Regulation of Immunoglobulin Promoter Activity by TFII-I Class Transcription Factors*

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Dean Tantin‡§, Maria Isabel Tussie-Luna¶, Ananda L. Roy§, and Phillip A. Sharp‡**

From the ‡Department of Biology and Center for Cancer Research, Massachusetts Institute of Technology, ‡McGovern Institute, Cambridge, Massachusetts 02139-4307, and the ¶Department of Pathology, Tufts University School of Medicine, Boston, Massachusetts 02111

The restriction of immunoglobulin variable region promoter activity to B lymphocytes is a well known paradigm of promoter specificity. Recently, a cis-element, located downstream of the transcription initiation site of murine heavy chain variable promoters, was shown to be critical for B cell activity and specificity. Here we show that mutation of this element, termed DICE (Downstream Immunoglobulin Control Element), reduces in vivo activity in B cells. Gel mobility shift assays show that DICE forms B cell-specific complexes that were also sensitive to DICE mutation. DICE mutation strongly reduces the ability of a distal immunoglobulin heavy chain intronic enhancer to stimulate transcription. We also identify a DICE-interacting factor: a TFII-I-related protein known as BEN (also termed Mus-TDR1 and WBSCR11). Dominant-negative and RNAi-mediated knockdown experiments indicate that BEN can both positively and negatively regulate IgH promoter activity, depending on the cell line.

During development, an organism must exclusively express some genes in particular tissues or cell types. A number of genes and their promoters have been studied in detail in order to obtain general principles from their mechanism of regulation. Among the most intensely studied are the immunoglobulin variable (V⁺) region promoters. Ig heavy chain (IgH) and κ light chain (Igκ) genes are selectively expressed in B lymphocytes, and their respective V region (VH and Vκ) promoters are normally inactive in all other tissues (reviewed in Ref. 1). The IgH and Igκ loci are sequentially activated during B lymphocyte maturation. Activation includes three principal events. Transcription initiates at various positions within each locus, including the VH and Vκ promoters (2, 3). Recombination of a V region gene segment with a joining (J) gene segment, in the case of the κ chain, and a diversity (D) and J segment, in the case of the heavy chain, forms an Ig variable region exon of a given specificity (4). Hyperacetylation of chromatin within the Ig loci also makes it less compact and more accessible to trans-acting factors (5, 6). The relative timing of these three events is unclear; however, it has been shown that non-coding (germ line) transcription precedes recombination for some VH segments (3, 7). The direct relationship between these events is also unclear; however, in the heavy chain locus, V-D-J recombination brings a downstream intronic enhancer into proximity with the VH promoter. The relocation of the enhancer greatly stimulates expression of the recombined IgH (reviewed in Ref. 8). These multiple interdependent layers of regulation serve to tightly control Ig expression and prevent activation outside of the normal B cell maturation pathway.

Studies from a number of laboratories have determined that in isolation, VH and Vκ promoters are preferentially active in B lymphocytes (9, 10). A feature of most VH and Vκ promoters is the octamer motif (5'-ATGCAAAT-3'), located 10–25 nucleotides upstream of the TATA box (and inverted in the case of Vκ) (9, 11–14). By using model constructs, the octamer motif appears to be a mediator of Ig promoter B cell specificity (15); however, the same sequence occurs in numerous other genes, most of which are not B cell-specific. For example, the U1 small nuclear RNA and histone H2B genes both contain functional octamer sequences and are ubiquitously expressed (16–18). Therefore, it is likely that the contribution of the octamer toward B cell specificity is dictated by other determinants within the promoter, otherwise octamer-containing genes such as U1 and H2B would also be expressed in a B cell-specific manner. In B cells, the octamer motif interacts with two POU domain transcription factors termed Oct-1 and Oct-2. Oct-1 (19–21) has a wide tissue distribution, whereas Oct-2 (22, 23) is more B cell-restricted. Genetic analyses have shown that Oct-2 is dispensable for B cell development and Ig transcription (24, 25). A similar finding has been made in this laboratory using Oct-1-deficient mice in this laboratory. A co-activator of Oct-1 and Oct-2, principally expressed in B cells, has also been described (26–28). This protein, termed OCA-B/Bob-1/OBF-1, is also dispensable for Ig gene expression (29–31).

Previously, we have shown that a widespread DNA sequence, located downstream of the transcription initiation site, is an important determinant of IgH promoter activity and B lymphoid selectivity (32). This sequence is present in many IgH and some Igκ promoters. Here we further characterize the properties of this element (termed DICE), and we show that...
DICE can interact with a protein known as BEN/MusTRD1. The prototypic member of this family, TFII-I (also known as BAP-135), can also bind DICE with high affinity in vitro. Surprisingly, dominant-negative and RNA, knockdown experiments reveal negative as well as positive roles for BEN. We also report that DICE helps mediate the activity of the IgH intronic enhancer, as mutation of the DICE sequence reduces enhancer activity.

**EXPERIMENTAL PROCEDURES**

**Plasmid Constructs**—The DICE sequences from the 186.2 promoter GenBank™ accession number J00530 were used in these experiments. The plasmid pGL3HgH154-1chima has been described (32). The corresponding point mutated plasmids were made by introducing mutated 186.2 DICE sequences into the plasmid pGL3HgH154-1chima that had been digested with HindIII and treated with alkaline phosphatase. Complementary oligonucleotides with HindIII overhangs and phosphate groups at their 5′ ends were annealed and ligated into the digested pGL3HgH1 plasmid, yielding the plasmids pGL3Vh17.22s5mut1 chimera and pGL3Vh17.22s5mut2 chimera. The 4 oligonucleotides used are shown in 5′ to 3′ as follows: Hin186.2mut1TOP, AGCTTGTAC-ATCGGAGATCCTTACAGTGGAAAGACACAGGAGA; Hin186.2mut1BOT, AGCTTGTACCATGCCAAGTCTTAACTGTAAAGA; Hin186.2mut2TOP, AGCTTGTACCATGCCAAGTCTTAACTGTAAAGA; Hin186.2mut2BOT, AGCTTCCAAGTCTTAACTGTAAAGA (boldface sequence indicates the positions of the mutations). Orientation and sequence were verified by DNA sequencing. The IgH intronic enhancer was introduced downstream of the DICE sequences into the plasmid pGL3IgH-154. The orientation was determined, and the sequence was verified by DNA sequencing. The IgH intronic enhancer was introduced downstream of the DICE sequences into the plasmid pGL3IgH-154. The orientation and sequence were verified by DNA sequencing.

**Nuclear Extract**—The DICE sequences from the 186.2 promoter were used as an internal transfection control. Activity was scored by the dual luciferase assay (Promega).

**Luciferase Assay**—Nuclear extracts were prepared from cells growing in suspension in 8-liter batches according to the method of Dignam et al. (33). 3T3 extracts were prepared from batches of 16-15 cm plates.

**EMSAs**—The double-stranded oligonucleotides described above were also used as probes in gel mobility shift assays. The HindIII overhangs were filled in with Klenow fragment using radiolabeled [α-32P]dATP and [α-32P]d(3′)TP. All probes were gel-purified. Gel mobility shift experiments were performed as described (34) with modifications. Briefly, 20-μl binding reactions were assembled on ice and contained 1 mg/ml bovine serum albumin, 1 mM dithiothreitol, 0.6× binding buffer (1× binding buffer: 20 mM Tris, pH 7.5, 100 mM NaCl, 1 mM EDTA, 20% [v/v] glycerol), either 400 ng of poly(dA·dT) or 700 ng of poly(dC·dC), −200 cpm (−10 pmol) of labeled double-stranded oligonucleotide probe, and the indicated amount of nuclear extract or purified protein. Subsequent to the addition of labeled probe, the samples were incubated at room temperature for 20 min, after which 10 μl of sample was electrophoresed through a 0.75-mm thick 5% polyacrylamide gel (29:1 acrylamide:bis-acrylamide) containing 1% glycerol and 0.5 μl of packed beads in a final volume of 500 μl containing 16 mM Hepes, pH 7.9, 200 mM KCl, 1 mM EDTA, 16% glycerol, 1 mM dithiothreitol, and 1 mg/ml bovine serum albumin for 20 min at room temperature, after which the mixture was centrifuged at 16,000 × g for 5 min at room temperature to pellet the beads. The supernatant was removed, and the beads were sequentially washed in buffer containing 0.01, 0.00, and 300 mM KCl. The beads were resuspended in 20 μl of SDS-PAGE loading buffer and heated to 90 °C for 2 min, and 10 μl were electrophoresed through a 10% SDS-polyacrylamide gel. Following silver staining and excision of the bands of interest, the silver grains were removed by treatment with 15 mM potassium ferricyanide and 50 mM sodium thiocyanate. The gel slice was digested with trypsin in 25 μl NH2HCO3, overnight (36, 37). Peptides were eluted with two extractions of CH3CN and combined. The tryptic peptides were dried, reconstituted with 8 μl of 0.1% trifluoroacetic acid, desalted on a C18 Zip Tip (Millipore), and eluted with 4 μl of 50% CH3CN, 0.1% trifluoroacetic acid. The eluate was dried and reconstituted in 1 μl of re-crystallized α-cyano-4-hydroxycinnamic acid matrix, and 0.5 μl was applied to separate matrix-assisted laser desorption/ionization-time of flight mass spectrometry (MALDI-TOF; Applied Biosystems model Voyager DESTR). Samples were analyzed in reflector mode, and the resulting spectra were screened against the NCBI data base (released June 6, 2002) using the Protein Prospector search engine (University of California, San Francisco).

**Immunoprecipitation and Western Blotting**—40 h post-transfection, COS7 cells were harvested, washed twice in phosphate-buffered saline, and lysed in lysis buffer (25 mM Tris-Cl, pH 8.0, 100 mM KCl, 1% glycerol, 5 mM NaF, 2 mM Na3VO4, 0.5% Nonidet P-40, and 0.1% Triton X-100) containing antiprotease mixture without EDTA (Roche Applied Sciences) for 30 min at 4 °C. After centrifugation at 16,200 × g for 15 min at 4 °C, the protein concentration of the supernatant was measured by the Bradford method (Bio-Rad). 250 μg of the lysate was used with the anti-GFP IP and 800 μg with the anti-TFII-I immunoprecipitation. The lysates were incubated with protein G-Sepharose (Amersham Biosciences) for 2 h of rocking at 4 °C. The Sepharose beads were then washed three times in 1 ml of lysis buffer lacking the Nonidet P-40 detergent. The beads were boiled in SDS-PAGE loading buffer for 2 min, and Western-blotted using antibodies to GFP GST-2 (Sigma) and GFP JL-8 (Clontech). The proteins were detected by chemiluminescence using ECL (Amersham Biosciences).

**RESULTS**

**Characteristics of DICE**—In transient transfection experiments, luciferase reporter constructs containing VH promoters are preferentially expressed in B cell lines. Promoter deletion and mutagenesis of the murine 7183 family 17.2.25 promoter revealed a DNA sequence downstream of the transcription initiation site that was critical for the high promoter activity in B cells. A Gibbs motif sampler (39) was used to show that this element is a feature of most VH and some Vλ downstream promoter regions (32). Here we term this element DICE for Downstream Immunoglobulin Control Element. DICE is composed of a 5′ end rich in pyrimidine residues and a 3′ end with a strong preference for ACAG. Interestingly, the best DICE matches were found in J558-class promoters, the most distal VH family with the most intrinsically active promoters. For example, the J558-class Vλ186.2 (B1–8) core promoter region contains three consensus sequences, two of which are downstream of the transcription initiation site. The first of these downstream sites is a perfect match (Fig. 1A). Searching the eukaryotic promoter database (residues −499 to +100 relative to the transcription initiation site) for DICE did not reveal any strong non-immunoglobulin matches to the consensus (not shown).

Deletion of DICE from the Vλ17.2.25 promoter results in a significant loss of promoter activity, which can be restored by introducing the downstream sequences from the Vλ186.2 promoter (32). To extend and expand upon these results, point mutations were introduced into the Vλ186.2 DICE consensus sequences. These mutant sequences were placed into the Vλ17.2.25 promoter and tested by transient transfection. In...
parallel, the same sequences were radiolabeled and used in EMSA to determine whether sequence- and B cell-specific nucleoprotein complexes could be identified. Fig. 1A shows the two DICE mutations used. Mutant 1 changes residues 3–5 in the 5’ end of the consensus to GGA, whereas mutant 2 changes residues 11–13 in the 3’ end to TTT. Fig. 1B depicts the aver-
aged results from three parallel transfections by using the human B cell line BJA-B. Insertion of VH17.2.25 promoter DNA (residues −154 to +35 relative to the transcription initiation site) into the backbone vector pGL3 strongly increases reporter expression. Removal of sequences downstream of the transcription initiation site from +2 to +35 resulted in significant down-regulation of promoter activity. As reported previously, replacing these residues with the corresponding sequences from the VH 186.2 promoter (IgH chimera) restores activity and results in a significant increase in activity over wild-type levels. This increase in activity may be explained by the fact that the VH186.2 downstream region contains two DICE sequences, one of which is a better match to the consensus than the DICE sequence present in 17.2.25. When mutant sequences replaced the wild-type DICE, promoter activity was reduced. In the case of mutant 1, promoter activity was markedly attenuated and approximately the activity of a construct containing no downstream promoter residues. Mutant 2 was less severe.

In the gel mobility shift experiment shown in Fig. 1C, DNA segments containing the wild-type and point-mutated 186.2 residues were labeled and incubated with fibroblast (3T3) or BJ-A-B B cell nuclear extracts. The 3T3 extracts formed limited complexes with the 186.2 DICE sequence, which were unaffected by point mutation (lanes 2 and 3, 7 and 8, and 12 and 13). B cell extracts formed more robust nucleoprotein complexes (lanes 4 and 5) composed of two bands: a major slower mobility band (asterisk) and a minor band of faster mobility (arrow). The major band was strongly reduced with mutant 1 (lanes 9 and 10), whereas the minor band was ablated using mutant 2 (lanes 14 and 15). Similar results were obtained with extracts from murine 70Z/3 B cells (not shown). Therefore, the severity of the mutations for gene expression in vivo mirrors the degree to which complex formation is impaired with B cell extracts in vitro. The presence of two bands that can be independently affected by point mutation also suggests that DICE is composed of at least two binding sites. The complex formation observed with DICE also is highly non-linear, as a 2-fold increase in protein concentration yields a much more than linear increase in intensity (compare lanes 4 and 5). The complex could be efficiently competed by a 20-fold excess of unlabeled DNA containing the wild-type 186.2 DICE sequence, much less well using the mutant 1 DICE sequence, but not with unrelated polylinker DNA of similar length, a sequence of 24 alternating A and T residues, or single-stranded DNA (not shown).

The IgH intronic enhancer is a potent B cell-specific control element (40–42) that interacts with numerous transcription factors, including those of the Oct, Ets, and helix-loop-helix families (8, 43). V-DJ recombination relocates the intronic enhancer from greater than 100 kb to less than 10 kb downstream from the selected V region. From this relatively proximal location, the enhancer strongly stimulates VH promoter activity. At least one report suggests that the intronic enhancer more effectively activates VH promoters than unrelated promoters (44). To assess the role of DICE in this promoter-enhancer interaction, the 700-bp core enhancer element was placed into an XbaI site 1.7 kb downstream of the reporter gene in plasmids containing wild-type and point mutant DICE sequences. These new constructs (termed ElgH chimera and ElgH mutant 1) were used in transient transfection assays with the BJ-A-B cells. As expected, addition of the intronic enhancer (Elg chimera) greatly stimulated reporter activity beyond that observed with the chimeric V promoter alone (IgH chimera; see Fig. 2). More importantly, the DICE point mutation eliminated the ability of a distal enhancer element to stimulate promoter expression, as the reporter activity from the promoter containing DICE mutant 1 (Ig mutant 1) displayed no increase in activity in the presence of the enhancer (Elg mutant 1).

**FIG. 2. Effect of DICE mutations on activity of the IgH intronic enhancer element.** Transient transfection assay using the cell line BJA-B. Ig promoter constructs used in Fig. 1A (Ig chimera and Ig mutant 1) were used in conjunction with plasmids in which the IgH intronic enhancer had been placed downstream (ElgH chimera and ElgH mutant 1). Averages from three experiments are shown. Error bars denote standard deviations.

**BEN/MusTRD1 Interacts with DICE**—To isolate factors that interact with DICE, latex microspheres coupled to the wild-type VH186.2 segment (“DICE WT”) were incubated with 70Z/3 B cell nuclear extracts and carrier DNA (35, 45). The beads were washed with 300 mM KCl, boiled in SDS loading buffer, electrophoresed through 10% SDS-PAGE gels, and silver-stained (Fig. 3A). In repeated experiments, a number of proteins were retained on the beads containing DNA. Most of these proteins were also retained using mutant 1 DNA sequences, but the degree of binding was much lower for an −110-kDa band (arrow). This band was excised from the gel for further analysis. Matrix-assisted laser desorption/ionization-time of flight (MALDI-TOF) mass spectroscopy of tryptic fragments from the −110-kDa protein identified 24 peptides. The Protein Prospector search engine (University of California, San Francisco) matched 11 of these peptides to a protein known as BEN/MusTRD1/GTF2IRD1/WBSCR11 (46–49). Re-calibration of the mass spectroscopy peak set using the masses of two matched BEN peptides improved the average 1 ppm of the remaining peptides to +0.42 (not shown). A non-overlapping set of peptides from the same band matched mMutS/MSH2, a 104-kDa protein involved in mismatch repair. This match was not studied further but may partially account for the background band seen in the mutant DNA sequence lane (lane 3).

Three of the peptides matched to BEN were predicted to be phosphorylated (Table I). The software algorithm NetPhos (50) was used to predict potential phosphorylation sites within the BEN primary sequence. Of the three peptides, two contained predicted phosphorylation targets. Conversely, only one of the remaining seven unphosphorylated peptides was predicted to be a phosphorylation target. Although indirect, these findings provide support for the validity of the phosphate modifications.

Several approaches were taken to assess the role of BEN in IgH promoter regulation. First, antibodies directed against BEN were incubated with nuclear extract in gel mobility shift experiments. BEN isoforms vary greatly in the C terminus (see for example Ref. 51); therefore, antibodies were raised against an N-terminal peptide of BEN. When sub-saturating amounts...
of 70Z/3 B cell extract were used, a significant increase in complex formation was evident when the extract was incubated with the antibody. The effect was greater in the presence of 7.5 mM MgCl₂ (Fig. 3B). BEN antibodies eliminated the faster migrating complex (arrow), and greatly intensified the slower mobility complex (asterisk). In contrast, antibodies against TFII-I (lane 4), Oct-1 (lane 5), TFIIH (lane 6), or pre-immune serum (lane 7) showed no effect on complex formation. Although TFII-I antibodies did not have any effect, differences in the epitopes recognized may be responsible, as the TFII-I an-
tobody was directed against the DNA binding domain whereas the BEN antibody recognizes the N terminus. A DNA segment containing the octamer site was used as a control. With this probe, the BEN antibody had no effect on nucleoprotein complexes (lane 10). In contrast, as expected an Oct-1 antibody resulted in an interaction (lane 12). The effect of the BEN antibody on the DICE complex was reduced when it was added after incubation of the probe with the nuclear extract (not shown). These experiments demonstrated that in 70Z/3 nuclear extracts, BEN plays a role in the formation of DICE complexes and are most consistent with a model in which antibodies against BEN promote formation of the slower mobility complex, and block formation of the faster mobility complex.

Full-length human BEN (β isoform) and the closely related TFII-I protein (δ isoform) were purified as histidine- and GST-tagged recombinant proteins by transient transfection and purification by nickel affinity chromatography (38). Fig. 3C shows a Coomassie Blue-stained polyacrylamide gel of the purified TFII-I (lane 1) and BEN (lane 2) proteins (arrow). The minor bars are contaminants and/or degradation products (brackets). The binding affinity of these partially purified recombinant proteins for the DICE sequence from the V<sub>1</sub>86.2 promoter was tested in gel mobility shift assays (Fig. 3D). By using 300 ng of protein, BEN formed a complex with DICE DNA, as well as a series of lower mobility oligomeric complexes (lane 2). In contrast, TFII-I failed to form any complexes at these concentrations (lane 4). However, at twice the concentration of protein, robust complexes were formed with both proteins, and TFII-I formed more intense complexes than BEN (lanes 3 and 5). Therefore, although BEN interacts with DICE DNA non-linearly, TFII-I displays even greater cooperativity. Substituting labeled mutant 1 DNA for the wild-type DICE sequence reduced binding of both BEN and TFII-I (lanes 8 and 10). Mutant 2 had little effect on complex formation (lanes 12–15). Competition studies showed that 20-fold excesses of unlabeled nonspecific DNA of similar length to the 186.2 DICE sequence did not significantly affect complex formation. In contrast, the wild-type sequence effectively competed for binding (data not shown). These data demonstrate that the BEN and TFII-I selectively form complexes with the 186.2 DICE sequences, and that the sensitivity to DICE mutation correlated with activity in vivo. Mixing experiments did not reveal a cooperative interaction between the two proteins under these conditions (data not shown).

Because both BEN and TFII-I interact with the DICE element in vitro, and because both proteins share isoforms of the same molecular weight, the mass spectroscopy peptides were tested specifically against mouse TFII-I. No significant matches were evident. Therefore, in 70Z/3 B cell nuclear extracts and using these conditions, TFII-I isoforms of this size are not detected on the beads.

Complex Regulation of IgH Promoter Activity by BEN—The leucine zipper domain of BEN was recently shown (52) to mediate dimerization of the protein. To address whether the interaction of BEN with DICE has functional consequences in B cell lines, a construct designed to disrupt the N-terminal leucine zipper domain of human BEN by introducing prolines in place of leucines at positions 38 and 45 was generated. This protein was predicted to be able to bind DNA but to be defective in its ability to form multimeric complexes with itself or other proteins. To test the ability of the protein to interact with itself and with the TFII-I protein, co-immunoprecipitations of COS7 cells transiently transfected with tagged versions of TFII-I and BEN were performed. Fig. 4A shows that precipitation of GFP-tagged BEN protein using anti-GFP antibodies and protein G-Sepharose beads efficiently co-precipitated GST-tagged wild-type BEN (lane 4) but not the L38/45P mutant protein (lane 6). Quantitation indicated that the homomeric interaction was reduced 85% under these conditions. Co-expression of TFII-I with BEN and precipitation using TFII-I antibodies resulted in efficient co-precipitation of BEN (lane 8) but not the double point mutant (lane 12). The results were similar when the cells were treated with recombinant epidermal growth factor (EGF), which is known to induce the phosphorylation of TFII-I on tyrosine residues (lanes 10 and 14). Although this protein was severely defective in its ability to interact with itself and with TFII-I, it could still interact with DICE sequences, as the purified mutant protein bound strongly to DICE in EMSA (Fig. 4B, arrow). However as compared with the wild-type protein, there was a significant decrease in the ability to form higher order complexes (asterisk).

The BEN L38/45P construct was overexpressed in murine M12 cells (a mature B cell plasmacytoma). Co-transfection of the dominant-negative BEN construct effectively reduced promoter activity, indicating a positive regulatory role for this protein (Fig. 4C). In this experiment, the most severe mutant DICE construct (ElgH mutant 1) was also tested. As expected, this construct was much less active, and importantly transfection of dominant-negative BEN resulted in little effect. These results indicated that the BEN protein functionally operates through the DICE sequence.

Promoter activity was also tested in the murine pre-B cell line HAFTL. Unlike M12, in this case promoter activity was up-regulated by co-transfected dominant-negative BEN (Fig. 4D). The activity of the mutant BEN protein was also tested in murine pre-B 70Z/3 cells. As depicted in Fig. 4E, titration of dominant-negative BEN resulted in a slight dose-dependent increase in Ig promoter activity, indicating that the wild-type protein also inhibits expression in this cell line. Other experiments indicated that BEN repressed gene activity in the non-B cell lines WERI-27 and 2017 as well (not shown).

The finding was somewhat surprising that a protein isolated through its ability to interact with a positively acting cis-element was capable of suppression of promoter activity. Therefore, to independently verify these data, siRNA hairpins were designed along the length of the mouse BEN coding sequence that would specifically target all known BEN isoforms but not the closely related TFII-I protein (Table II). U6 promoter-driven stem-loop constructs of similar design have been used successfully to reduce the expression of endogenous genes in culture. Four siRNA hairpins were initially tested for their ability to knock down a mouse BEN cDNA transfected into human 293T cells. A GST-tagged fragment of human TFII-I (p70, Ref. 38) was used as a co-transfected control. When only one cDNA construct was used, Western blotting detected the appropriate protein product (Fig. 5A, lanes 2 and 3). Of the four siRNA stem-loop constructs, construct 2 silenced the best, strongly and selectively reducing the expression of BEN protein (lane 6). Stem-loops 1 and 3 functioned much less well (lanes 5 and 7), whereas construct 4 and an siRNA stem-loop directed against the HIV Rev protein showed no activity (lanes 4 and 8). Stem-loop 2 was used for further experimentation.

In Fig. 5B, stem-loop 2 was transfected into 70Z/3 cells together with an IgH reporter construct containing a downstream enhancer element (ElgH chimera). The stem-loop directed against HIV Rev was used as a parallel control. In addition, a parallel set of transfections used the cell type non-specific H2B promoter. The siRNA stem-loop directed against BEN resulted in a statistically significant increase in IgH reporter activity; however, the same siRNA construct had no

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3. D. Dykhoom and P. A. Sharp, unpublished data.
FIG. 4. BEN positively and negatively regulates IgH promoter activity. A, interaction potential of L38 and 45P mutant version of BEN. Co-immunoprecipitation reactions were performed on lysates from COS7 cells that had been transiently co-transfected with BEN to test for homomeric interactions (lanes 1–6) or BEN and TFII-I to test for heteromeric interactions. The antibody used in the Western blot is shown to the right of each blot. For the lanes labeled +rhEGF, cells were serum-starved for 14–16 h and treated with 25 ng/ml recombinant human EGF for 20 min before harvesting the cells. IP, immunoprecipitation; WCE, whole-cell extract. B, DNA binding ability of BEN L38/45P. Increasing amounts of wild-type human BEN (lanes 2–5) or a double point mutant in which two of the leucine residues in the leucine zipper domain were changed to proline (lanes 6–9) were incubated with the DICE sequences from the 186.2 promoter. C, transient transfection assay of M12 cells using the ElgH chimera and ElgH mutant 1 constructs. Cells were co-transfected with either pEBG (empty vector) as a negative control or with pEBG containing a BEN cDNA double-point mutant sequence that disrupts the leucine zipper domain (L38/45P). D, similar transfection using the cells line HAFTL. M12 was transfected in parallel. E, transient transfection assay of 70Z/3 B cells with the ElgH chimera construct. Cells were co-transfected with either 750 ng of pEBG (empty vector), 750 ng of the BEN L38/45P construct, or a mixture of the two in which increasing amounts of the BEN construct were used.

| TABLE II | siRNAs directed against BEN |
|-----------|----------------------------|
| mBEN siRNA | Start position (β isoform, relative to start codon) | End position | cDNA location | Protein location | Note | Sequence |
| 1 | 358 | 379 | Exon 4 | 119–126 | HLH1 | AGCACUGCUUCGGAUGUGUAC |
| 2 | 1244 | 1265 | Exon 9 | 414–422 | HLH2 | CGAACAGCAUUCACUUCAU |
| 3 | 1555 | 1576 | Exon 14 | 518–526 | CUGACUCUUAUACCCUGGCAU |
| 4 | 2276 | 2297 | Exon 20 | 758–766 | HLH4 | GUUUCAGGUACCAGGCAU |
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Regions of the Ig promoter, including the V region 

Effect on the H2B promoter. Rev stem-loop constructs had no effect in either case. The other siRNA constructs had either a much smaller effect or no effect at all (not shown). Thus reduction of BEN levels results in increased IgH promoter activity in 70Z/3 cells, indicating a negative function for this protein in this cell line. These data support those generated with the dominant-negative construct, indicating that BEN can negatively regulate Ig promoter activity in 70Z/3 cells.

Discussion

Here we describe the initial biochemical characterization of DICE, a new promoter element located downstream of the transcription initiation site of Ig V region promoters. DICE is an ~14-residue sequence that was originally identified through a combination of experimental and statistical techniques (32) and is required for maximal Ig promoter expression in B cells. Deletion of DICE sequences within the 17.2.25 IgH promoter results in a significant decrease in promoter activity, both in the presence of the upstream octamer sequence (this study) as well as in its absence (not shown). It is therefore likely that these two elements act largely independently of one another. In transient transfection assays, we observed that the DICE sequence is an important mediator of distal IgH intronic enhancer activity, as mutation of DICE is much more severe in the context of the intronic enhancer. There have been reports that the intronic enhancer stimulates IgH promoters more effectively than heterologous promoters, although the basis of this effect has not been elucidated (44). A specific interaction with the DICE element may explain this interaction.

Formation of B cell-specific complexes on sequences downstream of the IgH core promoter has been described previously (53). Similar assays show that nuclear extracts from the mouse B cell lines BJA-B and 70Z/3 also contain activities that strongly and specifically interact with V11.186.2 DICE sequences. Mutant versions of DICE that impair transcription activity were also used directly as gel shift probes to show that these sequences are defective in complex formation. A series of specific complexes were observed indicating either that multiple sequence-specific factors bound to these probes or that multimeric complexes of a single protein bound. Formation of these complexes were strikingly concentration-dependent, perhaps more consistent with the latter.

Latex microsphere beads coated with the DICE sequences from the V11.186.2 promoter were used to purify DICE-interacting proteins. Latex microspheres are small in diameter, non-compressible, and form compact pellets. They have a much higher binding capacity than conventional supports and remain in suspension for long periods until high speed centrifugation packs the beads. In conjunction with MALDI-TOF mass spectroscopy, this technique allows proteins to be rapidly identified starting from nuclear extracts or crude fractions. Purification and mass spectrometry of proteins that interact with DICE resulted in the identification of a protein known as BEN/MusTRD1/CREAM-1/GTF2RD1/GTF3/WBSCR11.

BEN (Binds Enhancer) was originally identified in yeast one-hybrid assays as a protein that bound to a human slow twitch muscle-specific enhancer element (47), and subsequently to a site in the mouse Hox8 enhancer element critical for transcription activity in embryonic mesoderm (49). The yeast one-hybrid system was also used to isolate a Xenopus homologue of BEN that bound to a distal element in the goosecoid promoter (54). In several of these cases, part of the DNA sequence used to select BEN shows homology to DICE (not shown), but because these sequences were not defined by mutations, we do not know if binding to these sequences is involved in their activities. Additionally, BEN was isolated as an interaction partner of the retinoblastoma transcriptional repressor protein in a biochemical screen (46). BEN is closely related to TFII-I (55) and, like TFII-I, contains signature tandem helix-loop-helix motifs (also known as I-repeats) in a large central portion of the protein. The helix-loop-helix is a classic protein-protein interaction module (56). The fourth member of these repeats was shown recently (52) to mediate the DNA binding activity of BEN. BEN also contains a leucine zipper domain, important for multimerization, at its N terminus as well as a C-terminal region rich in serine residues. There are a number of BEN isoforms (at minimum 13) ranging in size from 65 to 160 kDa (51, 52, 57). Alternative splicing has been shown recently to alter the DNA binding properties of the protein (52). TFII-I is similar in primary structure but appears to be somewhat less subject to alternative splicing, with four well defined isoforms of 120–150 kDa (38). Both proteins are widely expressed and highly expressed in lymphoid tissues such as thymus and spleen.

Given the similarity between TFII-I and BEN, we tested whether TFII-I could also bind to DICE. Gel mobility shift experiments showed that purified human BEN and TFII-I both interact with DICE sequences. Of the two, purified TFII-I appears to interact with higher affinity in vitro. The lack of TFII-I binding to DICE in affinity selection of the extracts may be due to the low levels of TFII-I in 70Z/3 cells. Western blotting shows that TFII-I levels are much lower in 70Z/3 cells compared with
mature B cell lines such as Ba117. The complexes formed with either purified protein were highly cooperative; a 2-fold increase in protein concentration yielded a much more than linear increase in complex formation. This nonlinear titration effect was reminiscent of complexes formed using nuclear extracts. Also as with nuclear extract, binding of either BEN or TFII-I is reduced by DICE point mutations, further indicating that the binding is of functional consequence. BEN and TFII-I do not appear to bind cooperatively with one another (not shown). The complexes formed with nuclear extracts and the purified proteins have different mobilities in neutral gels, indicating that there may be additional binding partners in the extract and/or that DICE may be a compound site that is capable of interacting with other factors.

Pelletier et al. (58) described an element located 24–39 nucleotides downstream of the transcription initiation site of the mouse T1k promoter. In this study, mutation of this element was as deleterious to promoter function as mutation of the octamer site or TATA region. We have made similar findings with DICE and the 17.2.25 octamer sequence (not shown). The reverse complementary sequence of this element shows significant homology to DICE (not shown). More recently, downstream positive-acting cis-elements have also been identified in the V4 and κ-41 light chain promoters (59). Competition studies for BEN binding revealed that each of these promoter sequences could compete for binding of BEN to the 186.2 DICE sequence nearly as well as the 186.2 DICE probe, whereas a nonspecific competitor had no effect (data not shown). Interestingly, the V4 promoter sequence does not contain a significant match to the DICE consensus sequence, indicating that the specificity of BEN binding may be somewhat more relaxed than the consensus sequence depicted in Fig. 1A. Relaxed binding specificity appears to be a characteristic of this family since studies of TFII-I show it to be capable of interacting with E boxes, which have no obvious similarity to the consensus TFII-I-binding site (60).

Functional studies using a dominant-negative human BEN cDNA in which the leucine zipper motif has been disrupted resulted in both increased and decreased IgH reporter activity. In M12 cells, BEN activates IgH transcription, and this activation requires an intact DICE sequence. In this plasmacytoma cell line, the V1 promoter is strongly stimulated by the intron enhancer. Possibly, enhancer-promoter communication is aided by BEN, and overexpression of BEN L38/45P disrupts this interaction, resulting in a loss of activity in these cells. However, using both dominant-negative and siRNA constructs, we have also observed that in several cell lines, including 70Z/3, BEN functions not as an activator but as a repressor. Given the positive activity of the DICE sequence, the basis of the repressor activity in these cells may be due to BEN forming an inhibitory complex on DICE that interferes with the activity of another factor, which normally activates transcription. The recent finding that BEN inhibits transcription at the troponin I slow gene by antagonizing the activity of the transcriptional activation protein MEF2 (61) supports this idea.

Other recent accounts firmly establish the capacity of BEN to repress gene activity. BEN and TFII-I both interact with the HDAC-3 histone deacetylase, which represses gene expression by removing acetyl groups from lysine residues in the N-terminal tails of histone molecules (62). Co-expression of BEN with TFII-I results in exclusion of TFII-I from the nucleus and a loss in TFII-I-dependent activation of a c-fos promoter (63). Full-length BEN also represses the goosecoid gene through a trans-

forming growth factor-β signal-responsive Smad site within the goosecoid promoter.

Sub-nuclear localization of TFII-I and BEN may play a role in regulation of Ig genes. Both BEN and TFII-I were shown to interact with PIAS proteins, which mediate small ubiquitin-like modifier addition to target molecules (64). Phosphorylation of these proteins in response to signals is also an important mode of regulation. For example, TFII-I can be phosphorylated by Btk, a transducer of signals emanating from the B cell receptor, in a manner that promotes its ability to stimulate transcription (65, 66). In addition, the species of BEN associated with DICE in 70Z B cell extracts is phosphorylated on multiple residues.

In appropriate stage B cells, the activity of BEN may stimulate Ig promoter expression and therefore the recombination and expression of Ig pre-mRNA. The specificity of DICE activity for B cells (32) may confer some cell type specificity on promoters. The repressive functions of BEN may also serve to keep promoter activity restrained in certain stages of B cell maturation, such as before Ig expression takes place or during the silencing of germ line transcription in upstream V regions upon the successful completion of V(D)J recombination. Silencing of Ig expression also occurs when V-D-J rearrangement creates a non-functional protein, and the locus on the homologous chromosome becomes activated. In addition, there are data indicating that surface Ig is reduced in germinal center dark zone centroblasts, possibly to enhance selection of high affinity B cells during affinity maturation (67). These changes in Ig expression may, in part, be mediated by TFII-I factors.

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