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EBNA-2 Upregulation of Epstein-Barr Virus Latency Promoters and the Cellular CD23 Promoter Utilizes a Common Targeting Intermediate, CBF1

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The EBNA-2 protein is essential for the establishment of a latent Epstein-Barr virus (EBV) infection and for B-cell immortalization. EBNA-2 functions as a transcriptional activator that modulates viral latency gene expression as well as the expression of cellular genes, including CD23. We recently demonstrated that EBNA-2 transactivation of the EBV latency C promoter (Cp) is dependent on an interaction with a cellular DNA-binding protein, CBF1, for promoter targeting. To determine whether targeting via CBF1 is a common mechanism for EBNA-2-mediated transactivation, we have examined the requirements for activation of the cellular CD23 promoter. Binding of CBF1 to a 192-bp mapped EBNA-2-responsive region located at position −85 bp to −277 bp upstream of the CD23 promoter was detected in electrophoretic mobility shift assays. The identity of the bound protein as CBF1 was established by showing that the bound complex was competed for by the CBF1 binding site from the EBV Cp, that the bound protein could be supershifted with a bacterially expressed fusion protein containing amino acids 252 to 425 of EBNA-2 but was unable to interact with a non-CBF1-binding EBNA-2 mutant (WW323SR), and that in UV cross-linking experiments, the Cp CBF1 binding site and the CD23 probe bound proteins of the same size. The requirement for interaction with CBF1 was demonstrated in a transient cotransfection assay in which the multimerized 192-bp CD23 response region was transactivated by wild-type EBNA-2 but not by the WW323SR mutant. Reporter constructions carrying multimerized copies of the 192-bp CD23 response region or multimers of the CBF1 binding site from the CD23 promoter were significantly less responsive to EBNA-2 transactivation than equivalent constructions carrying a multimerized region from the Cp or multimers of the CBF1 binding site from the Cp. Direct binding and competition assays using 30-mer oligonucleotide probes representing the individual CBF1 binding sites indicated that CBF1 bound less efficiently to the CD23 promoter and the EBV LMP-1 promoter sites than to the Cp site. To investigate the basis for this difference, we synthesized a series of oligonucleotides carrying mutations across the CBF1 binding site and used these as competitors in electrophoretic mobility shift assays. The competition experiments indicated that a central core sequence, GTGGGAA, common to all known EBNA-2-responsive elements, is crucial for CBF1 binding. Flanking sequences on either side of this core influence the affinity for CBF1. The Cp has the most optimal binding site, with the CD23 site having an intermediate affinity and the LMP-1 site having a low affinity for CBF1. These differences in affinity can be directly correlated with changes in the flanking sequences. A search of databases using the CBF1 site defined in this study indicates that the number of cellular genes which can potentially be subject to EBNA-2 activation may be considerably larger than previously appreciated.

Epstein-Barr virus (EBV) is associated with a number of human B-cell malignancies. These include Burkitt's lymphoma, posttransplant lymphoma, and central nervous system lymphoma in AIDS (26, 27). In addition, a proportion of Hodgkin's lymphoma and systemic lymphoma in AIDS is also EBV associated (16, 19, 33, 49). In the laboratory, EBV infection of primary B cells leads to outgrowth of continually proliferating lymphoblastoid cell lines. This process is highly efficient, and the EBV-induced changes in B-cell growth are likely to play a significant role in lymphomagenesis.

The first genes expressed during EBV infection of primary B lymphocytes are EBNA-LP and EBNA-2 (2, 3, 29). The latency W promoter (Wp) is used initially, followed by a switch to the latency C promoter (Cp) that in turn leads to expression of EBNA-3A, -3B, -3C, and -1 (7, 31, 32, 47). Infection of primary B cells with P3HR-1 virus, which is deleted for the EBNA-2 gene, does not lead to activation of the Cp, implicating EBNA-2 as a mediator of the switch from Wp to Cp (30, 47). Cotransfection experiments confirmed the contribution of EBNA-2 by mapping an EBNA-2-responsive region between −331 and −380 of the Cp (20, 24, 25, 35). Similarly, both P3HR-1 infection and cotransfection experiments provided evidence for EBNA-2 upregulation of the promoters for the latency membrane proteins, LMP-1 and LMP-2A (TP1) (1, 15, 41, 45, 52, 53).

In addition to its role in the regulation of EBV latency gene expression, EBNA-2 also alters expression of cellular genes. The B-cell surface antigen CD23 is upregulated by EBNA-2 along with CD21 and c-*fgr* expression (9, 14, 21, 28, 42–44). Activation of CD23 may be particularly important, since only EBV-infected B cells expressing this protein go on to become immortalized (5, 39). CD23 functions as a low-affinity immu-

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noglobulin E receptor, and soluble cleavage products act as an autocrine growth factor (8, 38, 40, 51). Two species of CD23, types a and b, have been described. These differ by six amino acid residues in the cytoplasmic N terminus and also differ in tissue distribution (50). Type a is B cell specific, while type b is usually found in other cells of hematopoietic lineage. EBV-transformed B cells express both CD23 type a and type b (14). Studies by Wang et al. (44) identified a *cis*-acting EBNA-2-responsive region in the type a CD23 promoter (CD23p) that mapped from −89 to −275 relative to the mRNA start site.

EBNA-2 has been demonstrated to have an acidic activator domain located between amino acids 427 and 477 (10, 11, 25). However, determination of the underlying mechanism for EBNA-2 promoter targeting was originally frustrated by an inability to demonstrate direct binding to mapped EBNA-2 *cis*-acting responsive regions. Zimber-Strobl et al. (52) provided evidence for an interaction between EBNA-2 and a cell protein that bound to the LMP-2A (TP1) promoter (LMP-2Ap [TP1p]). Recent work in our laboratory has shown that a cellular protein, designated CBF1, binds to an EBNA-2-responsive enhancer element in the Cp (24). EBNA-2 is able to form a stable complex with Cp-bound CBF1 in an electrophoretic mobility shift assay (EMSA). This interaction can be mapped to a domain between amino acids 252 and 425 of EBNA-2. Sequence comparisons between EBNA-2A and -2B and EBNA-2 of the baboon virus herpesvirus papio revealed that this segment of EBNA-2 contains three highly conserved amino acid motifs (25). Mutation of amino acid residues 323 and 324 in the central motif from tryptophan-tryptophan to serine-arginine abolishes the ability of EBNA-2 to interact with CBF1, and this mutant fails to transactivate the Cp in transient transfection assays (24). We also mapped the CBF1 binding site to a 30-bp region in the Cp (−359 to −388) that, when fused to a heterologous minimal promoter, was able to confer EBNA-2 activation in transient cotransfection assays. Within this 30-bp sequence, there is a motif, GTGGGAA, that is also found in the mapped EBNA-2-responsive regions of the EBV LMP-1 promoter (LMP-1p) and LMP-2Ap and the cellular CD23p. Sequences flanking this core region, however, are somewhat heterogeneous in the different promoters. Having shown that EBNA-2 is targeted to the Cp through the CBF1 DNA-binding protein, we wished to verify that EBNA-2 transactivation of the CD23p and LMP-1p also involved interaction with CBF1. Demonstration of such an interaction would establish that CBF1 targeting is a generalized feature of EBNA-2-mediated transactivation.

MATERIALS AND METHODS

Plasmids. PCR was used to generate a DNA fragment containing promoter sequences from −85 to −277 from the CD23p. The 5′ primer sequence used in the PCR was 5′ CAGTAGATCTTTCCGTGGCTCCCCAGGG 3′, and the 3′ primer sequence was 5′ CAGTGGATCCCCGGGTGAAGTC CGTGTT 3′. The PCR template was derived from a plasmid obtained from H. Hofstetter, University of Basel, Basel, Switzerland (36, 37). The PCR product was cut with *Bgl*II and *Bam*HI and cloned into a modified pUC19 plasmid, pGH56, to generate pPDL140. The PCR DNA insert was sequenced to confirm that no errors had occurred during amplification. Tandemly repeated copies of this DNA fragment were generated as described previously (25). Plasmid pPDL178 containing eight tandem copies of the −85 to −277 CD23p DNA fragment was excised with *Bgl*II and *Bam*HI and cloned into the chloramphenicol acetyltransferase (CAT) reporter vector pGH262 to generate 8xCD23/CAT (pPDL181A). Plasmids pJH26A and pJH27A containing eight tandem copies of Cp (−359 to −389) and CD23p (−158 to −187) sequences were generated as described previously (25). These DNA fragments were cloned upstream of the minimal promoter in pGL2 (Promega), which contains the luciferase reporter gene. Plasmids SV-EBNA2 (pPDL151) and SV-EBNA2 (WW323SR) containing wild-type and mutant EBNA-2 coding sequences have been described previously (24). Wild-type and mutant EBNA-2 are expressed in equal amounts in cells transfected with these plasmids, as determined by Western blot (immunoblot) analysis (24), and localize appropriately to the nucleus, as determined by indirect immunofluorescence (data not shown).

CAT and luciferase assays. DG75 lymphoblastoid cells were maintained in RPMI supplemented with 10% fetal bovine serum (GIBCO) and incubated in 5% CO_2 at 37°C. Cells were transfected with 4.0 μg each of effector and target plasmid DNA unless otherwise specified and harvested at 48 h post-transfection. CAT activity was measured as described previously (25). For luciferase assays, cells were washed with 1× phosphate-buffered saline and resuspended in 100 μl of 0.25 M Tris (pH 7.8)–1 mM dithiothreitol. Cells were then sonicated, and the cell debris was removed by centrifugation. Cell lysates were added to 350 μl of reaction buffer A (25 mM glycylglycine [pH 7.8], 5.0 mM ATP, 15 mM $MgSO_4$, 4.0 mM EGTA). Samples were loaded into a luminometer (Lumat LB9501; Berthold Systems Inc., Pittsburgh, Pa.), and the reaction was started by addition of buffer B (1.0 mM luciferin [Sigma] in buffer A) and counted for 6 s. In all experiments, cell extracts were equalized for total protein. An internal control, SV2β-GAL, was also transfected, and β-galactosidase activity was used to standardize for transfection efficiency.

EMSA. Nuclear extracts and glutathione *S*-transferase–EBNA-2 fusion proteins were prepared as described previously (24). Protein-DNA complexes were formed in a reaction mixture containing 2 μl of buffer (100 mM HEPES [*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid], 500 mM KCl, 10 mM EDTA, 1 mM dithiothreitol, 1% Triton X-100, 25% glycerol [pH 7.5]), 2.0 μg of poly(dI-dC), 1.5 μl of CA46 extract, and H_2O to a final volume of 19 μl. After incubation for 5 min at 22°C, ^{32}P-labeled probe DNA (20,000 cpm) was added for 30 min, and reaction mixtures were then separated on 4.5% nondenaturing polyacrylamide gels (24). The sequences of 30-mer oligonucleotide pairs used for DNA binding assays are as follows:

Cp 5′ GATCTGGTGTAAACACGCCGTGGGAAAAAATTTATG 3′ (top)
5′ GATCCATAAATTTTTTCCCACGGCGTGTTTACACCA 3′ (bottom)

CD23p 5′ GATCTCCTCCTTCAGCCCTGTGGGAACTTGCTGCTG 3′ (top)
5′ GATCCAGCAGCAAGTTCCCACAGGGCTGAAGGAGGA 3′ (bottom)

LMP-1p 5′ GATCTCCGGGGGGCAAGCTGTGGGAATGCGGTGGCG 3′ (top)
5′ GATCCGCCACCGCATTCCCACAGCTTGCCCCCCGGA 3′ (bottom)

MUT 10 5′ GATCTGGTGTAAACACGAATTCGGAAAAAATTTATG 3′ (top)
5′ GATCCATAAATTTTTTCCGAATTCGTGTTTACACCA 3′ (bottom)

Oligonucleotides used in the competition assays were identical to the Cp oligonucleotide pair (see above) except for the mutations indicated in Table 1. Quantitation of results from the competition experiments was done with a Molecular Dynamics PhosphorImager.

UV cross-linking. Oligonucleotides containing the Cp or CD23p binding site sequences and a common 17-bp 3′ sequence homologous to the M13 forward sequencing primer (Cp/M13 [CTGGTGTAAACACGCCGTGGGAAAAAATT TATGACTGGCCGTCGTTTTAC] and CD23p/M13 [TCCT CCTTCAGCCCTGTGGGAACTTGCTGCTGACTGGC CGTCGTTTTAC]) were synthesized. After annealing of M13

primers, the oligonucleotides were converted to fully duplex forms with the Klenow fragment of DNA polymerase I (New England BioLabs) and Sequenase II (U.S. Biochemical) in the presence of dATP, dGTP, bromo-dUTP, and [^{32}P]dCTP. Duplex oligonucleotides were purified from polyacrylamide gels.

Protein-DNA binding reaction mixtures (100 μl) containing 10 mM HEPES-KOH (pH 7.4), 0.5 mM dithiothreitol, 1 mM EDTA, 0.1% Triton X-100, 2.5% glycerol, 25 mM KCl, 5 to 10 μl of protein extract, 5 μg of p(dI-dC), and 0.5 × 10^6 1.0 × 10^6 cpm of duplex DNA probe were incubated for 30 min at room temperature. The reactions were electrophoresed on 5% native polyacrylamide gels in 1× Tris-taurine buffer (89 mM Tris, 28.5 mM 2-aminoethanesulfonic acid, 0.5 mM EDTA). After electrophoresis, the polyacrylamide gels were placed directly on a shortwave UV light source (305-nm wavelength; Fotodyne) for 20 min. Protein-DNA complexes were identified by autoradiography and were recovered by excision from the wet gels. The protein-DNA complexes were eluted from the gel slices by maceration of the gels in 0.5 M ammonium acetate–1 mM EDTA–10 mM magnesium acetate–1% sodium dodecyl sulfate (SDS) and incubation overnight with mixing. Samples subjected to nuclease digestion were first passed over a P6 desalting column (Bio-Rad) that had been equilibrated in nuclease buffer (10 mM $MgCl_2$, 10 mM $CaCl_2$, 10 mM Tris-HCl [pH 7.5]), and the DNA was digested by the addition of 50 μg of DNase I and 50 μg of micrococcal nuclease with incubation for 20 min at room temperature. The eluted proteins were visualized after electrophoresis, fixing, drying, and autoradiography of the complexes on SDS–10% polyacrylamide gels.

RESULTS

The EBNA-2-responsive region in the CD23p binds CBF1. We have recently demonstrated that EBNA-2 activation of the latency Cp is mediated through a cellular protein, CBF1, that binds to a sequence containing the motif GTGGGAA (24). The cellular CD23a and EBV LMP-1 promoters are also known to be responsive to EBNA-2 (15, 41, 42), and inspection of the 100- to 200-bp upstream regions identified as mediating EBNA-2 responsiveness revealed that a GTGGGAA motif occurs in each promoter (Fig. 1). We wished to determine if EBNA-2 mediates activation of responsive promoters through a common mechanism involving CBF1. We therefore examined whether CBF1 binds to the −85 to −277 region of the CD23a promoter that was previously identified as being required for EBNA-2 responsiveness. Figure 2 shows an EMSA in which the −85 to −277 DNA probe from the CD23p was incubated with sequential fractions eluted from a heparin-agarose column loaded with EBV-negative CA46 lymphoblastoid cell nuclear extract. The major binding activity occurs in the column fractions used in lanes 8 and 9. The elution profile of this binding activity is identical to that previously described for CBF1 (24).

In competition experiments, a 30-mer oligonucleotide representing the sequences from −359 to −388 of the Cp that contain the Cp CBF1 binding site was able to compete for both major complexes from the −89 to −275 CD23 probe DNA, while a mutant oligonucleotide that does not bind CBF1 (MUT 10; see Table 1 and Materials and Methods) was unable to compete for these complexes (Fig. 3). A 30-mer oligonucleotide (−158 to −187) from the CD23p that contains the matching core element was also able to compete for the complexes, thus localizing the binding site (data not shown). The reason for the appearance of two complexes in the EMSA

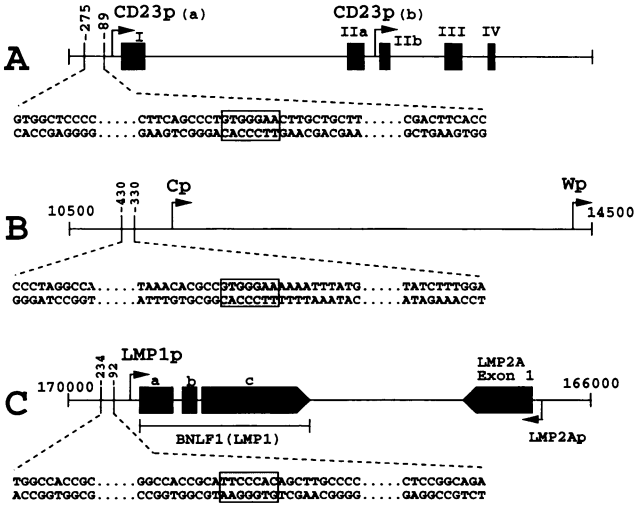

FIG. 1. CBF1 binding sites in EBNA-2-responsive promoters. Schematic representation of the cellular CD23 gene (A) and segments of the EBV genome containing the latency Cp (B) and the gene for LMP-1 (C). Promoters are indicated by bent arrows, and exons are represented by black rectangles. The locations of known EBNA-2-responsive regions are marked (numbering from the mRNA start site), and an expanded sequence showing the presence of CBF1 binding sites in each promoter (shaded boxes) is provided.

is not known. The faster-migrating band most likely represents a proteolytic degradation product, but a posttranslationally modified or alternatively spliced form of CBF1 may also be present in the extract.

To further establish that it was CBF1 binding to the CD23 EBNA-2-responsive region, we tested whether EBNA-2 could interact with this protein complex to form a supershifted complex in an EMSA. Figure 4 shows that bacterially expressed EBNA-2 [containing amino acids 252 to 425; hereafter referred to as EBNA (252-425)] interacts with the 192-bp CD23p-CBF1 complex to form a novel supershifted complex, while a mutant version of EBNA-2 (WW323SR) that is unable to interact with CBF1 is unable to supershift the complex. In another approach, UV cross-linking was used to identify the protein or proteins that bound to the Cp and CD23p sites. Bromo-rdUTP-containing probes representing the −359 to −388 Cp and −158 to −187 CD23p sequences were incubated

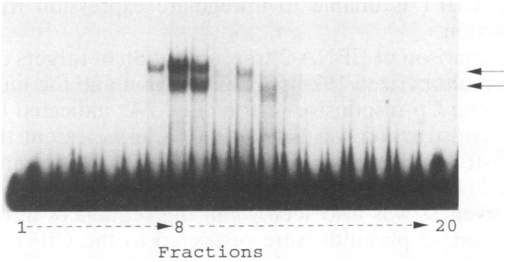

FIG. 2. Nuclear protein extracts containing CBF1 bind to sequences in the CD23p. Protein fractions eluted from a heparin-agarose column were bound to a 192-bp DNA fragment (−85 to −277) from the CD23p and analyzed by EMSA. The positions of the two major complexes detected are indicated by arrows. The fractions containing this activity are the same fractions previously shown to contain CBF1 (24).

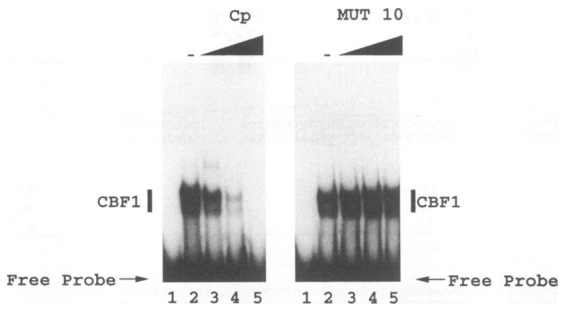

FIG. 3. An oligonucleotide representing the Cp CBF1 site competes for the bound complex from the 192-bp CD23 probe. A competition assay using the EBNA-2-responsive region (−85 to −277) from the CD23 promoter as a probe and the indicated 30-mer oligonucleotides as competitors was performed. In each panel, lane 1 contains probe alone, lane 2 contains added CBF1, and lanes 3 to 5 contain increasing amounts of unlabeled competitor (triangles) at 2.5-, 5.0-, 25-, and 125-fold excess. The position of the bound CBF1 complex is designated by a vertical bar.

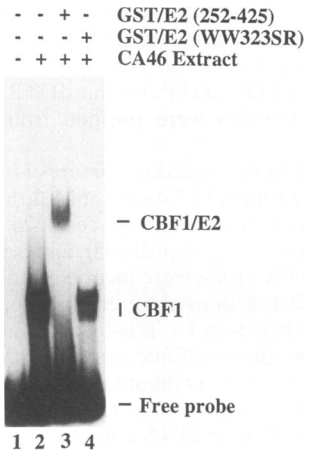

FIG. 4. EBNA-2 interacts with the CD23p-CBF1 complex. The fractions identified in Fig. 2 as containing CBF1 were bound to CD23p DNA alone (lane 2) or with wild-type EBNA-2 (252-425) (lane 3) or a mutant version (WW323SR) that is unable to interact with CBF1 (lane 4). Lane 1 contains the radiolabeled probe without added protein. GST, glutathione *S*-transferase.

with fractionated CA46 extract, and an EMSA was performed. After electrophoresis, the gel was exposed to a shortwave UV light source to cross-link the labeled DNA to the bound protein. The protein-DNA complexes were located by autoradiography and excised from the gel, and the proteins present in the complex were characterized by denaturing polyacrylamide gel electrophoresis. The −359 to −388 Cp probe and the −158 to −187 CD23p probe selectively interacted with proteins of the same electrophoretic mobility (Fig. 5A). Less CD23p probe than Cp probe was cross-linked in the bound complex, and it required a longer exposure to detect the protein in the CD23p complex. To more accurately determine the size of the bound protein (presumably CBF1), the experiment was repeated with the inclusion of a nuclease digestion step to remove flanking DNA. A single protein of approximately 65 kDa was observed cross-linked to the Cp probe (Fig. 5B).

Activation of the CD23p by EBNA-2 is mediated through CBF1. Having established that CBF1 binds to the EBNA-2-responsive region within the CD23p, we tested whether there was a correlation between the in vitro interaction of EBNA-2 and CBF1 and the ability of EBNA-2 to transactivate the CD23p in transient transfection assays. In a representative CAT assay (Fig. 6), wild-type EBNA-2 transactivates expression from a CAT reporter plasmid containing eight upstream copies of the 192-bp EBNA-2-responsive region up to 14-fold, while a mutant version of EBNA-2 (WW323SR) that is unable to bind CBF1 is unable to upregulate expression from this construction.

A comparison of EBNA-2 transactivation of targets containing the multimerized 192-bp CD23p region and the multimerized 100-bp Cp-responsive region (Fig. 7A) indicated that the CD23p construction was activated to a lesser extent than the Cp construction even at the highest levels of cotransfected EBNA-2 (15-fold versus >46-fold). This difference in EBNA-2 responsiveness was also seen when the sequences introduced into the target plasmids were restricted to the CBF1 binding sites from the Cp and the CD23p (Fig. 7B). In this case, the activation of luciferase expression from the plasmid containing the Cp CBF1 binding site was ninefold, compared with twofold for the equivalent plasmid carrying the CBF1 binding site from the CD23p.

The Cp, CD23p, and LMP-1p differ in binding affinity for CBF1. Both the CD23p and the Cp bind CBF1, and yet the extents of EBNA-2 responsiveness of targets containing the Cp and CD23p elements differed. To examine whether the transactivation effect was related to differences in CBF1 binding affinity, EMSAs were performed with 30-mer oligonucleotide probes containing the CBF1 core motif along with the natural flanking sequences from each promoter (Table 1). As shown in Fig. 8, incubation of the CD23p and LMP-1p probes with the CBF1 nuclear extract led to the formation of a shifted complex that migrated in the same position in the gel as the complex formed with the Cp probe. No complex was formed in the presence of a previously described mutant Cp probe (MUT Cp [MUT 10]) that contains four base substitutions. On addition of EBNA-2 (252-425), a supershifted complex (CBF1-E2) was detected with the Cp and CD23p and, on a longer exposure, with the LMP-1p (inset in Fig. 8). A noticeable feature of this assay was the apparent reduction in the amount of CBF1 complex formed with the CD23p and LMP-1p probes compared with the Cp probe. To examine this difference more quantitatively, we performed a competition experiment in which a radiolabeled Cp 30-mer probe was incubated with a

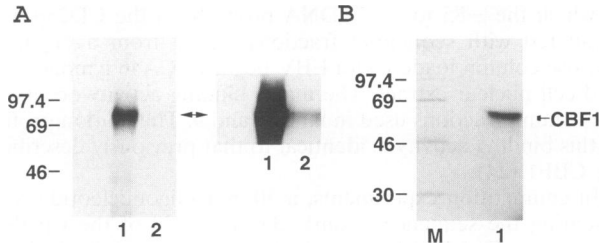

FIG. 5. The Cp and CD23p CBF1 binding sites interact with a 65-kDa protein. (A) UV cross-linking assays demonstrating that the Cp (lane 1) and the CD23p (lane 2) sites interact with a protein which has the same electrophoretic mobility in an SDS-polyacrylamide gel. A longer exposure was needed to visualize the protein bound to the CD23p probe (inset). (B) After nuclease digestion to remove non-cross-linked DNA, the bound protein (lane 1) migrated with an apparent molecular mass of 65 kDa relative to the size markers (M). Sizes are indicated in kilodaltons.

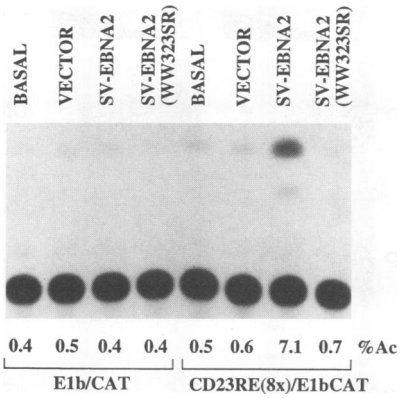

FIG. 6. CBF1 mediates EBNA-2 transactivation of the CD23p. The cotransfection assay in DG75 lymphoblastoid cells demonstrates that the EBNA-2 mutant WW323SR, which is unable to bind CBF1, is unable to transactivate a heterologous promoter containing eight copies of the 192-bp CD23-responsive region [CD23RE(8x)/E1bCAT]. This target is activated by wild-type EBNA-2. The effector DNA constructions used are shown above the lanes, and the corresponding target constructions are shown below. The percentage conversion of [^{14}C]chloramphenicol (%AC) is indicated.

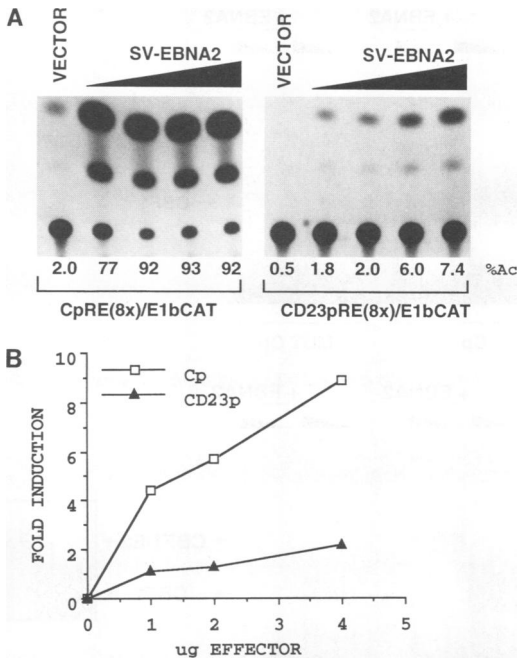

FIG. 7. EBNA-2 transactivates CD23p constructions less effectively than Cp constructions. (A) Dose response comparing EBNA-2 transactivation of reporter plasmids containing eight tandem copies of a Cp response region (−330 to −430) (25) or CD23p response region (see Fig. 6 and Materials and Methods) cloned upstream of the minimal promoter in an E1bCAT vector. DG75 cells were transfected with 2 μg of target DNA and increasing amounts (0.25, 0.5, 1.0, or 2.0 μg) of effector DNA expressing EBNA-2 (triangles). Controls were cotransfected with SG5 vector DNA. The effector DNA constructions used are shown above the lanes, and the corresponding target constructions are shown below. The percentage conversion of [^{14}C]chloramphenicol (%AC) is indicated. (B) Dose response comparing EBNA-2 transactivation of reporter plasmids containing eight tandem copies of the Cp or CD23p CBF1 binding site cloned upstream of the minimal promoter in the luciferase vector pGL2. DG75 cells were transfected with 2 μg of target DNA and 1, 2, or 4 μg of effector DNA expressing EBNA-2. Controls were cotransfected with SG5 vector DNA.

fixed amount of CBF1-containing extract and the bound CBF1 complex was then competed for with increasing amounts of unlabeled 30-mer Cp, CD23p, LMP-1p, or mutant Cp oligonucleotide. Both the CD23p and LMP-1p oligonucleotides competed for CBF1 binding less effectively than the Cp oligonucleotide, with the LMP-1p oligonucleotide being the least effective (Fig. 9). To obtain a 50% level of competition required a 12 nM concentration of the Cp oligonucleotide, an 86 nM concentration of the CD23p oligonucleotide, and a 214 nM concentration of LMP-1p oligonucleotide.

Mutagenesis of the CBF1 recognition element revealed an essential core motif and modifying flanking sequences. CBF1 bound with considerably different affinities to the 30-mer oligonucleotides containing the CBF1 recognition sequence from the Cp, CD23p, and LMP-1p. Each of these oligonucleotides contain a common core motif, GTGGGAA, but the sequences flanking this motif are not constant in the three promoters. To examine the requirements for CBF1 binding, we synthesized a series of 30-mer Cp oligonucleotides carrying mutations across the core and flanking sequences. Competition assays were performed with the same ^{32}P-labeled Cp probe that was used in the experiment shown in Fig. 9 and increasing amounts of the different unlabeled mutant oligonucleotides (Fig. 10; Table 1). Paired mutations across the core GGGAA sequence (Fig. 10A and D) eliminated CBF1 binding, and oligonucleotides carrying these mutations (MUT 5, 6, and 7) did not compete except at the highest competitor concentration, where there was a nonspecific competitor effect. Mutation of the first two bases of the core sequence from GT to TG reduced CBF1 binding to barely detectable levels, and in the competition assay, the oligonucleotide carrying this mutation (MUT 4) competed at only 3% of the wild-type Cp levels. Thus, the entire GTGGGAA core sequence is essential for effective CBF1 binding.

Each of the paired mutations introduced into the flanking sequences had some effect on the affinity of CBF1 (Fig. 10B and E). The most drastic effect seen in the first set of mutations was the conversion of the immediate flanking bases on the 5′ side of the core sequence from CC to GG (MUT 3). This change reduced the effectiveness of the competitor oligonucleotide to 20% of that of the wild type. Changes made on the four adjacent 5′ bases (MUT 1 and 2) and on the four 3′ flanking bases (MUT 8 and 9) reduced competitor efficiency to 63 to 79% of the wild-type levels.

The LMP-1p had the lowest affinity for CBF1. To determine if this reduced affinity could be directly correlated with the alterations in flanking sequence present in the LMP-1p, we tested four additional mutant oligonucleotides, each of which carried a substitution that occurred in LMP-1 (Fig. 10C and F). Again, the substitutions immediately adjacent to the core sequence had the greatest effect. The C-to-T change (MUT 12) 5′ of the core reduced the competitor effectiveness to 35% of that of the wild-type Cp, while the AA-to-TG change (MUT 13) on the 3′ flank resulted in a competitor that was 17% as effective as the wild type. Curiously, the G-to-C change (MUT 11) in the 5′ flanking sequence of the LMP-1p slightly increased binding efficiency. Overall, mutagenesis of the CBF1 response element provided evidence for the essential nature of the core motif, GTGGGAA, and indicated that the sequence immediately flanking this motif influences the affinity with which CBF1 binds.

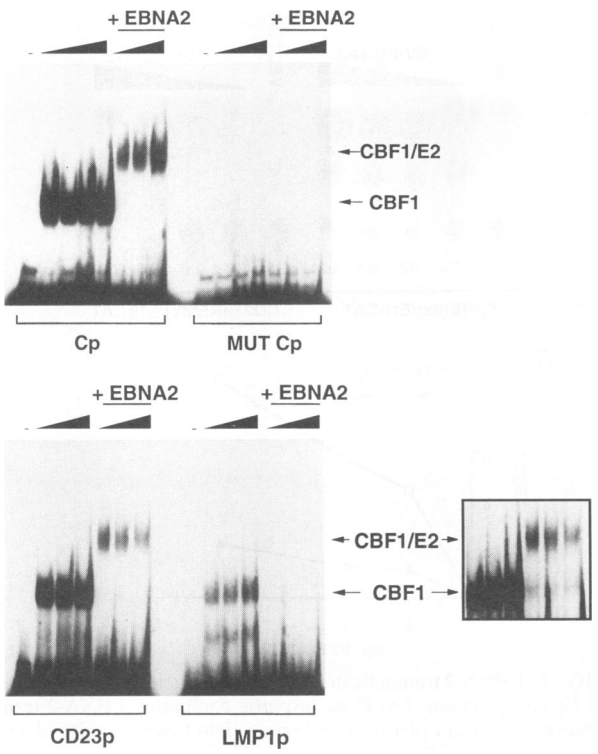

FIG. 8. CBF1 binds to a core motif found in EBNA-2-responsive regions of the CD23p and LMP-1p. The EMSA shows binding of increasing amounts (triangles) of nuclear extract containing CBF1 alone or in the presence of glutathione *S*-transferase–EBNA-2 (252 to 425) (+ EBNA-2) to 30-mer oligonucleotide probes containing CBF1 recognition elements from the EBV Cp and LMP-1p and the cellular CD23p. The MUT Cp oligonucleotide is MUT 10 in Table 1. The positions of the CBF1 and CBF1–EBNA-2 complexes are indicated. The inset is a longer exposure of the LMP-1 CBF1 binding probe showing that an EBNA-2–CBF1 complex is formed.

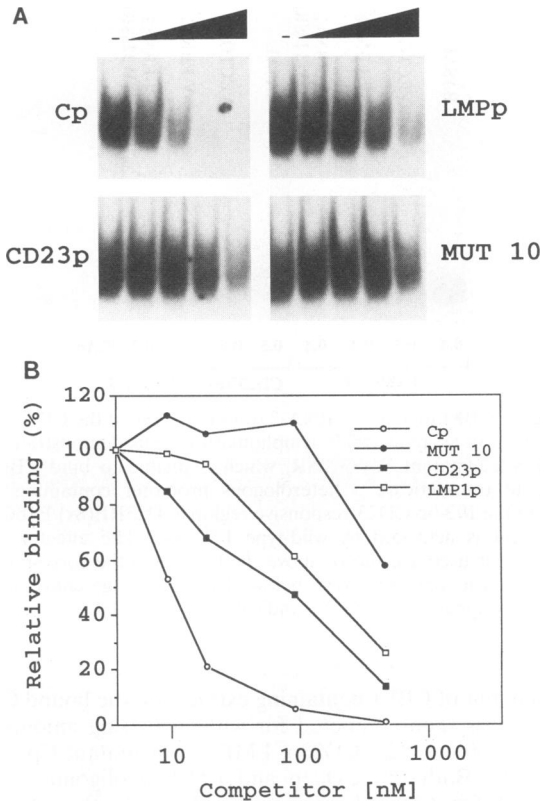

FIG. 9. Comparison of the relative binding affinity of CBF1 for the sites within the Cp, CD23p, and LMP-1p. (A) Competition assay using the Cp 30-mer oligonucleotide as a probe and the indicated unlabeled 30-mer oligonucleotides as competitors. Only the shifted CBF1 complex is shown. −, no competitor added; triangle, increasing amounts of unlabeled competitor added at 2.5-, 5.0-, 25-, and 125-fold excess. (B) Quantitative analysis of the competition assays shown in panel A.

DISCUSSION

The EBNA-2 protein is a transcriptional activator that regulates two aspects of the EBV infection process. On primary infection of B cells, there is a temporal order of viral gene expression which involves initial transcription of the EBNA-2 and EBNA-LP genes from the Wp followed by a switch to the Cp that is EBNA-2 mediated (20, 25, 30, 35, 47). Each of the EBNA latency genes is then expressed from the Cp. Since the latency membrane genes are also responsive to EBNA-2 (15, 41, 43, 52, 53), this places the entire pattern of latency gene expression under EBNA-2 regulation. Not only does EBNA-2 modulate viral latency gene expression, but it is also essential for EBV-driven B-cell immortalization (12, 13, 17). The contribution of EBNA-2 to this latter process is most likely through modification of cellular gene expression. One of the cellular genes known to be upregulated by EBNA-2 is CD23 (42).

We recently demonstrated that EBNA-2 transactivation of the EBV latency Cp is mediated via a cellular DNA-binding protein, CBF1, which targets EBNA-2 to the Cp (24). In this study, we examined whether the mechanism of EBNA-2 transactivation of the Cp was common to EBNA-2 regulation of the cellular CD23p. Using the same heparin-agarose-fractionated nuclear extract that had been previously used to identify CBF1 (24), we were able to demonstrate CBF1 interaction with a 192-bp EBNA-2-responsive region (44) from the CD23p. In EMSA, the shifted complex formed with the CD23p was competed for both by a 30-mer CBF1 binding site from the Cp and by a 30-mer oligonucleotide from the CD23p (−158 to −187) containing a matching core element. As had been previously demonstrated for CBF1 (25), the DNA-bound protein interacted with EBNA-2 (253-425) but not with the EBNA-2 WW323SR mutant. Further, UV cross-linking showed that the CBF1 binding site from the Cp and the 30-mer CD23p oligonucleotide each bound a protein with the same electrophoretic mobility in a denaturing polyacrylamide gel. This analysis additionally identifies CBF1 as a protein of approximately 65 kDa. Not only did CBF1 bind to the CD23 EBNA-2-responsive region, but CBF1 mediated EBNA-2 transactivation of this promoter. An EBNA-2 mutant (WW323 SR) that is unable to interact with CBF1 was unable to transactivate a CD23p-CAT construction. A related sequence located between −203 and −232 in the EBV LMP-1p was also shown to bind CBF1 (Fig. 8), thus additionally implicating CBF1 in the EBNA-2 responsiveness of this viral promoter.

A feature of the direct binding experiments was the apparent difference in affinity of CBF1 for the binding sites within the Cp, the LMP-1p, and the cellular CD23p, and this difference was confirmed and quantified in competition experiments. It is possible that the differences in affinity for CBF1 may allow a graded promoter response to EBNA-2 with

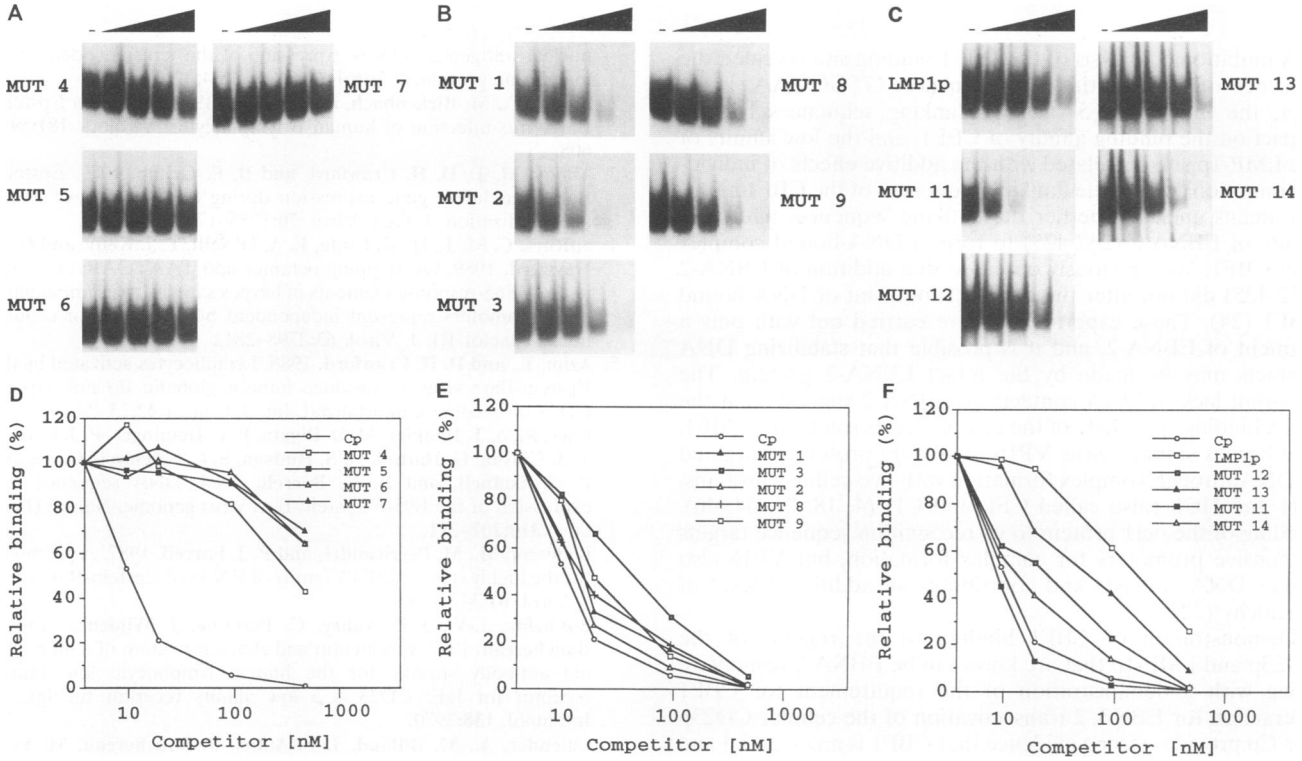

FIG. 10. Effects of mutations across the CBF1 binding site. (A to C) Competition assays showing only the shifted CBF1 complex. The Cp oligonucleotide probe and the competitor conditions were identical to those described for Fig. 9. The unlabeled competitors are identified alongside each panel, and their sequences are shown in Table 1. (D to F) Quantitative analysis of the results shown in panels A to C, respectively). For comparative purposes, the analysis is subdivided into core mutations (A and D), flanking mutations (B and E), and substitutions found in the LMP-1p (C and F).

TABLE 1. Summary of the mutagenesis analysis of the CBF1 binding site[a]

			Competitor[b] []	Relative[c] competition %
	LMP1p	CAAGCTGTGGGAATGCG	214	5.6
	CD23p	AGCCCTGTGGGAACTTG	86	14
	Cp	CACGCCGTGGGAAAAAA	12	100
MUT 1	Cp	TCCGCCGTGGGAAAAAA	15	79
MUT 2	Cp	CAGTCCGTGGGAAAAAA	15	79
MUT 3	Cp	CACGGGGTGGGAAAAAA	60	20
MUT 4	Cp	CACGCCTGGGGAAAAAA	412	3.0
MUT 5	Cp	CACGCCGTTTGAAAAAA	>470	<2.5
MUT 6	Cp	CACGCCGTGTTAAAAAA	>470	<2.5
MUT 7	Cp	CACGCCGTGGGCCAAAA	>470	<2.5
MUT 8	Cp	CACGCCGTGGGAAGCAA	15	79
MUT 9	Cp	CACGCCGTGGGAAAAGC	19	63
MUT 10	Cp	CACGAATTCGGAAAAAA	>470	<2.5
	LMP1p	CAAGCTGTGGGAATGCG	214	5.6
	Cp	CACGCCGTGGGAAAAAA	12	100
MUT 11	Cp	CACCCCGTGGGAAAAAA	9.4	128
MUT 12	Cp	CACGCTGTGGGAAAAAA	34	35
MUT 13	Cp	CACGCCGTGGGAATGAA	72	17
MUT 14	Cp	CACGCCGTGGGAAAACG	16	75
CONSENSUS SITE		-GTGGGAA-		

[a] The sequences shown represent the central 17 bp of the 30-mer oligonucleotide probes. Additional flanking sequences are identical to the Cp oligonucleotide probe described in Materials and Methods or, in the cases of CD23p and LMPp, to their natural flanking sequences.

[b] Concentration in nanomoles of unlabeled competitor oligonucleotide required for 50% competition of CBF1 binding to the wild-type Cp oligonucleotide probe.

[c] The ability of each oligonucleotide to compete for CBF1 relative to the Cp (set at 100%).

promoters such as the Cp and LMP-2Ap (52), which have high-affinity CBF1 sites, having the potential for a greater response than promoters such as the CD23p, with its intermediate-affinity CBF1 binding site. Our CD23p-CAT and Cp-CAT constructions are not completely comparable since they contain different amounts of natural flanking sequences (192 and 100 bp, respectively). However, CD23p-CAT gave a significantly weaker response to EBNA-2 transactivation than Cp-CAT (a 10-fold difference in the linear response range). This reduced response appeared to be related to the binding affinity of CBF1, since a cotransfected target plasmid carrying multiple copies of the lower-affinity CBF1 site from the CD23p was less responsive to EBNA-2 transactivation than an equivalent construction carrying the higher affinity CBF1 site from the Cp. Another aspect of the cotransfection data is the reduction in overall responsiveness to EBNA-2 that occurs when naturally occurring sequences from the Cp or CD23p are removed, leaving only the CBF1 binding sites. Thus, while CBF1 binding is absolutely required for EBNA-2 transactivation of both these promoters and is a factor in the relative levels of EBNA-2 response, the overall level of response is markedly influenced by the presence of other *cis*-acting elements. Whether this results from a synergistic interaction between other DNA-bound transcription factors and CBF1 or between these factors and the tethered EBNA-2 remains to be explored. The LMP-1p binding site has a very low affinity for CBF1, suggesting that the overall response of this promoter may be especially dependent on the presence of other transcriptional regulatory factors.

A mutational analysis of the CBF1 binding site revealed the presence of an essential core sequence, GTGGGAA. However, the immediate 5′ and 3′ flanking sequences had an impact on the binding affinity of CBF1, and the low affinity of the LMP-1p site correlated with the additive effects of individual mutations within the flanking sequences of the CBF1 motif. It remains unclear whether the flanking sequences affect the ability of EBNA-2 (252-425) to form a DNA-bound complex with CBF1. We previously observed that addition of EBNA-2 (252-425) did not alter the DNase I footprint of DNA-bound CBF1 (24). These experiments were carried out with only a segment of EBNA-2, and it is possible that stabilizing DNA contacts may be made by the intact EBNA-2 protein. The apparent lack of DNA contacts by EBNA-2 suggests that the DNA binding specificity of the complex rests solely with CBF1. The herpes simplex virus VP16 regulatory protein is targeted to DNA through complex formation with two cellular proteins, oct1 and HCF (also called CFF and C1) (4, 18, 23, 34, 46). Binding of the oct1 protein to its recognition sequence targets responsive promoters for complex formation, but VP16 also makes DNA contacts and contributes an additional level of specificity (22).

Demonstration of CBF1 binding to the regions of the CD23p and LMP-1p that are known to be EBNA-2 responsive along with a demonstration of the requirement for CBF1 interaction for EBNA-2 transactivation of the cellular CD23p and Cp provides strong evidence that CBF1 is an essential and ubiquitous targeting intermediate for EBNA-2. We defined a core sequence, GTGGGAA, that is important for CBF1 binding. Any seven-base sequence will have a random match every 16,384 bp, which precludes a useful database search using this sequence. However, we also found that the immediate flanking sequences had a significant effect on CBF1 binding and that an extended CBF1 binding site, CYGTGGGAA, would occur randomly at a reduced frequency of once per 131,072 bp. A search of the EBV genome (6) for matches to this sequence revealed that, in addition to the already mapped Cp, LMP-1p and TP1p (LMP-2Ap) sites, this sequence also occurs in the EBV genome at five other positions: upstream of three early promoters (−680 of BFRF3, −210 of BLLF2, and −360 of BBLF3), −80 of an undefined TATA box in *Bam*HI-G, and at position 3605, which does not appear to be promoter related. The biological relevance of these CBF1 binding sites is presently unknown.

A search of primate databases for the CYGTGGGAA sequence identified more than 100 matches. It thus seems likely that CBF1 may be involved in the regulation of expression of a relatively large number of cellular genes, including other B-cell activation antigens. By extension, the expression of these same genes would potentially be subject to modulation by EBNA-2, implying that EBNA-2 may have a much greater impact on B-cell gene expression than was appreciated from the two known examples of EBNA-2-responsive cellular genes, c-*fgr* and CD23.

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