ZEB1 and CtBP form a repressive complex at a distal promoter element of the BCL6 locus

Vasiliki PAPADOPOULOU*, Antonio POSTIGO†‡, Ester SÁNCHEZ-TILLÓ†, Andrew C. G. PORTER* and Simon D. WAGNER§

*Department of Haematology, Imperial College London, Hammersmith Hospital, Du Cane Road, London W12 0NN, U.K., †Transcriptional Regulation Group, Department of Oncology and Haematology, IDIBAPS, Villarroel 170, 08036 Barcelona, Spain, ‡ICREA, Passiege Lluís Companys 23, 08010 Barcelona, Spain, and §Department of Cancer Studies and Molecular Medicine, MRC Toxicology Unit, University of Leicester, Leicester LE1 9HN, U.K.

INTRODUCTION

BCL6 is a zinc finger transcription factor that is highly expressed in normal germinal centre B-cells [1] and is essential for the production of high-affinity antibodies [2,3]. BCL6 is also involved in chromosomal translocations in 20–30% of human diffuse large B-cell lymphomas [4] and is expressed in follicular lymphoma, Burkitt’s lymphoma and nodular lymphocyte predominant Hodgkin’s lymphoma.

The protein is subject to several post-translational modifications that may promote its degradation [5,6]. Transcriptional regulation is also important [7–9], especially in the transition from germinal centre B-cell to terminally differentiated plasma cell.

BCL6 inhibits its own transcription through a site in exon 1 of the gene, but the necessary mechanisms to overcome this effect and allow sustained expression have not been described [7,9]. BCL6 transcription is responsive to growth-factor signalling through STAT5 (signal transducer and activator of transcription 5) in primary tonsillar B-cells [10]. The forkhead transcription factor FoxO3a, which is downstream of PI3K (phosphoinositide 3-kinase), can also drive BCL6 transcription [11], and FoxO3a-binding sites have been identified in the BCL6 promoter [12]. Recently we showed that CD40 signalling both represses BCL6 transcription [through NF-κB (nuclear factor κB) and IRF4 (interferon regulatory factor 4)] and induces it by activating p38 MAPK (mitogen-activated protein kinase) [12a]. We conclude that BCL6 transcription integrates strong repressive signals and weak positive signals such that the balance tends towards reducing BCL6 expression, and we suggest that it may only be switched on transiently in small populations within the germinal centre.

In the B-cell lineage, BCL6 is only expressed in germinal centre cells and not in naïve B-cells or plasma cells. BCL6 is also expressed in other cell lineages. It is found in many types of epithelial cell and cardiac myocytes. BCL6 is expressed in normal breast epithelium and breast cancer, and high BCL6 expression correlates with histologically aggressive disease. BCL6 is therefore tightly controlled temporally and in a lineage-specific manner. We sought to discover cis-acting sites which may contribute to these specific patterns of control.

We implicate the transcriptional repressor ZEB1 (zinc finger E-box-binding homeobox 1) in the control of BCL6. ZEB1 binds DNA through zinc finger DNA-binding regions at both the N- and C-termini. ZEB1 recognizes a subset of E-box sequences [13], as well as other less-defined sequences [14]. ZEB1 accomplishes repression through interactions with the co-repressor, CtBP (C-terminal binding protein), which interacts with PLDLS (Pro-Leu-Asp-Leu-Ser) and PLDLS-like sequences [15], as well as other co-repressors [16]. ZEB1 represses E-cadherin expression, and other proteins required to maintain epithelial cell polarity, and induces an EMT (epithelial-to-mesenchymal transition) that may be important in promoting cancer invasiveness and metastasis [17–19]. ZEB2 (also called SIP1) has a high degree of homology with ZEB1 and there is some degree of overlap in their expression [17]. ZEB1 is highly expressed in thymus and is required for T-cell differentiation. Its down-regulation accompanies leukemogenesis in ATLL (adult T-cell leukaemia/lymphoma).

Key words: BCL6, C-terminal binding protein (CtBP), germinal centre, lymphoma, transcription, zinc finger E-box-binding homeobox 1 (ZEB1).

Abbreviations used: ABC-DLBCL, activated B-cell-like diffuse large B-cell lymphoma; BLIMP1, B-lymphocyte-induced maturation protein 1; ChIP, chromatin immunoprecipitation; CtBP, C-terminal binding protein; EBV, Epstein–Barr virus; EMT, epithelial-to-mesenchymal transition; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; GC-DLBCL, germinal centre diffuse large B-cell lymphoma; HEK, human embryonic kidney; RT, reverse transcription; siRNA, small interfering RNA; STAT5, signal transducer and activator of transcription 5; ZEB1, zinc finger E-box-binding homeobox 1.

1 To whom correspondence should be addressed (email sw227@le.ac.uk).
Both ZEB1 and ZEB2 are expressed in B-cells [17], and ZEB1 regulates transcription from the immunoglobulin heavy chain locus [13]. In the model put forward in the present paper, ZEB1 competes for E-box binding sites in the IgH enhancer with the transcriptional activator, E2A.

Using DNase I-hypersensitive site mapping we found a distal promoter sequence at the BCL6 locus that has been shown to recruit CtBP [19]. We show that an E-box at this site binds ZEB1, and that ZEB1 overexpression and siRNA (small interfering RNA)-mediated knockdown cause changes in BCL6 expression.

**MATERIALS AND METHODS**

**Cell lines and tonsils**

Germinal-centre-representative Burkitt’s lymphoma cell lines DG75 and Ramos, plasma cell line KMS11, T-cell line Jurkat and HEK (human embryonic kidney)-293 cells were grown in RPMI 1640 (Lonza) supplemented with 10% (v/v) fetal calf serum (Lonza), 2% L-glutamine and 5% penicillin/streptomycin (Invitrogen). DG75 cells for siRNA-mediated knockdown were obtained from Dr Antonio Parrado (Immunology Unit, University Hospital Virgen de la Arrixaca, Murcia, Spain).

Single-cell suspensions were made from tonsils (obtained with permission of the Local Research Ethics Committee). B-cells were separated using a Robosep immunosensitive cell separator with the EasySep Human B-cell Enrichment kit (Stem Cell Technologies, catalogue number 19054). The B-cell population was ~95% pure as assessed by staining with an anti-CD19 antibody.

**DNase I-hypersensitive site mapping**

Approx. $50 \times 10^6$ exponentially growing cultured cells or $30 \times 10^6$ freshly isolated tonsillar B-cells were harvested by centrifugation (10 min at 4°C at 250 g) on the day of the experiment. The cell pellet was washed twice in ice-cold PBS and finally resuspended in 1 ml of ice-cold RSB buffer [10 mM Tris/HCl (pH 7.4) 10 mM NaCl and 3 mM MgCl$_2$, hypotonic buffer] per $5 \times 10^6$ cells. After DNase I digestion (described in the Supplementary Materials and methods section at http://www.BiochemJ.org/bj/427/bj4270541add.htm), 20 μg of the resulting DNA was used for each restriction enzyme digest. Digestion was carried out in a total volume of 50 μl, using 100 units of a restriction endonuclease (New England Biolabs). After overnight digestion, the samples were loaded on to a 0.8% agarose gel and electrophoresed at 55 V overnight. Southern blot transfer was carried out overnight. Nylon filters were hybridized with probes (Figure 1a and Supplementary Table S1 at http://www.BiochemJ.org/bj/427/bj4270541add.htm).

**Luciferase reporter assays**

Genomic fragments of the BCL6 promoter region including HSS-4.4 (Supplementary Table S2 at http://www.BiochemJ.org/bj/427/bj4270541add.htm) were amplified using Pfu DNA polymerase (Promega) (200 ng of template DNA, 1 × Pfu buffer, 300 nM of each primer, 0.06 unit of Pfu and 200 nM of each dNTP) in a 50 μl reaction volume. Cycling conditions were 30 cycles of 95°C for 1 min, 62°C for 30 s and 72°C for 1 min. In order to mutate the E-box sequence, 5’-CTTCTG-CATGGTTTAACATAACAGGGCG-3’ was used. The PCR
product was purified, restriction enzyme digested and cloned into the pGL3Promoter vector (Promega). Fragments (4–7 kb long) (Supplementary Table S2) were amplified using a long template PCR system (Expand Long Template PCR, Roche) in a 50 μl reaction mixture (40 ng of template DNA, 300 nM of each primer, 0.4 μl of DNA polymerase mix and 200 nM of each dNTP) and cloned into the pGL3Basic vector. Thermal cycling conditions were 98°C for 1 min, followed by 30 cycles of 98°C for 30 s, 60°C for 30 s and 68°C for 10 min, and a final 10 min at 68°C.

ChiP (chromatin immunoprecipitation)
Cultured cells (KMS11 and DG75; 10 × 10⁶ cells) per antibody to be used were cross-linked in their culture medium with 1% formaldehyde at 37°C for 10 min. Cross-linking was stopped with the addition of glycine solution to a final concentration of 0.125 M. Cells were then harvested by centrifugation (10 min at 4°C at 250 g) and washed twice with ice-cold PBS. Sonication and ChiP were then carried out as described in the Supplementary Materials and methods section.

Transfection
The adherent HEK-293 cell line was plated in six-well plates in 2.5 ml of complete medium at a density of 5 × 10⁶ cells/well 1 day prior to transfection. On the day of transfection, 5 μl of Lipofectamine™ 2000 (Invitrogen; catalogue number 11668-019) was mixed with the appropriate amount of DNA in 400 μl of serum-free medium followed by incubation for 30 min at room temperature (20°C). The transfection mixture was then added to the plated cells (400 μl of mixture to each well of a six-well plate) and the lipofected adherent cells were harvested by trypsination 18 h post-transfection or 36 h post-transfection if an expression plasmid, i.e. pCS2-MT-ZEB1 (ZEB1 cDNA), was transfected simultaneously with the reporter plasmids. DG75 cells were transfected by means of a Nucleofector (Lonza Amaxa) using program O-006 and solution V.

In total, 2 μg of plasmid with cloned HSS-4.4 was transfected, but 4 μg of plasmids containing the longer constructs (4–7 kb) were transfected. Renilla luciferase expression vector (1 μg), pRL-TK (thymidine kinase promoter–Renilla luciferase reporter plasmid; Promega) was co-transfected in all experiments.

Gel-shift assays
Ramos or KMS11 cells (20 × 10⁶ cells) were harvested and nuclei were prepared as described for DNase I-hypersensitive site assays. The nuclear pellet was resuspended in 500 μl of RSB buffer for 20 × 10⁶ cells and then brought to a volume of 2 ml by the addition of 25% sucrose in RSB. Cell lysates were produced and binding reactions carried out as described in the Supplementary Materials and methods section.

Real-time RT (reverse transcription)–PCR
RNA was extracted using the Qiagen RNeasy kit and the Qiagen RNase-free DNase I set or TRIzol® (Invitrogen). For each RT reaction, 2 μg of RNA was used (OmniScript RT kit; Qiagen). Reaction mixtures were incubated at 37°C for 1 h and then stored as total cDNA sample at −20°C or used immediately for real-time PCR as the cDNA template. For siRNA knockdown, cDNA was generated using the Transcriptor High Fidelity cDNA Synthesis kit (Roche) and real-time PCR was carried out with SYBR Green qPCR Master Mix (Fermentas).

For real-time PCR, specific assays were purchased from Applied Biosystems: BCL6, Hs00153368_m1; BLIMP1 (B-lymphocyte-induced maturation protein 1), Hs00153357_m1; and HPRT (hypoxanthine phosphoribosyltransferase), Hs99999909_m1. The PCR primers used for ZEB1 and CtBP have been published previously [20–22]. GAPDH (glyceraldehyde-3-phosphate dehydrogenase) was used as a housekeeping control gene to normalize data [23]. Annealing conditions for all primers were 60°C for 45 s. Each sample was analysed in triplicate. Expression levels were normalized to GAPDH. Relative values are represented.

siRNA knockdown
DG75 cells were transfected with siRNA oligonucleotides at 50 nM using Lipofectamine™ RNAiMAX (Invitrogen). siRNA oligonucleotides were ordered from Invitrogen using sequences described previously: ZEB1 [24] and CtBP [25]. As a negative control we used Low GC-Stealth RNAi Negative Control (Invitrogen).

RESULTS
A DNase I-hypersensitive site 4.4 kb upstream of the transcription start site
In order to discover cis-acting sites responsible for these specific patterns of control we carried out DNase I-hypersensitive site mapping of the BCL6 locus. A total of 35 kb of the locus (from 19.5 kb upstream of the transcription start site to 15.1 kb downstream) were mapped (Figure 1a) and strong DNase I-hypersensitive sites were found in DG75 human EBV (Epstein–Barr virus)-negative Burkitt's lymphoma cells at +0.3 kb and +0.5 kb from the transcription start site and at −4.4 kb (HSS-4.4) (Figure 1b and Supplementary Figure S1 at http://www.BiochemJ.org/bj/427/bj4270541add.htm).

The Jurkat human T-cell line possesses a weak hypersensitive site (HSS-1.9) within the Swai restriction fragment, but does not have HSS-4.4, demonstrating that this site is B-cell-specific (Figure 1b). Purified human tonsillar B-cells also demonstrated HSS-4.4 (Figure 1c).

We conclude that there is a DNase I-hypersensitive site at the BCL6 locus in primary human B-cells 4.4 kb upstream of the BCL6 transcription start site. A sequence of 356 bp flanked by 5'-polyT and 3'-polyA (forming a partial inverted repeat) is present at this location, of which the core sequence of 240 bp shows homology with the dog and mouse sequences (Figures 2a and 2b).

We carried out a genomic comparison [26], between human, mouse and dog, across a 30 kb region comprising intron 1, exon 1 and approx. 20 kb of upstream sequence. Pairwise comparisons (Figure 2b) reveal sections of non-coding sequence homology. HSS-4.4 corresponds to a site of >50% sequence homology, separated from the proximal promoter region by sequence showing no homology between species. Other hypersensitive sites, HSS+3.8 and HSS+5.8, reported in the present paper also appear to correspond with regions of sequence homology.

Single-stranded sections of promoter regions can be recognized by transcription factors and contribute to recruitment of specific proteins and tight gene regulation [27,28]. The partial inverted repeat sequences suggested that DNA at this site could form a secondary structure. Utilizing M-fold (http://mfold.bioinfo.rpi.edu/) [29,30] we found that the 356 bp sequence can be folded (Figure 2c) and the lowest free energy form (Figure 2d) appears to form a cruciate structure. HSS-4.4 is therefore a potential site...
for a cis-acting control region and we focused on this for further investigation.

**ZEB1 binds at HSS-4.4**

ChIP–ChIP data has shown that the co-repressor CtBP is recruited to exon 1 of BCL6 and also to a site upstream of the coding sequence at approximately the location of HSS-4.4 [19]. CtBP associates with several transcription factors including the two-handed zinc finger ZEB1 [15]. This protein binds to E-boxes and its binding is facilitated by the additional consensus sequence GTTTC [14], both of which are present in HSS-4.4 (Figure 2a).

In order to analyse potential protein-binding sites we carried out gel-shift assays using overlapping oligonucleotides across the core sequence. Oligonucleotides 3, 4, 6 and 7 showed shifted bands. We chose to focus on oligonucleotide 7, which showed two strong shifted bands and possessed an E-box sequence (CAGGTGT) (Figure 3a). In gel-shift assays protein binding was competed less well by an oligonucleotide bearing mutations in the E-box sequence, demonstrating its importance in forming the

---

**Figure 2 Sequence at HSS-4.4**

(a) Comparison of human (Hs), mouse (Mm) and dog (Cf) sequence at HSS-4.4. Shared bases are shaded in grey. The oligonucleotide used in the gel-shift assay (Figure 3c) is indicated by a black line. (b) Pairwise comparison of mouse with human (upper panel) and mouse with dog (lower panel). The position of exon 1 is indicated by a black rectangle above the panels with intron 1 to the left and the proximal promoter (grey bar) to the right. Sites of sequence homology corresponding to HSS + 5.8, HSS + 3.8 and HSS-4.4 are indicated. (c) Dot plot shows base pairing for the optimal energy for folding of 356 bp at HSS-4.4 (lower triangle) and base pairing for all four predicted structures (upper triangle). (d) Structure for optimal energy folding of the 356 bp sequence. The consensus (CAGGTGT) and additional consensus ZEB1-binding site (GTTTC) are indicated.
protein–DNA complex. We therefore chose to test the hypothesis that HSS-4.4 is the site of binding of a ZEB1–CtBP complex.

The ZEB1 protein complex involves CtBP and E2A

ZEB1 was cloned from a B-cell line and found to compete with E2A to regulate transcription at the Ig H-chain locus, although subsequently most work has been carried out on its role in epithelial cells and T-cells. Using supershift antibodies (Figure 3b) we showed that in the Burkitt’s lymphoma cell line Ramos (which does not express ZEB1, but does express E2A), an anti-E2A antibody produced a supershifted band with concomitant reduction in intensity of the upper band obtained with oligonucleotide 7 (grey arrowhead, Figure 3b). E2A is not expressed in the plasma cell line KMS11, which does express ZEB1. An anti-ZEB1 antibody produced a decrease in intensity of the upper shifted band. Both ZEB1 and E2A can therefore bind to oligonucleotide 7.

Next we used ChIP to analyse in vivo protein binding to HSS-4.4 (Figure 3c). We again compared a germinal centre representative, DG75 cells, with the plasma cell line KMS11. DG75 expressed ZEB1 and we were therefore able to immunoprecipitate the endogenous protein. CtBP and ZEB1 were immunoprecipitated from both cell lines, and E2A was only present at HSS-4.4 in DG75 cells. We conclude that HSS-4.4 is the previously identified site of CtBP recruitment, and that it is also capable of binding ZEB1 and E2A.

Transcriptional activity of HSS-4.4

Luciferase reporter assays were carried out in order to measure the transcriptional activity of the isolated HSS-4.4 sequence. An 800 bp fragment which included HSS-4.4 showed a 12-fold
increase in transcription (as compared with empty vector) in DG75, but not in Jurkat, cells or the HEK-293 cell line (Figure 3d), demonstrating that this sequence responds to B-cell-specific transcription factors. Next we analysed position- and orientation-dependence of transcription (Figure 3e), and showed that only when this sequence was upstream of the luciferase reporter gene and in the correct orientation did it have transcriptional activity. We conclude that HSS-4.4 is a distal element of the BCL6 promoter.

Role of HSS-4.4 in the BCL6 promoter

Elements regulating BCL6 transcription have been found in the proximal part of intron 1 [8,31], exon 1 [7,9] and approx. 1.3 kb upstream of the transcription start site [12]. CtBP is recruited to the BCL6-binding site in exon 1 and we therefore used luciferase reporter assays to assess the transcriptional effects of HSS-4.4 in the context of the whole BCL6 promoter in a B-cell line expressing BCL6 (Figure 3f).

Deletion of the HSS-4.4 sequence up to the E-box [Figure 3f (i)] produced a non-significant increase in transcription. However, mutation of the E-box sequence almost abolished transcription. Changing the mutated site to the wild-type sequence restored transcriptional activity, demonstrating that this specific change was responsible for the effect observed. Binding to the E-box sequence is therefore essential for activity.

Expression of ZEB1 and BCL6

Previous work suggested a reciprocal relationship between ZEB1 and E2A in B-cells [13]. We therefore analysed protein expression in cell lines (Figure 4a). ZEB1 is highly expressed in the human T-cell line, Jurkat, and not expressed in HEK-293 cells. It is expressed at low levels in the human plasma cell line, KMS11 and the Burkitt’s lymphoma cell line DG75. However, Ramos, another Burkitt’s lymphoma cell line showed absent ZEB1 and high expression of E2A. CtBP was detectable in all cell lines and BCL6 was only expressed in Ramos and DG75 cells. In purified human tonsillar B-cells, BCL6 and E2A associated with low ZEB1. Therefore the germinal centre representative cell line, Ramos, and primary tonsillar cells support the association of E2A with BCL6 and absent or reduced ZEB1.

To further analyse the correlation between ZEB1, E2A and BCL6, we turned to gene expression microarray data using primary cells [32] (http://llmpp.nih.gov/lymphoma/) (Figures 4b and 4c). We found a reciprocal relationship between ZEB1 expression (high in peripheral blood B-cells and low in germinal centre B-cells) and BCL6. ZEB1 expression was higher in peripheral blood B-cells (median mRNA level relative to the whole microarray = 1.5) than in germinal centre cells (median = 0.74). BCL6 expression is associated with the germinal centre type of human diffuse large B-cell lymphoma in a ‘cell of origin’ classification [32], and with the BCR (breakpoint cluster region) cluster in a functional classification [33]. ZEB1 expression is suppressed to normal germinal centre levels (median = 0.69) in GC-DLBCL (germinal centre diffuse large B-cell lymphoma), and is higher in ABC-DLBCL (activated B-cell-like diffuse large B-cell lymphoma) (median = 1.15); however, the difference is not significant (Mann–Whitney U test) and there is no correlation with overall survival. E2A expression is low in resting B-cells and higher in normal germinal centre B-cells (similar to that of BCL6), but in the cell lines tested on the microarray there seems to be high E2A with low BCL6.

Overexpression of ZEB1 represses BCL6 transcription

Using the Burkitt’s cell line DG75, we next tested whether overexpression of ZEB1 can regulate BCL6 (Figure 5a). Both 1 μg and 5 μg of ZEB1 cDNA produced a reduction in BCL6 mRNA. A statistically significant reduction (P = 0.03, Mann–Whitney U test) was obtained with 5 μg of ZEB1 cDNA. Western blot analysis showed that the reduction in BCL6 mRNA was sufficient to produce a decrease in BCL6 protein expression.

Figure 4 Expression of ZEB1 and BCL6

(a) Western blot analysis of ZEB1, BCL6, E2A, CtBP and GAPDH (loading control) in Burkitt’s cell lines (Ramos and DG75), plasma cell line (KMS11), T-cell line (Jurkat) and HEK-293 cells. In addition, expression of these proteins in purified human tonsillar B-cells is shown. (b) Scatter plot showing gene expression analysis (using data from http://www.llmpp.nih.gov/lymphoma/) of BCL6 mRNA in normal peripheral blood B-cells (PB), normal germinal centre cells (GC) and the two subtypes of DLBCL (GC-DLBCL and ABC-DLBCL). Average across the whole microarray = 1. (c) Scatter plot showing gene expression analysis of ZEB1 mRNA levels. The left-hand panel shows expression in peripheral blood and the right-hand panel shows expression in GC and GC-DLBCL and ABC-DLBCL. (d) Scatter plot showing gene expression analysis of E2A mRNA levels in peripheral blood, GC and GC-DLBCL and ABC-DLBCL.
Figure 5  ZEB1 represses BCL6 mRNA and protein and requires its CtBP sequence

(a) Taqman RT–PCR (representative of three experiments) showing BCL6 mRNA expression following transfection of DG75 cells with wild-type ZEB1 cDNA (ZEB1) or ZEB1 bearing mutations in its CtBP-binding site (ZEB1-CID). ZEB1 cDNA (5 µg) produces a significant (P = 0.03, Mann–Whitney U test) reduction in BCL6 mRNA. (b) Western blot analysis (representative of three experiments) showing the effect of transfection of ZEB1 or ZEB1-CID cDNA on expression of endogenous BCL6 protein. ZEB1 and ZEB1-CID transfection was consistently associated with the appearance of lower-molecular-mass material (grey arrowhead), which may represent degradation. A non-specific band on the BCL6 Western blot is indicated by ‘ns’. (c) Taqman RT–PCR (representative of three experiments) showing BLIMP1 mRNA expression following transfection with ZEB1 and ZEB1-CID. (d) DG75 cells were transfected with negative control siRNA or siRNA against ZEB1 or CtBP. ZEB1, CtBP or BCL6 mRNA was then measured and results are expressed relative to expression using the control siRNA. Results are representative of three experiments. (e) Model of the ZEB1 complex at the BCL6 locus. BCL6 transcription is associated with E2A expression and E2A is capable of binding the HSS-4.4 E-box. Other transcription factors, e.g. STAT5, induce BCL6 expression and this model does not imply that E2A is either necessary or sufficient for transcription. (f) BCL6 transcription is repressed. CtBP recruitment to exon 1 and HSS-4.4 has been proven [19], as has BCL6 binding to exon 1 [9]. We have shown ZEB1 binding to HSS-4.4. We speculate that these proteins form a repressive complex at the BCL6 locus.

(Figure 5b). BLIMP1 is a transcription factor that is required for B-cell terminal differentiation to plasma cells, and is also directly repressed by BCL6. Therefore, in order to show that the reduction in BCL6 protein was functionally important, we measured BLIMP1 mRNA (Figure 5c). Transfection of 5 µg of ZEB1 cDNA produced a 5-fold increase in BLIMP1 mRNA, demonstrating an effect on downstream BCL6 targets. Finally, in order to show that the ZEB1 effect on BCL6 was mediated through an interaction with CtBP, we employed a mutated form of ZEB1, ZEB1-CID, in which the CtBP interaction sites in ZEB at residues 705, 734 and 767 were mutated from PLDLS/T to ASASA thus preventing CtBP binding. The ZEB1 effect was
reversed by mutation of the CtBP-binding sites as ZEB1-CID failed to repress BCL6 or induce BLIMP1 mRNA expression (Figure 5b). We conclude that ZEB1 repressed BCL6 transcription in a mechanism which involved binding of the CtBP co-repressor.

**siRNA knockdown of ZEB1 or CtBP increases BCL6 mRNA expression**

As a further test of the repressive effects of the ZEB1–CtBP complex on BCL6 expression, we used siRNA-mediated knockdown (Figure 5d) in DG75 cells. Reduction of either ZEB1 or CtBP produced a ∼2.5-fold increase in BCL6 mRNA. We conclude that under basal conditions the ZEB1–CtBP complex contributes to repression of transcription at the BCL6 locus.

**DISCUSSION**

BCL6 represents a model gene for analysing lineage- and stage-specific regulation of gene expression. It is expressed in primary epithelial cells [34] and in breast cancer [35], although most work has been carried out on its targets and function in germinal centre B-cells and lymphomas derived from these cells.

In the present study we used DNase I-hypersensitive site assays to discover potential cis-acting control regions. By this means we discovered HSS-4.4, a site which appeared to correspond to a site of CtBP recruitment, whose relevance was unknown [19]. CtBP is an important co-repressor for ZEB1 [15], a transcription factor that was cloned by its ability to bind to the delta 1-crystallin core enhancer [36] and subsequently from a λ-phage expression library through its ability to bind to E-box sequences at the IgH enhancer in B-cells [13]. Most work has been carried out on the role of ZEB1 in the EMT process, a process in which polarized epithelial cells lose expression of some epithelial markers, including E-cadherin, and acquire a more motile (mesenchymal) phenotype. An EMT-like process is believed to take place in carcinomas as they invade surrounding normal tissues. ZEB1 represses E-cadherin and is one of several transcription factors (together with ZEB2, SNAI1 and SNAI2) that are up-regulated during EMT [37]. Target genes of ZEB1, or those that prevent EMT, are anticipated to be down-regulated during EMT. It is interesting that in one study of gene expression changes in EMT in epithelial cells, BCL6 mRNA was down-regulated [38], consistent with increased levels of ZEB1 in such a process. BCL6 is expressed in diverse epithelial tissues [34] and may have a role in epithelial development, as well as B-cell development. A function for ZEB1 in the regulation of BCL6 in B-cells may represent a further link between BCL6 and epithelial cell development.

Others have shown that CtBP is a co-repressor for BCL6 at its binding site within exon 1 [19]. This binding site has a powerful inhibitory effect on BCL6 transcription and, whereas mutations of the BCL6-binding site are associated with constitutive BCL6 expression in lymphomas [7], it is not clear how the consequences of binding are normally avoided to allow sustained BCL6 expression. We suggest a model (Figures 5e and 5f) in which repressive complexes involving both the BCL6-binding site in exon 1 and HSS-4.4 regulate BCL6 transcription. Both ZEB1 and BCL6 are known to use CtBP as a co-repressor and we speculate that a complex is formed that involves binding of transcription factors to HSS-4.4 and exon 1 (ZEB1 and BCL6 respectively) that are linked through binding of a common co-repressor, CtBP. In the absence of ZEB1 the HSS-4.4 site may be occupied by E2A, which forms part of an activator complex.

We carried out most of our experiments using the Burkitt’s lymphoma cell line DG75. This line expresses both ZEB1 and E2A and may potentially be able to assemble both activating and repressive complexes, although the activating E2A complex is present in basal conditions (because BCL6 protein is expressed). Evidence for the presence of an activating complex under basal conditions comes from the luciferase reporter assays (Figure 3a) which demonstrate an activating effect of HSS-4.4. However, transient transfection of ZEB1 cDNA (Figure 5b) shows repression of BCL6 expression. This result is compatible with our model: overexpression of ZEB1 caused the repressive complex to form and the consequent decrease in BCL6 expression.

ZEB1 binds to a subset of E-boxes. Does the E-box site at HSS-4.4 confer to a consensus ZEB1 site? A systematic approach to discovering the sequence requirements for ZEB1 binding showed that the zinc fingers at the N- and C-termini can bind separately to a consensus CACCTGT sequence. The entire protein can also bind to this sequence together with an ‘additional consensus sequence’ GTTTC/G which is spaced and oriented variably with respect to the consensus [14]. More recently it has been demonstrated that each zinc finger domain of ZEB1 binds to separate and inverted target sites [39], such as are found in several ZEB1 target promoters. However, ZEB1 also binds to single CACCT sites [13,40–44] and exerts transcriptional repression, suggesting that context-dependent factors have a role. A CACCTGT motif is present at HSS-4.4 (Figure 3), but there is no related inverted site, although 150 bp upstream there is a GTTTTC site. Although the single CACCTGT is sufficient for in vitro binding, these two sites may co-operate in vivo.

We found higher ZEB1 expression in ABC-DLBCL than in GC-DLBCL, with BCL6 showing the opposite association. This is consistent with a role for ZEB1 as a repressor of BCL6. There was no correlation of ZEB1 expression with overall survival, but, in the present study, and unlike others [45], there is also no correlation of BCL6 expression with survival. Therefore a larger study to analyse expression of ZEB1 in DLBCL is required. Another factor reducing the impact of ZEB1 is that a proportion of these lymphomas will have mutation or translocation of the BCL6 locus to cause constitutive expression and therefore bypass regulation by ZEB1.

The present study is the first to suggest a germinal centre B-cell function for ZEB1 through repression of BCL6, a gene essential for germinal centre formation. Previous work showed that a B-cell role for ZEB1 is repression of transcription at the IgH enhancer [13] and ZEB1 in B-cells has also been implicated in repression of BZLF1, an EBV immediate early gene which controls the switch between latent and lytic infection [41,46]. In epithelial cells ZEB1 expression is regulated by TGFβ (transforming growth factor β) signalling [47] through SMAD-dependent and independent pathways. The miR-200 family also has a controlling role in EMT [48,49], in part through the regulation of ZEB1. There are major differences in ZEB1 expression between peripheral blood and germinal centre B-cells, but the factors regulating this protein in B-cell development are unknown and will give insights into germinal centre formation and lymphomagenesis.

**AUTHOR CONTRIBUTION**

Simon Wagner designed experiments, analysed the data and wrote the paper. Antonio Postigo designed experiments and edited the paper before acceptance. Andrew Porter designed experiments and edited the paper before acceptance. Ester Sánchez-Tilló carried out experiments. Vasiliki Papadopoulou designed and carried out experiments, and analysed the data.

**FUNDING**

This work was supported by the Lymphoma Research Trust (grant to S.D.W.); Amgen (Ph.D. Studentship to V.P.); and the Spanish Ministry of Science and Innovation [grant number MICINN-BUFI2007-60302 (to A.P.)].
V. Papadopoulou and others


Received 14 October 2009/1 February 2010; accepted 23 February 2010
Published as BJ Immediate Publication 23 February 2010, doi:10.1042/BJ20091578
SUPPLEMENTARY ONLINE DATA
ZEB1 and CtBP form a repressive complex at a distal promoter element of the BCL6 locus

Vasiliki PAPADOPOULOU*, Antonio POSTIGO†‡, Ester SÁNCHEZ-TILLÓ†, Andrew C. G. PORTER* and Simon D. WAGNER§†

*Department of Haematology, Imperial College London, Hammersmith Hospital, Du Cane Road, London W12 0NN, U.K., †Transcriptional Regulation Group, Department of Oncology and Haematology, IDIBAPS, Villarroel 170, 08036 Barcelona, Spain, ‡ICREA, Passeig Lluís Companys 23, 08010 Barcelona, Spain, and §Department of Cancer Studies and Molecular Medicine, MRC Toxicology Unit, University of Leicester, Leicester LE1 9HN, U.K.

Figure S1 DNase I-hypersensitive site mapping of the BCL6 locus

(a) DNase I-hypersensitive site mapping of EcoRV, ApaLI, XhoI and HindIII restriction fragments in DG75. Probes used are indicated above the autoradiographs (Pr1 and Pr3–Pr8). The black arrowhead indicates the germline band and labelled grey arrows indicate the hypersensitive sites. Black wedges above the autoradiograph indicate the use of increasing amounts of DNase I.
(b) Summary of DNase I-hypersensitive site mapping. Sites considered to be strong are in black and weaker sites in grey.

1 To whom correspondence should be addressed (email sw227@le.ac.uk).

© The Authors Journal compilation © 2010 Biochemical Society
at 37°C dissolved in RSB) was added and the samples were incubated in RSB buffer in a volume not greater than 2 ml for an initial 5 min. After 5 min of incubation on ice, 0.25 ml of cold RSB buffer containing 0.5% Nonidet P40 was added to the cell suspension [RSB: 10 mM Tris/HCl (pH 7.4), 10 mM NaCl and 3 mM MgCl₂]. After mixing and incubating on ice for a further 5 min, the suspension was diluted 10-fold in RSB in 50 ml Falcon tubes and then centrifuged at 250 × g for 5 min at 4°C. After discarding the supernatant, the nuclear pellets were resuspended in RSB buffer in a volume not greater than 2 ml for an initial 4°C. The supernatant was diluted to 900 μl with ChIP Dilution Buffer (Millipore) and pre-cleared with 100 μl of 50% Protein A–agarose slurry. Pre-cleared lysate (300 μl) was used for each immunoprecipitation reaction and 50 μl was kept as input chromatin. The volume of each immunoprecipitation reaction was brought to 1 ml with the addition of 700 μl of ChIP Dilution Buffer (so the SDS concentration in the sample was reduced even further) and the amounts of antibodies in each reaction used were: 10 μg of anti-ZEB1 antibody (Santa Cruz Biotechnology; sc-25388), 10 μg of anti-E2A antibody (BD Biosciences; 554199), 10 μg of anti-CtBP1 antibody (Santa Cruz Biotechnology; sc-11390) and 10 μg of normal rabbit IgG (Millipore; 12-370). Collection of the immunocomplexes, washes after ChIP and elution of the bound fractions were performed according to standard protocols (Millipore ChIP assay kit). DNA–protein cross-links caused by formaldehyde were reversed with overnight heating of the final ChIP sample at 65°C prior to DNA purification. DNA pellets were hydrated in a volume of 20 μl for each ChIP sample. The DNA concentration in the samples was quantified with the use of the Pico-Green kit (Molecular Probes, Invitrogen; catalogue number P7581). Primers used to amplify from HSS-4.4 were: forward 5′-TGGTTTAACA TACA-3′ and reverse 5′-GAGCTTGAA TTTCAGGCAGCTGAAAG-3′. Template DNA (500 pg) was used for each ChIP sample and 100 ng for the input DNA. Prior to the amplification of experimental samples, the linearity of the PCR was confirmed using sonicated genomic DNA as the template. A 20 μl aliquot of the 50 μl experimental PCR products were electrophoresed on a 1.5% agarose gel.

Gel-shift assays

After centrifugation at 1500 g (~3500 rev/min) in a microcentrifuge for 5 min at 4°C, the supernatant was aspirated and the nuclear pellet lysed in 60 μl of Buffer C [20 mM Hepes (pH 7.9), 20% (v/v) glycerol, 0.1 M KCl and 0.2 mM EDTA, with 1/100 volume of protease inhibitor cocktail (Sigma)]. After a 20 min incubation on ice, nuclear lysates were centrifuged at 1500 g for a further 20 min.

Probes used were: wild-type sequence Oligo1 5′-TGGTTTAACA TACA-3′, Oligo2 5′-TGGTTTAACA TACA-3′ and reverse 5′-GAGCTTGAA TTTCAGGCAGCTGAAAG-3′. Oligo3 5′-GACCTTGAAATTTCGCGACCTGAAAG-GTTTTCTCGCCTGGA-3′, Oligo4 5′-GGTTAAGGTGTTAAG-GTTTTCTCGCCTGGA-3′, Oligo5 5′-GA-TGGAATGGAATTGGCCCGCTCTCGGGAGAG-3′, Oligo6 5′-TTCCGCTCTCGAGCTCTTCGAG-3′, Oligo7 5′-GAGCTTGAAATTTCGCGACCTGAAAG-GTTTTCTCGCCTGGA-3′. The E-box mutated probe was Oligo1 5′-GAGCTTGAAATTTCGCGACCTGAAAG-GTTTTCTCGCCTGGA-3′.

DNA pellets were resuspended in 60 μl of TE buffer [10 mM Tris (pH 8.0) and 1 mM EDTA].

ChIP

The cell pellet was lysed in 500 μl of SDS lysis buffer (Millipore, ChIP assay kit) and the lysate incubated on ice for 10 min. Sonication was then carried out on ice at an intensity of 4 microns (Sanyo MSE Soniprep 150) for ten pulses of 10 s with 30 s intervals. The sonication protocol was optimized as described above in order to shear genomic DNA to random fragments of an average size 400–500 bp. The sonicated lysate was centrifuged at 1500 g for 20 min at 4°C. The supernatant was diluted to 900 μl with ChIP Dilution Buffer (Millipore) and pre-cleared with 100 μl of 50% Protein A–agarose slurry. Pre-cleared lysate (300 μl) was used for each immunoprecipitation reaction and 50 μl was kept as input chromatin. The volume of each immunoprecipitation reaction was brought to 1 ml with the addition of 700 μl of ChIP Dilution Buffer (so the SDS concentration in the sample was reduced even further) and the amounts of antibodies in each reaction used were: 10 μg of anti-ZEB1 antibody (Santa Cruz Biotechnology; sc-25388), 10 μg of anti-E2A antibody (BD Biosciences; 554199), 10 μg of anti-CtBP1 antibody (Santa Cruz Biotechnology; sc-11390) and 10 μg of normal rabbit IgG (Millipore; 12-370). Collection of the immunocomplexes, washes after ChIP and elution of the bound fractions were performed according to standard protocols (Millipore ChIP assay kit). DNA–protein cross-links caused by formaldehyde were reversed with overnight heating of the final ChIP sample at 65°C prior to DNA purification. DNA pellets were hydrated in a volume of 20 μl for each ChIP sample. The DNA concentration in the samples was quantified with the use of the Pico-Green kit (Molecular Probes, Invitrogen; catalogue number P7581). Primers used to amplify from HSS-4.4 were: forward 5′-TGGTTTAACA TACA-3′ and reverse 5′-GAGCTTGAA TTTCAGGCAGCTGAAAG-3′. Template DNA (500 pg) was used for each ChIP sample and 100 ng for the input DNA. Prior to the amplification of experimental samples, the linearity of the PCR was confirmed using sonicated genomic DNA as the template. A 20 μl aliquot of the 50 μl experimental PCR products were electrophoresed on a 1.5% agarose gel.

Gel-shift assays

After centrifugation at 1500 g (~3500 rev/min) in a microcentrifuge for 5 min at 4°C, the supernatant was aspirated and the nuclear pellet lysed in 60 μl of Buffer C [20 mM Hepes (pH 7.9), 20% (v/v) glycerol, 0.1 M KCl and 0.2 mM EDTA, with 1/100 volume of protease inhibitor cocktail (Sigma)]. After a 20 min incubation on ice, nuclear lysates were centrifuged at 1500 g for a further 20 min.

Probes used were: wild-type sequence Oligo1 5′-TGGTTTAACA TACA-3′, Oligo2 5′-TGGTTTAACA TACA-3′ and reverse 5′-GAGCTTGAA TTTCAGGCAGCTGAAAG-3′. Oligo3 5′-GACCTTGAAATTTCGCGACCTGAAAG-GTTTTCTCGCCTGGA-3′, Oligo4 5′-GGTTAAGGTGTTAAG-GTTTTCTCGCCTGGA-3′, Oligo5 5′-GA-TGGAATGGAATTGGCCCGCTCTCGGGAGAG-3′, Oligo6 5′-TTCCGCTCTCGAGCTCTTCGAG-3′, Oligo7 5′-GAGCTTGAAATTTCGCGACCTGAAAG-GTTTTCTCGCCTGGA-3′. The E-box mutated probe was Oligo1 5′-GAGCTTGAAATTTCGCGACCTGAAAG-GTTTTCTCGCCTGGA-3′.
Table S1  Probe sequences for Southern blots
The restriction enzyme fragment to which the probe hybridizes are represented in Figure 1(A) of the main text.

<table>
<thead>
<tr>
<th>Probe</th>
<th>Position relative to transcription start site</th>
<th>Direction</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>5’ End of EcoRV fragment</td>
<td>Forward</td>
<td>5’-CCTGGGTGTGAACCTCCTTCATC-3’</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Reverse</td>
<td>5’-CTGATCCAACACTGAAGTGAG-3’</td>
</tr>
<tr>
<td>2</td>
<td>5’ End of SwaI fragment</td>
<td>Forward</td>
<td>5’-CTAGGGGATGGTTCAAGAC-3’</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Reverse</td>
<td>5’-TGAGGATGACCTCAC-3’</td>
</tr>
<tr>
<td>3</td>
<td>5’ End of ApaLI fragment</td>
<td>Forward</td>
<td>5’-CTCCACCTCCACCGG-3’</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Reverse</td>
<td>5’-CAGGGGATGGTGACCT-3’</td>
</tr>
<tr>
<td>4</td>
<td>3’ End of ApaLI fragment</td>
<td>Forward</td>
<td>5’-CAAGCCTTACGAGAAAGG-3’</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Reverse</td>
<td>5’-ATGAGGATGACCTCAC-3’</td>
</tr>
<tr>
<td>5</td>
<td>5’ End of XhoI fragment</td>
<td>Forward</td>
<td>5’-AGAGTGTTGAGGCTTGAATG-3’</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Reverse</td>
<td>5’-CGGCCACACACAGGAC-3’</td>
</tr>
<tr>
<td>6</td>
<td>3’ End of XhoI fragment</td>
<td>Forward</td>
<td>5’-TGCAAGGGAGGTGACCT-3’</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Reverse</td>
<td>5’-ACATGGGAGGTGACCT-3’</td>
</tr>
<tr>
<td>7</td>
<td>5’ End of HindIII fragment</td>
<td>Forward</td>
<td>5’-CAGGGGATGGTGACCT-3’</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Reverse</td>
<td>5’-AAGCCTTACGAGAAAGG-3’</td>
</tr>
<tr>
<td>8</td>
<td>3’ End of HindIII fragment (+6354 to +15069)</td>
<td>Forward</td>
<td>5’-GAGGGTTTGGGAGGGTC-3’</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Reverse</td>
<td>5’-GAGGGTTTGGGAGGGTC-3’</td>
</tr>
</tbody>
</table>

Table S2  Primers used to clone fragments of the BCL6 locus into luciferase reporter constructs
The E-box sequence within HSS-4.4 is included within one of the primers and is underlined.

<table>
<thead>
<tr>
<th>Description</th>
<th>Position relative to the transcription start site</th>
<th>Direction</th>
<th>Restriction site</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>800 bp around HSS-4.4</td>
<td>−4.9 to −4.1</td>
<td>Forward</td>
<td>NheI</td>
<td>5’-GCTAGCCGCTCTCCACCTCCCTTG-3’</td>
</tr>
<tr>
<td>6.9 kb including HSS-4.4 and including 1.5 kb of intron 1</td>
<td>−4.9 to +2.0</td>
<td>Reverse</td>
<td>HindIII</td>
<td>5’-GAAGGGTTTGGGAGGGTC-3’</td>
</tr>
<tr>
<td>6.3 kb from the E-box in HSS-4.4 and including 1.5 kb of intron 1</td>
<td>−4.3 to +2.0</td>
<td>Forward</td>
<td>NheI</td>
<td>5’-GCTAGCCGCTCTCCACCTCCCTTG-3’</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Reverse</td>
<td>BglII</td>
<td>5’-GCTAGCCGCTCTCCACCTCCCTTG-3’</td>
</tr>
</tbody>
</table>

buffer [10 mM HCl (pH 7.5), 1 mM EDTA and 50 % glycerol] to a final volume of 19 μl. For cold competitor assays, 2, 5 and 10 pmol of non-labelled double-stranded probe was added to the binding reaction. For antibody supershift assays, the lysate was incubated with 1–5 μl of specific antiserum or 5 μl of normal rabbit serum prior to adding the other components and incubated on ice for 30 min. E2A rabbit antiserum (a gift from Professor Richard Baer, Columbia University, New York, U.S.A.) and ZEB1 rabbit antiserum (a gift from Professor Hisato Kondoh, Osaka University, Osaka, Japan) were used as indicated.

After preparing the binding reaction the labelled probe stock was diluted 1:30 and 1 μl of the diluted labelled probe was added to the binding reaction. The 20 μl binding reaction volume was incubated for 30 min at room temperature, loaded on to a 5 % vertical non-denaturing poly-acrylamide gel and electrophoresed for 3 h at 150 V.