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ORIGINAL ARTICLE

The immunoglobulin heavy-chain gene 3′ enhancers deregulate *bcl*-2 promoter usage in t(14;18) lymphoma cells

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In t(14;18) lymphomas, *bcl*-2 is juxtaposed to the immunoglobulin heavy-chain gene (IgH), resulting in increased *bcl*-2 transcription and resistance to apoptosis. Regulatory elements of both the *bcl*-2 promoter and the IgH enhancers are believed to play a role in the increased expression of *bcl*-2 in t(14;18) lymphoma cells. In addition, transcription of the translocated *bcl*-2 allele is deregulated with activation of the normally minor *bcl*-2 P2 promoter. The mechanisms involved in the promoter shift from P1 to P2 are not known. We found that the murine IgH 3′ enhancers increased *bcl*-2 P2 promoter activity in an episomal model of the translocation, and IgH enhancer region HS12 had the greatest effect. Quantitative chromatin immunoprecipitation (ChIP) assays revealed that localized histone H3 hyperacetylation of the P2 promoter was observed on the translocated allele in t(14;18) DHL-4 cells and also on the stably transfected *bcl*-2 promoter-IgH enhancer episomal construct. Analysis of the HS12 enhancer region revealed that a previously identified nuclear factor-κB (NF-κB) site and a previously uncharacterized downstream Cdx site, both of which are conserved in the human and murine IgH enhancers, were important for its enhancer activity and promoter activation. ChIP assays showed that C/EBPβ bound to the HS12 Cdx site *in vivo*, and mutation of this site abrogated the binding of C/EBPβ. Reduced expression of C/EBPβ by transfection of small interfering RNA or interference with NF-κB activity decreased transcription from the *bcl*-2 promoters. These results demonstrate that the IgH 3′ enhancers, particularly HS12, are important for the deregulation of *bcl*-2 promoter usage in t(14;18) lymphomas.
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Introduction

The t(14;18)(q32;q21) is the most common chromosomal translocation in human low-grade lymphomas. As a result of the translocation, one allele of the antiapoptotic *bcl*-2 gene from chromosome 18 is juxtaposed to the immunoglobulin heavy-chain (IgH) locus on chromosome 14 (Tsujimoto *et al*., 1985; Cleary *et al*., 1986). Transcription of the *bcl*-2 gene is increased, presumably owing to regulatory elements in the IgH locus. The increased cell survival owing to elevated Bcl-2 expression has been shown to contribute to the development of B-cell lymphomas and confer resistance to a variety of anticancer therapies (Hockenberry *et al*., 1990; Desoize, 1994; Reed *et al*., 1994; Schmitt and Lowe, 2001).

Two promoters mediate transcriptional control of the *bcl*-2 gene (Seto *et al*., 1988). In normal, B-cells *bcl*-2 transcripts are primarily derived from the 5′ (P1) promoter, whereas the 3′ (P2) promoter shows no to minimal usage. Previous studies from our laboratory and others have shown that the P2 promoter is activated in t(14;18) lymphoma cells (Seto *et al*., 1988; Wu *et al*., 2001). However, the regulation of P1 and P2 promoter usage has not been precisely defined, and the underlying mechanisms of the activation of the P2 promoter by the translocation have not been explored.

It is believed that regulatory elements of the IgH gene play critical roles in the deregulated expression of the translocated *bcl*-2 allele. Enhancers (HS1-4) are located downstream of the murine IgH gene, and similar but not identical elements are located downstream of the two human Cα genes (reviewed by Khamlichi *et al*., 2000). Our recent studies showed that the murine IgH 3′ enhancers HS1-4 increased *bcl*-2 expression when they were linked to the *bcl*-2 promoter (Heckman *et al*., 2003a). However, it was unclear whether the IgH 3′ enhancers increased transcription from the P1 or P2 promoter or from both promoters. In addition, the regulatory elements in the IgH enhancers that contributed specifically to promoter activation are not known.

In this study, we use an episomal *bcl*-2 promoter construct to study the effect of the murine IgH enhancers on *bcl*-2 transcription, and we demonstrate that this model reproduces many aspects of the translocated *bcl*-2 allele. We specifically focused on the regions of the IgH enhancers that are conserved in the mouse and human genes. Our results show that the IgH 3′ enhancers

upregulate transcription from both the *bcl*-2 P1 and P2 promoters, but the impact on transcription from P2 is more profound. Increased histone H3 acetylation is associated with the aberrant upregulation of the *bcl*-2 P2 promoter by the IgH enhancers, and nuclear factor-κB (NF-κB) and Cdx sites within the HS12 enhancer region play important roles in *bcl*-2 promoter activation. These results provide insight into the mechanisms of *bcl*-2 deregulation in t(14;18) lymphomas.

Results

Activation of bcl-*2 transcription in t(14;18) lymphoma cells*

To precisely measure the activation of *bcl*-2 transcription from the two promoters, quantitative real-time reverse transcriptase–polymerase chain reaction (qRT–PCR) analysis was performed to compare the *bcl*-2 transcripts in t(14;18) DHL-4 cells and in DHL-9 cells that lack the translocation. Two primer/probe sets within the *bcl*-2 gene corresponding to the P1 and P2 regions were used to measure the *bcl*-2 transcripts (Figure 1a). As shown in Figure 1b, a sevenfold increase in P1 transcription was observed in DHL-4 cells compared to DHL-9 cells. However, a more dramatic 17-fold increase of total *bcl*-2 transcription was observed in DHL-4 cells versus DHL-9 cells. The P2/P1 ratio was approximately 2 in DHL-4 cells, whereas it was less than 0.2 in DHL-9 cells (Figure 1c). Taken together, these results indicate that transcripts from both *bcl*-2 promoters are enhanced in t(14;18) cells, and increased transcription from the normally minor *bcl*-2 P2 promoter contributes more significantly to *bcl*-2 upregulation in t(14;18) cells.

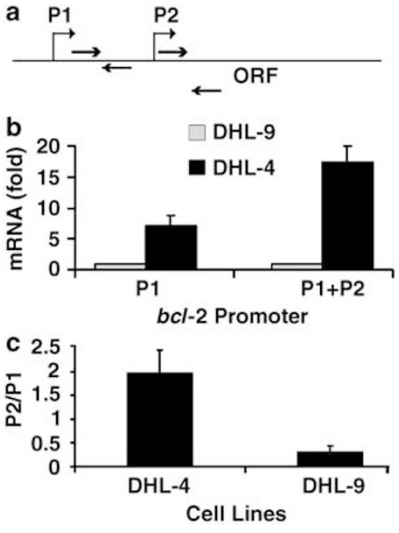

Figure 1 Transactivation of the *bcl*-2 gene in t(14;18) lymphoma cells. (**a**) Diagram of the *bcl*-2 gene and the location of primer/probe sets used for qRT–PCR analysis of the P1 and P2 *bcl*-2 transcripts. (**b**) Analysis of *bcl*-2 transcription in t(14;18) DHL-4 cells and DHL-9 cells that lack the translocation. *Bcl-2* transcripts from the P1 promoter (P1) or both promoters (P1 + P2) were determined by qRT–PCR and normalized to GAPDH expression in each sample. The relative *bcl*-2 mRNA transcription was represented as a fold increase compared to *bcl*-2 mRNA expression in DHL-9 cells. (**c**) Analysis of the relative *bcl*-2 promoter usage in DHL-4 and DHL-9 cells. The *bcl*-2 transcripts from the P1 and the P1 + P2 promoters were determined by qRT–PCR. The absolute level of transcripts from the P1 promoter and both P1 and P2 promoters was determined from a standard curve generated with a construct containing the *bcl*-2 5′ region. Subtraction of the quantity of P1 transcripts from the P1 + P2 transcripts was performed to determine the quantity of transcripts originating from the P2 promoter.

Increased histone H3 acetylation is associated with bcl-*2 P2 promoter activation in t(14;18) cells*

Evidence from a number of systems indicates that an important aspect of enhancer function is to increase histone acetylation and counteract repressive chromatin structure to facilitate transcription (Krumm *et al*., 1998; Madisen *et al*., 1998). To determine the role of histone acetylation in the activation of *bcl*-2 transcription by the IgH enhancers, we performed quantitative chromatin immunoprecipitation (ChIP) assays to examine the binding of acetylated histones to the *bcl*-2 promoter and exon regions in DHL-9 and DHL-4 cells. The location of the primer/probe sets is shown in Figure 2a.

Consistent with the predominant usage of the *bcl*-2 P1 promoter in the cells without the t(14;18) translocation, we found that histone H3 was highly acetylated around the P1 promoter region in DHL-9 cells (Figure 2b). In contrast to the hyperacetylation of the P1 promoter, the binding of acetylated histone H3 to the P2 promoter was only slightly above the background level, which is consistent with the minor usage of the P2 promoter (Figure 2c). Interestingly, no significant binding of acetylated histone H4 was observed to either promoter. These results indicate that histone H3 acetylation is correlated with *bcl*-2 P1 promoter activity in cells that lack the t(14;18) translocation. Although both the P1 and P2 promoters are active in t(14;18) cells as determined by endogenous mRNA transcription and reporter gene assays, only the P2 promoter showed greatly increased binding of acetylated histone H3 (Figure 2d and e). The binding of acetylated histone H3 to the P1 promoter was clearly above the background level, but much lower compared to the P2 promoter on the translocated allele or to the P1 promoter in DHL-9 cells. Similar to the observation in DHL-9 cells, no significant binding of acetylated histone H4 to either promoter was observed in DHL-4 cells. These results suggest that histone H3 acetylation is uniquely involved in the transactivation of the *bcl*-2 P1 and P2 promoters in t(14;18) cells. Although histone H3 acetylation is closely correlated with elevated transcription from the P2 promoter, histone acetylation is not sufficient to explain the increased transcription from the P1 promoter. Furthermore, we found that histone H3 acetylation was observed locally at the *bcl*-2 promoter regions and not downstream in exons 2 and 3 (Figure 2f).

The acetylation and methylation status of histone H3 within the endogenous IgH enhancers was also examined by quantitative ChIP assays in DHL-4 cells. The structure of the human IgH locus is shown in Supplementary Figure 1a. As shown in Supplementary Figure 1b, all of the IgH enhancers were associated with

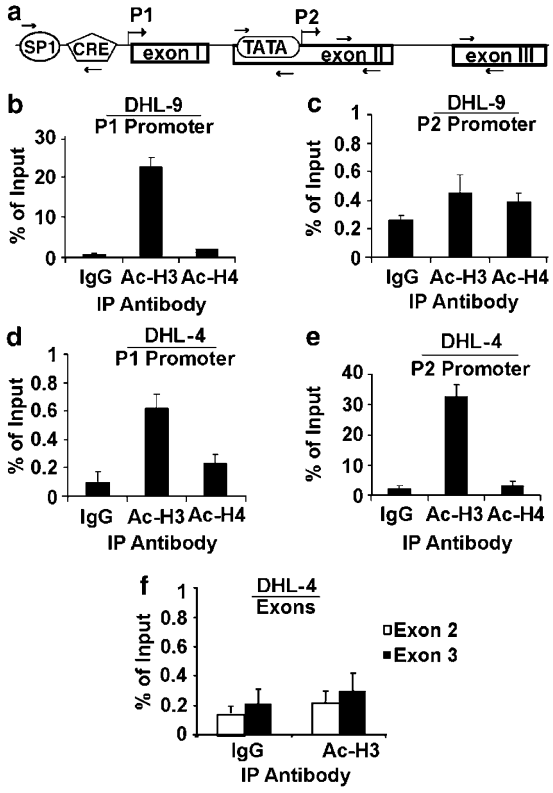

Figure 2 Increased histone H3 acetylation is associated with the active *bcl*-2 P2 promoter in t(14;18) cells. (**a**) Diagram of the *bcl*-2 gene and the primer/probe sets used for quantitative ChIP analysis of acetyl-histone binding to the P1 and P2 promoters and exons 2 and 3. (**b**–**c**) Quantitative ChIP assay of acetyl-histone binding to the *bcl*-2 P1 (**b**) and P2 (**c**) promoters in DHL-9 cells. The cross-linked chromatin was precipitated with specific antibodies as indicated. The results are displayed as the percentage of input DNA. (**d**–**e**) Quantitative ChIP assay of acetyl-histone binding to the *bcl*-2 P1 (**d**) and P2 (**e**) promoters in DHL-4 cells. (**f**) Quantitative ChIP assay of acetyl-histone binding to *bcl*-2 exons 2 and 3 in DHL-4 cells.

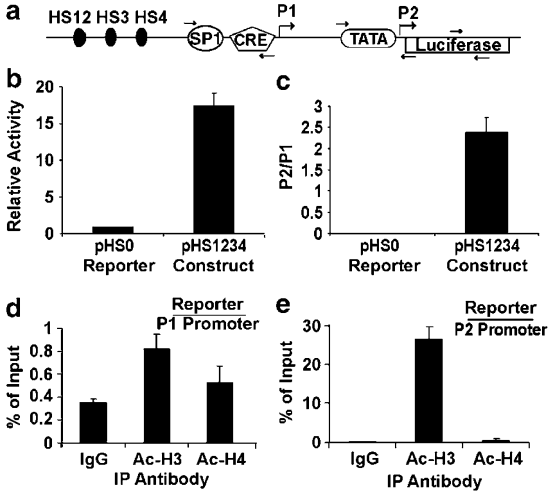

Figure 3 Increased histone H3 acetylation is associated with the *bcl*-2 P2 promoter in the episomal vector stably maintained in t(14;18) cells. (**a**) Diagram of the episomal murine IgH enhancer HS1-4-*bcl*-2 promoter reporter gene construct and the primer/probe sets used to determine the P1 and P1 + P2 transcripts and the primers/probe sets used for the quantitative ChIP assay. (**b**) Relative luciferase activity of the episomal reporter gene without the IgH enhancers (pHS0) or with the murine IgH enhancers HS1-4 (pHS1234). The reporter gene constructs were stably transfected into DHL-4 cells. The relative luciferase activity in each stable cell line was normalized to the amount of protein and transgene copy number and represented as the fold increase compared to the luciferase activity of the reporter construct without the IgH enhancers. (**c**) *Bcl*-2 promoter usage in the episomal constructs with and without the IgH enhancers. The P1 and P1 + P2 transcripts were determined by qRT–PCR. The absolute amount of P1 and P1 + P2 transcripts was derived from a standard curve generated with a *bcl*-2 promoter-luciferase construct. The P2 transcript level was calculated by subtracting the P1 transcripts from the P1 + P2 transcripts. (**d**–**e**) Quantitative ChIP assay of acetylated histone binding to the *bcl*-2 P1 (**d**) and P2 (**e**) promoters in the episomal context. To quantify the acetylated histone binding to the P2 promoter in the reporter gene, the 5′ primer was selected in the *bcl*-2 P2 promoter region and the 3′ primer was selected within the luciferase DNA region. The acetylated histone binding to the P1 promoter in the reporter gene was calculated by deducting the amount of binding of acetylated histone to the endogenous *bcl*-2 promoter in DHL-4 cells from the binding in the episomal gene transfected DHL-4 cells.

a substantial level of histone H3 acetylated at lysines 9 and 14 (acH3-K9,14) and histone H3 dimethylated at lysine 4 (mH3-K4). Compared to background binding, the acK9,14-H3 bound to HS3, HS12 and HS4 were enriched by 10-, 22- and 15-fold, respectively. An even greater increase in binding of mH3-K4 was found at the HS3, HS12 and HS4 regions with enrichments of 35-, 42- and 59-fold, respectively. The acetylation and methylation patterns of the histones at the *bcl*-2 promoter region and the human IgH 3′ enhancers suggest that these regions are transcriptionally competent.

Deregulation of the bcl-*2 P1 and P2 promoters by the murine IgH enhancers in an episomal model of the translocation*

To further investigate the role of the IgH 3′ enhancers in the activation of the *bcl*-2 promoters, an episomal *bcl*-2 promoter-luciferase construct was generated and stably transfected into DHL-4 cells (Figure 3a). Constructs with (pHS1234) or without (pHS0) the murine IgH enhancer regions HS1-4 were utilized to measure the relative luciferase activity from the *bcl*-2 promoter. As expected, the enhancers increased expression from the *bcl*-2 promoters (Figure 3b). To examine the effect of the different IgH enhancers on *bcl*-2 individual promoter usage, qRT–PCR was performed to measure transcripts from each promoter in the stable cell lines (as described in the Materials and methods). As shown in Figure 3c, the P2 promoter was activated to a greater extent than the P1 promoter such that the P2/P1 ratio increased to 2.4. This promoter usage is quite similar to that of the endogenous translocated *bcl*-2 gene (Figure 1c).

The binding of acetylated histones to the *bcl*-2 promoters in the episomal reporter gene was determined by quantitative ChIP assays. We found that the *bcl*-2 P1 promoter in the episomal vector was hypoacetylated as it is in the endogenous *bcl*-2 gene (Figure 3d). As shown in Figure 3e, the binding of acetylated histone H3 was highly enriched at the P2 promoter in the episomal construct. Again, this is similar to the binding of

acetylated histone H3 to the endogenous *bcl*-2 P2 promoter on the translocated allele. There was no significant histone H4 acetylation on either the *bcl*-2 P1 or P2 promoters in the episomal vector. These results indicate that the murine IgH enhancer region HS1234 mimics the function of the endogenous human IgH locus and confers a unique histone acetylation pattern on the *bcl*-2 promoter regions.

Episomal *bcl*-2 promoter-luciferase constructs with different regions of the IgH 3′ enhancers were generated and stably transfected into DHL-4 cells. The constructs contain the enhancer regions HS12, HS3 and HS4 individually or as a unit (Supplementary Figure 2a). Analysis by qRT–PCR revealed that HS12 and HS4 had the greatest activity with the *bcl*-2 promoter, whereas HS3 alone had a smaller effect (Supplementary Figure 2b). The HS12 region increased *bcl*-2 promoter activity by ninefold, whereas the HS4 region induced a 13-fold increase.

The presence of any of the enhancers increased the transcription from the P2 promoter to a level greater than the transcription from P1 and resulted in a P2/P1 ratio greater than 1 (Supplementary Figure 2c). Interestingly, HS12 had the greatest effect on the *bcl*-2 promoter usage shift from P1 to P2 with a P2/P1 ratio of 3.2. The effect of HS12 on the P2/P1 ratio was greater than that of HS1234 because the four IgH enhancer regions increased P1 promoter transcription more than HS12 did. HS3 and HS4 also increased the P2/P1 ratio. These results indicate that the regulation of *bcl*-2 promoter usage by IgH enhancers is complex and that different regions of the IgH enhancers have differential effects on each *bcl*-2 promoter.

NF-κB and Cdx sites within HS12 are involved in the deregulation of bcl-*2 promoter usage by the IgH enhancers*

Because HS12 had the most dramatic effect on *bcl*-2 promoter usage, we wished to locate the important elements that contribute to the enhancer activity of HS12 on the *bcl*-2 promoters. Episomal reporter gene constructs containing a series of 5′ deletions of the HS12 enhancer in the presence of the HS3 and HS4 regions were generated (Figure 4a). Analysis of these episomal reporter gene activities revealed two regions that contributed significant regulatory activity to the *bcl*-2 promoter (Figure 4b). One region between bases 800 and 817 contains a NF-κB binding site, which has been described previously (Heckman *et al*., 2003a). The other active region was located between bases 858 and 886. We identified a potential binding site for Cdx transcription factors in this region, and this site is conserved in the human IgH enhancer HS12. Further analysis revealed that mutation of the Cdx site decreased *bcl*-2 promoter activity (Figure 4b), indicating that the site is involved in the HS12-mediated upregulation of *bcl*-2 promoter activity.

The functional role of the NF-κB and Cdx sites in the HS12 region on *bcl*-2 promoter usage was examined with episomal reporter gene constructs with mutation of the NF-κB site (CCC to GAG) or the Cdx site (ATTT to CGCC) stably transfected into DHL-4 cells (these mutations were tested in electrophoretic mobility shift assay, and no specific protein binding was observed). Mutation of the NF-κB site decreased the P2/P1 ratio from 2.4 to 1.6, and mutation of the Cdx site decreased the P2/P1 ratio to 1.8 (Figure 4c). These results indicate that the NF-κB and Cdx sites within HS12 are involved in the deregulation of *bcl*-2 promoter usage.

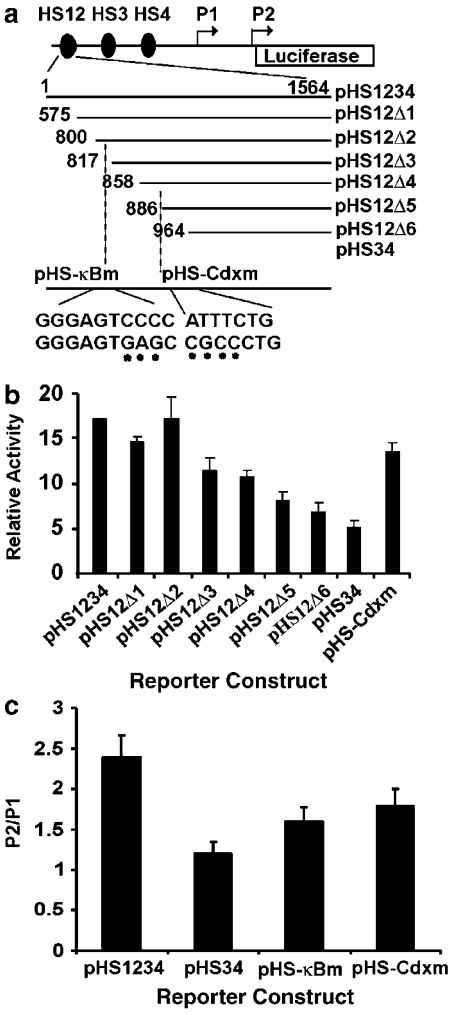

Figure 4 Deletion and mutation analysis of the IgH enhancer HS12 region. (**a**) Diagram of the HS12 deletion constructs and the constructs with the mutated NF-κB and Cdx sites. pHS-κBm and pHS-Cdxm are the same as pHS1234 except that the NF-κB and Cdx sites have been mutated as indicated. (**b**) Relative activity of the *bcl*-2 promoter episomal constructs with different HS12 deletions. *Bcl-2* promoter-luciferase reporter gene constructs were transfected into DHL-4 cells. The luciferase activity was determined at day 5 after transfection and normalized to the amount of protein in each sample. (**c**) The effect of NF-κB and Cdx site mutations on the *bcl*-2 promoter usage. The P2/P1 ratio in DHL-4 cells stably transfected with pHS-κBm and pHS-Cdxm episomal constructs was determined by qRT–PCR as described in Figure 3c.

Interference with NF-κB function decreases the bcl-*2 promoter shift*

We have previously shown by ChIP analysis that NF-κB family members bind to HS12 (Heckman *et al*., 2003a). To further confirm the involvement of NF-κB in the

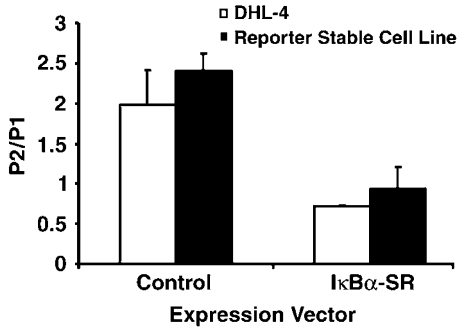

Figure 5 Interference with NF-κB activity alleviates the deregulation of *bcl*-2 promoter usage. DHL-4 cells or cells stably expressing pHS1234 were co-transfected with the IκBα-SR expression vector or an empty expression vector and an EGFP expression vector. At 24 h after transfection, the cells were sorted for GFP expression, and the positive cells were collected, qRT–PCR was performed, and the ratio of P2 transcripts to P1 transcripts was determined as described in Figures 1 and 3c.

deregulation of *bcl*-2 promoter usage, a dominant negative inhibitor, inhibitor κBα-SR (IκBα-SR), was used to inhibit the function of NF-κB factors. Transfection of the IκBα-SR into DHL-4 cells resulted in a 98% decrease in activity from a reporter gene driven by five NF-κB sites (Supplementary Figure 3). The effect of inhibition of NF-κB activity was examined on both the endogenous *bcl*-2 gene in DHL-4 cells and on the episomal vector with the murine IgH enhancer HS1234 linked to the *bcl*-2 promoter. As shown in Figure 5, expression of IκBα-SR significantly relieved the P2/P1 *bcl*-2 promoter shift from 2.4 to approximately 1.0 in the episomal vector. A similar finding was observed with the endogenous *bcl*-2 promoter with a promoter shift of 0.7 in the presence of the IκBα-SR.

C/EBPβ *binds to the Cdx site within HS12 and is involved in the deregulation of* bcl-*2 promoter usage*

To further characterize the transcription factors that bind to the Cdx site of HS12, quantitative ChIP assays were performed using the two cell lines stably transfected with either the wild-type or Cdx site mutant episomal reporter gene constructs (pHS1234 and pHS-Cdxm). We have previously observed that C/EBP family members bind to a Cdx site in the *bcl*-2 promoter (Heckman *et al*., 2003b), so we examined whether C/EBP factors interact with the HS12 region. As shown in Figure 6a, C/EBPβ showed significant binding to HS12 of the episomal vector, and this interaction was decreased by mutation of the Cdx site. By ChIP analysis, we also confirmed binding of C/EBPβ to the endogenous HS12 Cdx site in DHL-4 cells (Figure 6b). The regulatory role of C/EBPβ on *bcl*-2 promoter usage was examined by the use of small interfering RNA (siRNA) to C/EBPβ. As shown in Figure 6c, transfection of siRNA specifically targeting C/EBPβ decreased the *bcl*-2 promoter P2/P1 ratio from 2.4 to 1.9, whereas a scrambled siRNA had no significant effect on *bcl*-2 promoter usage. Furthermore, transfection of the C/EBPβ siRNA into the stable cell line with the pHS-Cdxm construct, which has the mutation in the Cdx site

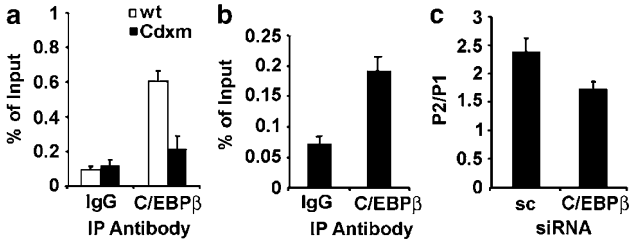

Figure 6 C/EBPβ binds to the Cdx site in IgH enhancer HS12 and is involved in the deregulation of *bcl*-2 promoter usage by the IgH enhancers. (**a**) Quantitative ChIP assay of C/EBPβ binding to HS12 in DHL-4 cells stably transfected with pHS1234 or pHS-Cdxm (mutated Cdx site) *bcl*-2 reporter gene constructs. (**b**) Quantitative ChIP assay of C/EBPβ binding to the human IgH enhancer HS12 region in DHL-4 cells. The primer/probe set used is shown in Supplementary Figure 1. (**c**) C/EBPβ siRNA transfection decreases the *bcl*-2 promoter shift. DHL-4 cells stably transfected with the episomal pHS1234 reporter gene construct were co-transfected with a scrambled siRNA (sc) or siRNA targeting C/EBPβ and an EGFP expression vector. At 24 h after transfection, the cells were sorted for GFP expression, and positive cells were collected and qRT–PCR was performed to determine the transcription from the *bcl*-2 P1 and P2 promoters as described in Figure 3c. The siRNA to C/EBPβ decreased the C/EBPβ mRNA to 18% of its normal level.

in HS12, did not lead to a change in the *bcl*-2 P2/P1 ratio (data not shown). These results indicate that C/EBPβ binds to the HS12 Cdx site and is involved in the shift in *bcl*-2 promoter usage from P1 to P2, although the effect is not as great as that of the NF-κB site.

Discussion

In normal B cells, transcription of *bcl*-2 is primarily derived from the P1 promoter of the gene. However, in cells with the t(14;18) translocation, *bcl*-2 expression is increased with transcription also occurring from the P2 promoter. Little is known of the mechanisms involved in this alteration of promoter usage. In the present work, we investigated *bcl*-2 promoter usage in t(14;18) cells by studying both endogenous *bcl*-2 mRNA transcription and a model system consisting of stably transfected episomal *bcl*-2 promoter reporter genes linked to regions of the murine IgH 3′ enhancers. Quantitative RT–PCR analysis of endogenous *bcl*-2 mRNA levels revealed a sevenfold increase in *bcl*-2 P1 promoter transcription and a 17-fold increase in *bcl*-2 transcription from both promoters in the t(14;18) DHL-4 cells compared to DHL-9 cells which lack the translocation. These results suggest that increased transcription from the normally minor *bcl*-2 P2 promoter significantly contributes to *bcl*-2 upregulation in t(14;18) cells.

We also showed that acetylation of histone H3 was associated with the *bcl*-2 promoter regions during transcription. In DHL-9 cells, the acetylation status of histone H3 correlated well with *bcl*-2 promoter transcriptional activity at both the P1 and P2 promoters. In contrast to the correlation between histone H3 acetylation with *bcl*-2 transcription, no appreciable histone H4 acetylation was observed at either *bcl*-2 promoter region in DHL-9 cells. However, significant histone H4

acetylation at the *bcl*-2 promoter has been reported in epithelial cells (Decary *et al*., 2002), suggesting that the regulatory roles of histone acetylation for *bcl*-2 expression may be cell-type specific.

In this study, we found that the IgH enhancer specifically increased the acetylation of histone H3 within the normally minor *bcl*-2 P2 promoter in B-cell lymphoma cells with the t(14;18) translocation. This histone acetylation pattern was also reproduced in the episomal reporter gene with the *bcl*-2 promoters linked to the murine IgH enhancers, suggesting that the IgH enhancers play critical roles in the establishment of the histone H3 acetylation status of the *bcl*-2 P2 promoter region. Moreover, our previous study with histone deacetylase inhibitors showed that trichostatin A repressed *bcl*-2 transcription and dramatically decreased *bcl*-2 P2 promoter histone H3 acetylation in DHL-4 cells (Duan *et al*., 2005). Taken together, these studies support the idea that the IgH enhancers increase *bcl*-2 P2 promoter transcription, at least in part, by the acetylation of histone H3 at this promoter region.

We found that although transcription from the P1 promoter was also increased by the IgH enhancers, histone H3 was hypoacetylated in this region, suggesting that mechanisms other than histone acetylation are involved in the increased *bcl*-2 P1 transcription in t(14;18) cells. The simian virus 40 enhancer has been reported to increase gene expression by modulation of the elongation competence of RNA polymerase (Krumm *et al*., 1995), and this is also the case for the IgH enhancer-mediated c-*myc* P1 promoter activity in Burkitt's lymphoma cells (Madisen and Groudine, 1994). Therefore, it is possible that some components of the IgH enhancer specifically load histone acetyltransferase activity to the *bcl*-2 P2 promoter, whereas other components are involved in increasing the transcriptional competence of the P1 promoter.

Quantitative RT–PCR analysis of the *bcl*-2 P1 and P2 promoters in the episomal reporter gene-transfected stable cell lines showed that the murine IgH enhancer deregulated *bcl*-2 promoter usage. In the absence of the IgH enhancers, *bcl*-2 transcription was predominantly derived from the P1 promoter. Each of the individual enhancers increased *bcl*-2 transcription from the normally minor P2 promoter to a level close to or greater than that from the P1 promoter. HS12 was the most active IgH enhancer region mediating *bcl*-2 promoter usage deregulation. Deletion and mutation analysis showed that the NF-κB site within HS12 was critically involved in the HS12-mediated *bcl*-2 promoter transactivation as well as in the deregulated promoter usage. We have shown previously that NF-κB is constitutively active in t(14;18) lymphoma cells and acts to upregulate *bcl*-2 expression through cyclic AMP responsive element binding and Sp1 binding sites in the *bcl*-2 P1 promoter and in the IgH enhancer HS4 region (Heckman *et al*., 2002, 2003a). Mutation of the NF-κB site in the HS12 region decreased *bcl*-2 promoter activity and also decreased the P2/P1 ratio. The involvement of NF-κB in the *bcl*-2 promoter usage deregulation was further supported by inhibition of NF-κB activity with the IκBα-SR. Overexpression of the IκBα-SR decreased the P2 to P1 ratio to a greater extent than that of mutation of the NF-κB site in HS12 alone, suggesting that additional NF-κB sites are involved in the promoter activation. We have found that mutation of the HS4 NF-κB site also decreases the P2 to P1 ratio (data not shown).

Further deletion and mutation analysis of the murine HS12 enhancer region revealed that a Cdx site downstream of the NF-κB site was also important for *bcl*-2 transcription and promoter usage. Quantitative ChIP assays revealed that C/EBPβ bound to this region in the endogenous human IgH enhancer in DHL-4 cells and in the episomal reporter gene construct. Mutation of this site abrogated the binding of C/EBPβ to this region, downregulated *bcl*-2 promoter activity and decreased the IgH enhancer-mediated *bcl*-2 promoter shift. Transfection of C/EBPβ siRNA also resulted in a decreased *bcl*-2 promoter shift from 2.4 to 1.9. We have previously shown that C/EBPα and C/EBPβ upregulated *bcl*-2 expression in t(14;18) cells through sites in the *bcl*-2 promoter (Heckman *et al*., 2003b). Our current study further revealed that C/EBPβ bound to a Cdx site in HS12 and participated in the IgH enhancer-mediated *bcl*-2 promoter shift that is observed with the t(14;18) translocation.

Bcl-2 overexpression in t(14;18) cells plays a central role in the development of B-cell lymphoma. The results presented here show that the IgH enhancers deregulate *bcl*-2 transcription by influencing its promoter usage, although the effects are different on the two *bcl*-2 promoters, P1 and P2. Furthermore, we demonstrate that an episomal construct with the murine IgH enhancers is a faithful model of the *bcl*-2 translocation in t(14;18) lymphomas, and this construct can be used to examine in detail the mechanisms involved in the activation of *bcl*-2 expression by the IgH enhancers. A better understanding of the mechanisms of *bcl*-2 deregulation by the translocation will be useful in the development of new directed therapies for t(14;18) lymphomas.

Materials and methods

Plasmid constructs

The generation of the episomal *bcl*-2 promoter-luciferase constructs with the IgH enhancers (HS1234) has been described previously (Duan *et al*., 2005) with the regions of the IgH enhancers as described (Madisen and Groudine, 1994). Reporter constructs with the *bcl*-2 P1 or P2 promoter and individual IgH enhancer regions or serial deletions of HS12 were generated from this construct. The NF-κB reporter gene construct, the IκBα-SR, and the control expression vectors, and the mutation of the HS4 NF-*k*B site have been described (Heckman *et al*., 2002, 2003a).

Site-directed mutagenesis

Mutagenesis of the Cdx site of the HS12 region was performed by the oligo-directed mutagenesis method with the Quick Change kit from Stratagene (La Jolla, CA, USA). The following primer was used for mutagenesis with the mutated bases underlined:

GGGGGAGTCACTCATGCTCGCCCTGGAAACAACC TCAGAAAG.

Cell lines, transfection and reporter gene activity analysis
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). Stable transfections were performed as described previously (Duan *et al*., 2005). The copy numbers of the plasmids were determined by Southern blot analysis.

For short-term stable transfections, 2×10^7 cells were transfected with 10 μg of plasmid DNA by electroporation. The transfected cells were allowed to recover for 24 h before selection with 400 μg/ml of hygromycin B. Reporter gene activity was determined at day 5 after transfection. All transfection results are represented as the average and s.d. from at least six independent transfection experiments.

Selection of transfected cells
A total of 5×10^6 cells at mid-log phase were transfected with 2 μg of IκBα-SR or 100 nM siRNA targeting C/EBPβ and 1 μg of an enhanced green fluorescent protein (EGFP) expression vector using Amaxa Kit R. At 24 h after transfection, the cells were sorted by GFP expression on a FACSVantage SE cell sorter.

qRT–PCR analysis
ToTTLY RNA and RETROscript kits from Ambion (Austin, TX, USA) were used for the isolation of RNA and the generation of cDNA according to the manufacturer's protocol. Real-time PCR of cDNA was performed on the ABI Prism 7900-HT Sequence Detection System using the Universal PCR Master Mix. Assay-on-Demand primer/probe sets for the detection of transcripts from both *bcl*-2 promoters and a primer/probe set for the detection of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) transcripts were from Applied Biosystems. When discrimination of *bcl*-2 transcripts from the different promoters was needed, primer/probe sets listed in Supplementary Table 1 were used. All quantitative real-time PCR results are presented as the average and s.d. from at least three independent experiments with duplicate PCR analysis.

Quantitative ChIP assay
The ChIP assay was performed as outlined previously (Heckman *et al*., 2003b; Duan *et al*., 2005). Antibodies for the histones were from Upstate Biotechnologies and the other antibodies were from Santa Cruz Biotechnology. Real-time PCR was performed to quantify the amount of immunoprecipitated DNA using the TaqMan primers and probes in Supplementary Table 1. All quantitative ChIP assay results are presented as the average and s.d. from at least three independent immunoprecipitations followed by duplicate real-time PCR analysis.

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Supplementary Information accompanies the paper on the Oncogene website (http://www.nature.com/onc).