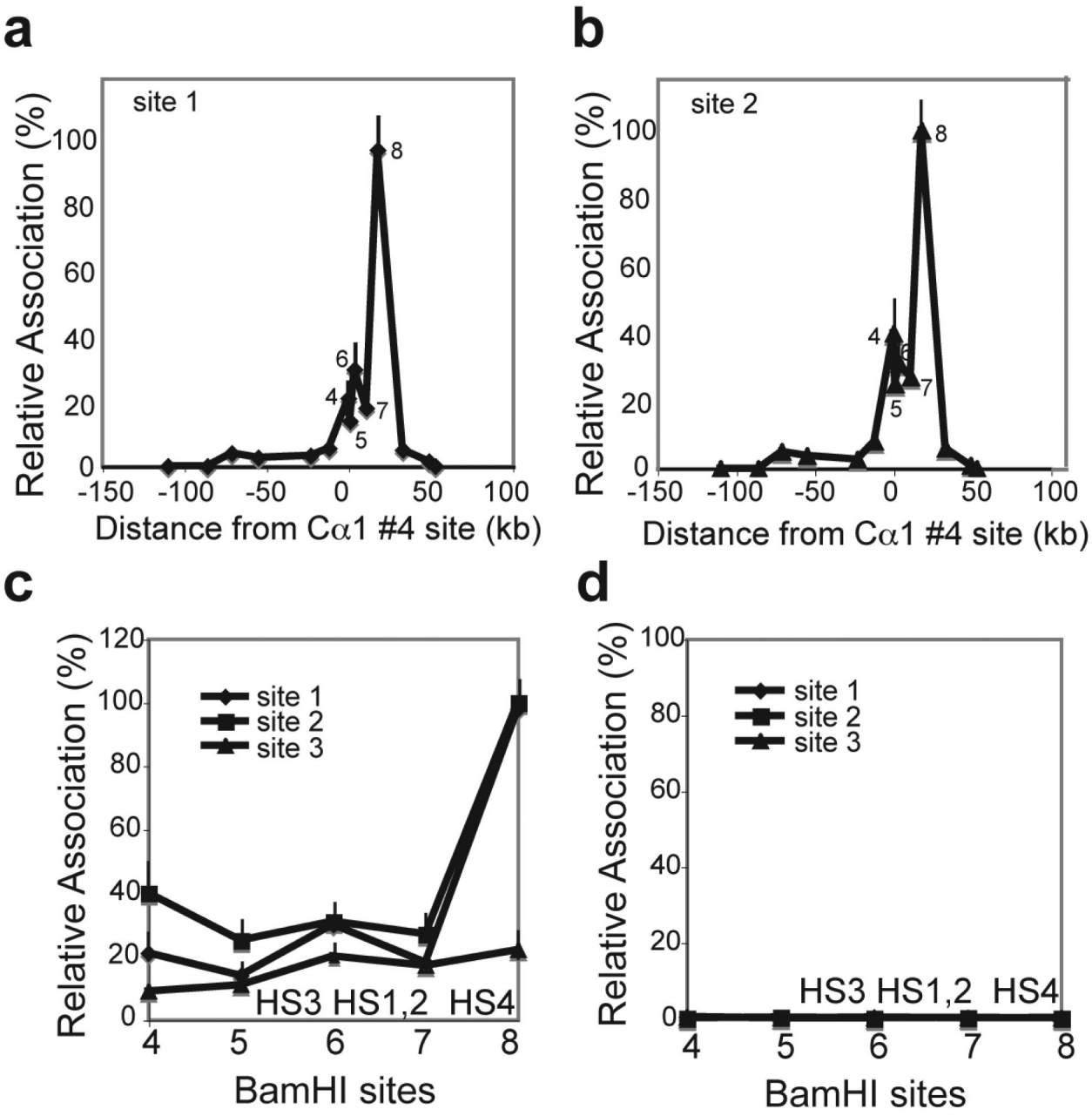

Figure 3. Interactions between the IgH 3' enhancers and*bcl*-2 promoter region are observed in human t(14;18) DHL-4 follicular lymphoma cells but not in non-t(14;18) DHL-9 cells

(**a**) 3C analysis was performed in DHL-4 cells with real-time PCR using the primer and probe at *bcl*-2 promoter region BamHI site 1 and the other set of primers located at the IgH enhancers (BamHI sites 4, 5, 6, 7, 8, labeled on the graph) and regions 5' and 3' of the enhancers. Analysis of 3C samples of BAC DNA RP11-495C15 and RP11-815P20 or RP11-495C15 and RP11-731F5 was utilized to optimize the PCR conditions, and the relative association was determined relative to the interactions at BamHI sites at the GAPDH locus. The x-axis represents the genomic distance from Cα1 #4 site, which was arbitrarily defined as 0. The flanking sequences are 5' and 3' of the Cα1 region. No interaction was observed with sequences

5' and 3' to the Cα2 region although these results are not shown. The relative association is the average of three independent 3C analyses.

(**b**) 3C analysis was performed in DHL-4 cells with real-time PCR using the primer and probe at *bcl*-2 promoter region BamHI site 2 and the other set of primers located at the IgH enhancers and regions 5' and 3' of the enhancers as described for (**a**).

(**c**) 3C analysis was performed in DHL-4 cells with real-time PCR using the sets of primers and probes anchored at the *bcl*-2 promoter region (BamHI sites 1, 2, and 3) and the other set of primers located at the IgH enhancers (BamHI sites 4, 5, 6, 7, 8). The BamHI sites 4 through 8 at the IgH enhancers are shown on the x-axis, and the location of the IgH enhancer HS sites is shown above the x-axis.

(**d**) 3C analysis was performed in DHL-9 cells with real-time PCR using the primers and probes at the *bcl*-2 promoter region (BamHI sites 1, 2, and 3) and the primers at the IgH enhancers (BamHI sites 4, 5, 6, 7, and 8) as described for (**c**).

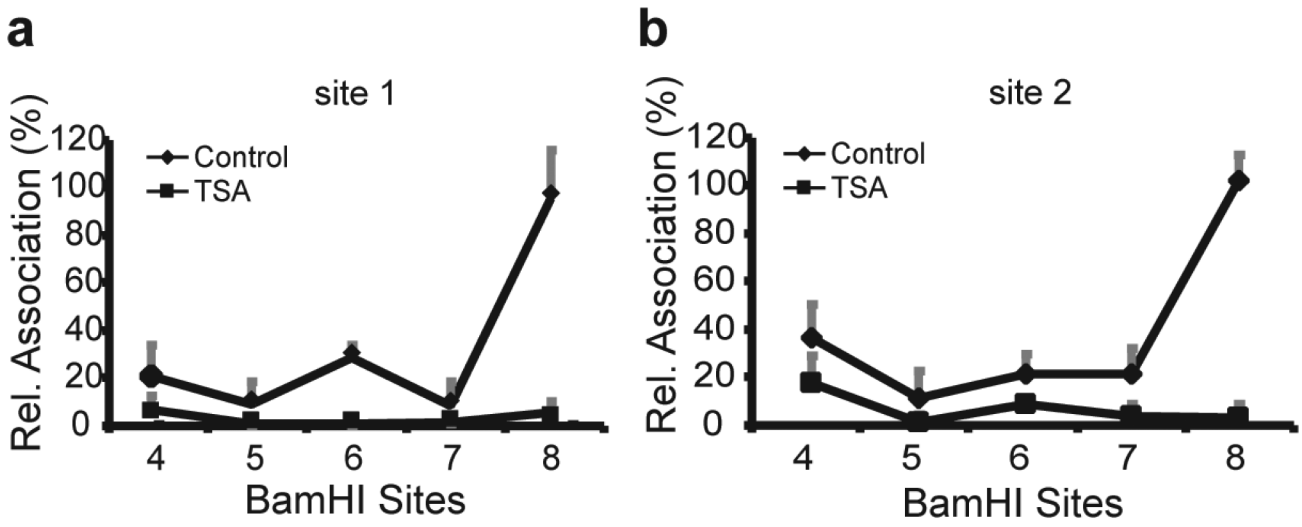

Figure 4. Treatment with TSA markedly decreases the interactions of the IgH enhancers with the*bcl*-2 promoter region

(**a**) The effect of TSA treatment on the interactions of the IgH 3' enhancer BamHI sites 4 through 8 with the *bcl*-2 promoter region BamHI site 1 in DHL-4 cells was determined by 3C analysis. DHL-4 cells were treated with 500 ng/ml TSA for 18 hours. The PCR signal was normalized to the PCR efficiency derived from a parallel analysis of 3C samples of BAC DNA clones as well as to the association of the BamHI sites at the GAPDH locus in the untreated or treated samples.

(**b**) The effect of TSA on the interactions of the IgH 3' enhancer BamHI sites with the *bcl*-2 promoter region BamHI site 2 in DHL-4 cells was determined by 3C analysis as described for (**a**).

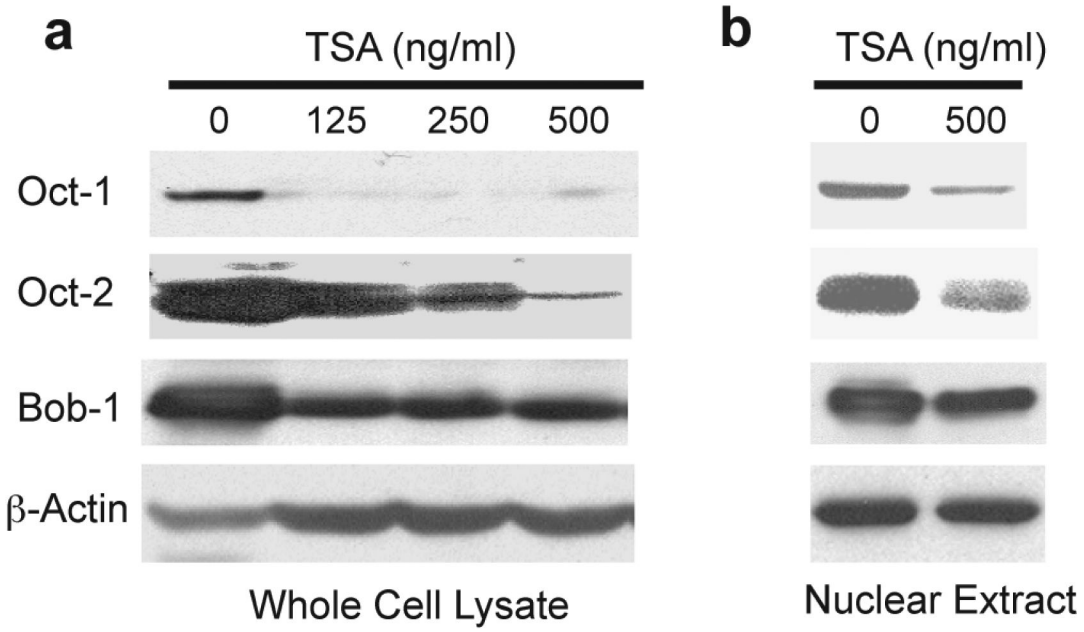

Figure 5. Treatment with TSA decreases the expression of Oct-1 and Oct-2 in DHL-4 cells

(**a**) TSA decreases Oct-1 and Oct-2 expression in whole cell lysates in DHL-4 cells. Cells were incubated with or without TSA for 18 hours. The expression of Oct-1, Oct-2, and Bob-1 was determined by Western blot analysis, and β-actin was used as loading control.

(**b**) TSA decreases Oct-1 and Oct-2 expression in the nuclear extracts of DHL-4 cells.

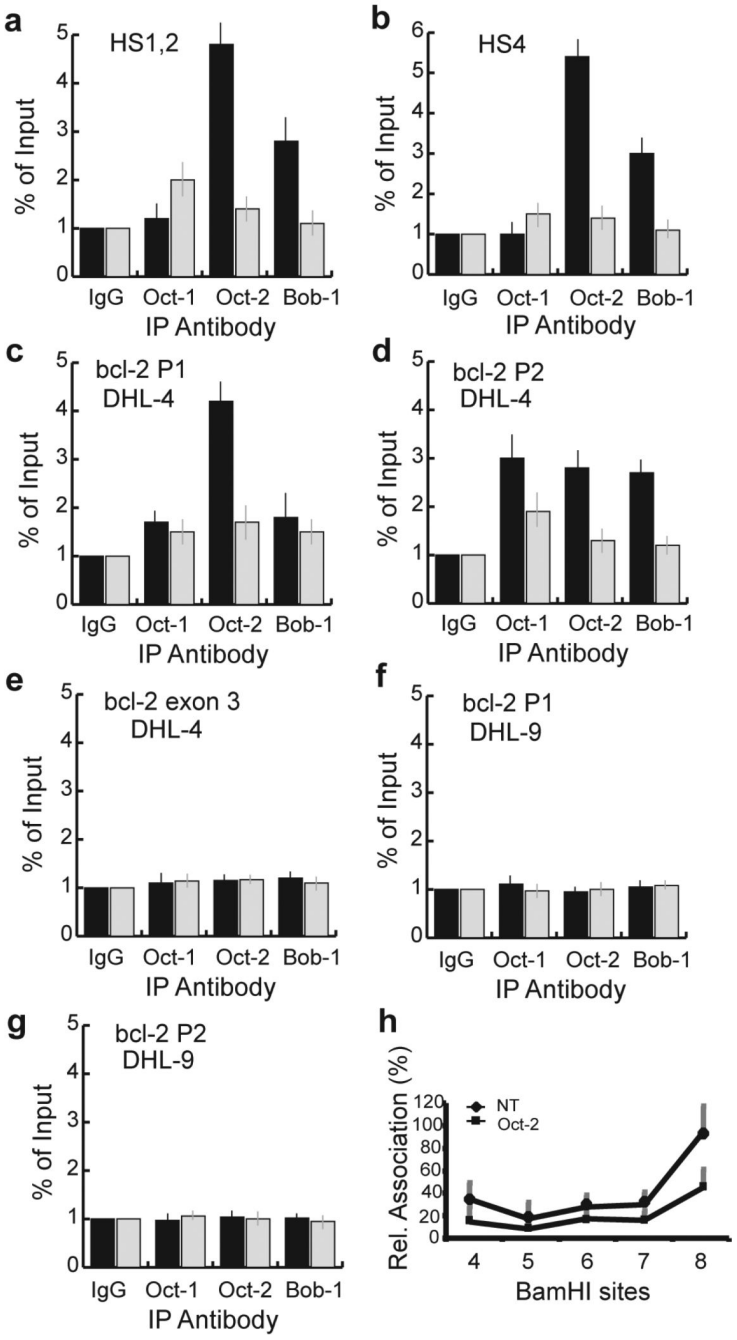

Figure 6. Effect of TSA on Oct factor binding to the IgH enhancers and*bcl*-2 promoter region

(**a**) TSA decreases Oct-2 and Bob-1 binding to the IgH HS1, 2 region as determined by quantitative ChIP assay. DHL-4 cells were untreated (black bars) or treated (gray bars) with 500 ng/ml of TSA for 18 h. Chromatin proteins and DNA were cross-linked by formaldehyde. The cross-linked chromatin was sheared and then fractionated using specific antibodies as indicated. Purified immunoprecipitated DNA was quantified using primer/probe sets corresponding to the IgH HS1, 2 region.

(**b**) TSA decreases Oct-2 and Bob-1 binding to the IgH HS4 region in DHL-4 cells as determined by quantitative ChIP assay.

(**c**) TSA decreases Oct-2 binding to the *bcl*-2 P1 promoter in DHL-4 cells as determined by quantitative ChIP assay.
(**d**) TSA decreases Oct-2 binding to the *bcl*-2 P2 promoter in DHL-4 cells as determined by quantitative ChIP assay.
(**e**) Oct-1, Oct-2, and Bob-1 do not bind to the *bcl*-2 exon 3 region in DHL-4 cells.
(**f**) Oct-1, Oct-2, and Bob-1 do not bind to the *bcl*-2 P1 promoter in DHL-9 cells.
(**g**) Oct-1, Oct-2, and Bob-1 do not bind to the *bcl*-2 P2 promoter in DHL-9 cells.
(**h**) siRNA targeting Oct-2 decreases the interactions of the IgH enhancers and *bcl*-2 promoter region as determined by 3C analysis. DHL-4 cells were transfected with siRNA targeting Oct-2 or a non-targeting siRNA. 24 hours after transfection, the cells were harvested, and dead cells were excluded by Ficoll purification. The relative associations of BamHI site 2 at the *bcl*-2 promoter region with the IgH enhancer BamHI sites were determined and quantified as described in Figure 4.