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Epstein-Barr Virus Nuclear Protein 2 Transactivation of the Latent Membrane Protein 1 Promoter Is Mediated by Jκ and PU.1

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Expression of the Epstein-Barr virus (EBV) latent membrane protein 1 (LMP-1) oncogene is regulated by the EBV nuclear protein 2 (EBNA-2) transactivator. EBNA-2 is known to interact with the cellular DNA-binding protein Jκ and is recruited to promoters containing the GTGGGAA Jκ recognition sequence. The minimal EBNA-2-responsive LMP-1 promoter includes one Jκ-binding site, and we now show that mutation of that site, such that Jκ cannot bind, reduces EBNA-2 responsiveness by 60%. To identify other factors which interact with the LMP-1 EBNA-2 response element (E2RE), a −236/−145 minimal E2RE was used as a probe in an electrophoretic mobility shift assay. The previously characterized factors Jκ, PU.1, and AML1 bind to the LMP-1 E2RE, along with six other unidentified factors (LBF2 to LBF7). Binding sites were mapped for each factor. LBF4 is B- and T-cell specific and recognizes the PU.1 GGAA core sequence as shown by methylation interference. LBF4 has a molecular mass of 105 kDa and is probably unrelated to PU.1. LBF2 was found only in epithelial cell lines, whereas LBF3, LBF5, LBF6, and LBF7 were not cell type specific. Mutations of the AML1- or LBF4-binding sites had no effect on EBNA-2 transactivation, whereas mutation of the PU.1-binding site completely eliminated EBNA-2 responses. A gst–EBNA-2 fusion protein specifically depleted PU.1 from nuclear extracts and bound in vitro translated PU.1, providing biochemical evidence for a direct EBNA-2–PU.1 interaction. Thus, EBNA-2 transactivation of the LMP-1 promoter is dependent on interaction with at least two distinct sequence-specific DNA-binding proteins, Jκ and PU.1. LBF3, LBF5, LBF6, or LBF7 may also be involved, since their binding sites also contribute to EBNA-2 responsiveness.

Epstein-Barr virus (EBV) nuclear protein 2 (EBNA-2) regulates virus and cell gene transcription and is essential for primary B-lymphocyte growth transformation (8, 18). One critical EBNA-2 function is to regulate transcription of the EBV latent membrane protein 1 oncogene (LMP-1) (1, 44), whose expression is also essential for primary B-lymphocyte growth transformation (21). EBNA-2 response elements (E2REs) have been characterized upstream of the EBV LMP-1, LMP-2A, and Cp promoters (12, 20, 41, 51), as well as the CD23 promoter (45). Each E2RE includes MNYYGTGG GAA, which includes the cognate sequence for the cellular DNA-binding protein Jκ (16, 42). Jκ interacts with EBNA-2 in vitro (16, 19) and in vivo (48) and thus can recruit EBNA-2 to E2REs. While lacking direct sequence-specific DNA-binding activity, EBNA-2 has an acidic activator domain that can interact with TFIIB and TAF40 and increase transcription from heterologous promoters (7, 40).

However, E2REs are more complex than expected from a simple model whereby only Jκ cognate sequences are necessary to direct EBNA-2-responsive promoters. Jκ is ubiquitously expressed in cell lines and tissues (14, 16, 17, 38), but the LMP-1 E2RE is responsive in B cells but not T or epithelial cells (12). Furthermore, the LMP-1, LMP-2, Cp, and CD23 minimal E2REs all include one or two Jκ sites but cannot be limited to those sites and still maintain EBNA-2 responsiveness (12, 20, 41, 46, 51). Moreover, reporter constructs containing four tandem Jκ sites or two tandem Cp E2REs were not EBNA-2 responsive, although higher-order oligomers did exhibit E2RE activity (25).

The promoter-distal end of the LMP-1 E2RE has been mapped to either −234 bp (41) or −214 bp (12) relative to the mRNA cap site. The two likely LMP-1 E2RE Jκ-binding sites are at −298 to −290 or −223 to −213. One of these sites lies within a −234 E2RE, and neither is within a −214 E2RE. The promoter-proximal end of the LMP1 E2RE has been defined to be −145 (12) or −92 (41). The objectives of these experiments are to more precisely define the role of Jκ in the LMP-1 E2RE and to identify other cellular factors that may be involved.

MATERIALS AND METHODS

Cell culture. Cell lines were maintained in either RPMI 1640 (B- and T-cell lines) or Dulbecco's modified Eagle's medium DMEM (293 and HeLa cell lines) supplemented with 10% fetal calf serum.

Plasmids. Plasmids p−512/LMP, p−205/LMP, p−147/LMP, and p−55/LMP, which contain inserts beginning at the indicated upstream positions (relative to the mRNA cap site) of the B95-8 LMP-1 gene cloned into the *Bam*HI site of the promoterless reporter plasmid pCAT3M, have been described previously (24). p−236/LMP and p−215/LMP were constructed by PCR amplification of the LMP-1 upstream region with the 3′ (+39) LMP primer (CCGGATCCCAGG GCAGTGTGTCAGG) and either the −236 (GCGGATCCTTGGCCACCGCA TTCCCA) or −215 (CCGGATCCGCTTGCCCCCCGGGGACC) 5′ primer. Plasmid p−236ΔJκ contained a −236/LMP insert mutated at nucleotides −219 to −217 (CAC→AGA) cloned into the *Bam*HI site in pCAT3M. The 5′-mutated primer (−236ΔJκ) used in the PCR was GCGGATCCTTGGCCACCGCATTC-C**AGA**AGCTTGCCCC. Plasmid p−236ΔLBF1 was constructed in a similar fashion with a CAC→ACA mutation at positions −230 to −228, using the 5′ −236ΔLBF1 primer, GCGGATCCTTGGC**ACA**CGCATTCCCA. Plasmid p−236 ΔPU.1 was constructed by using overlapping primers (GTAGAAAGGG**TCC**G TAGAAAGCG and CGCTTTCTAC**GGA**CCCTTTCTAC) to generate overlapping products mutated at nucleotides −166 to −164 (TTC→GGA), which were then extended with the −236 and LMP primers and cloned into the *Bam*HI site of pCAT3M. Plasmid p−215ΔPU.1 was constructed in a similar manner to

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p−236ΔPU.1, except that the −215 primer was used as the 5′ primer in the second PCR step.

Plasmids pTK−236/−145, pTK−215/−145, pTK−236/−196, pTK−215/−174, and pTK−187/−145 contain inserts from the indicated upstream positions of the B95-8 LMP-1 gene cloned as PCR products or synthetic oligonucleotides into the *Bam*HI site of pBLCAT2, which contains a minimal thymidine kinase (TK) promoter upstream of the chloramphenicol acetyltransferase (CAT) gene (27). The 5′ primers used in these PCRs are described above (primers −236 and −215); the 3′ −145 primer was GCGGATCCTCTAGAATGTAAGCGTAGAAG GGG. Plasmids pTK−236ΔJκ, pTK−236ΔLBF1, pTK−236ΔPU.1, and pTK−215 ΔPU.1 contain the above-described mutations within the −236/−145 insert that was PCR amplified with the above-described mutant 5′ primers and cloned into the *Bam*HI site of pBLCAT2. The 3′ primer for these products was the −145 primer except for pTK−236ΔPU.1 and pTK−215ΔPU.1, for which it was GCGGATCC TCTAGAATGTAAGCGTAGAAAGGG**TCC**GTAGAAAGCG. Plasmid pTK−236ΔLBF4 contains a heptanucleotide mutation (GCTTTCT→TAGG GAG) at positions −175 to −169 within the −236 to −145 insert and was PCR amplified with the −236 and the LBF4Δ (GCGGATCCATGTAAGCGTAG AAAGGGGAAGT**CT**CCC**TA**GTGTGTTTGT) primers before being cloned into the *Bam*HI site of pBLCAT2. All LMP-1 promoter constructs were verified by dideoxynucleotide sequencing.

Nuclear extracts and EMSA. Nuclear extracts were prepared by a modified Dignam method as previously described (48). Probes were prepared from annealed synthetic oligonucleotides or from the 92-bp *Bam*HI inserts in pTK−236 or pTK−236ΔPU.1. Plasmids were digested with *Bam*HI, and GATC overhangs were filled in with Klenow by using a [^{32}P]GTP. For electrophoretic mobility shift assays (EMSAs) 5 μg of nuclear extract was incubated for 5 min at room temperature in reaction buffer [10 mM *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid (HEPES; pH 7.9), 5% glycerol, 0.5 mM EDTA, 1 mM dithiothreitol, 125 mM phenylmethylsulfonyl fluoride, 100 μg of bovine serum albumin per ml, 40 μg of poly(dI-dC) per ml, 50 mM KCl] with or without a 100-fold molar excess of specific competitor oligonucleotide. Probe was then added to the reaction mixture (1 to 2 ng or 5×10^4 to 10×10^4 cpm per reaction), which was incubated for 15 min at room temperature. For supershift assays, antibody was preincubated with nuclear extract in reaction buffer on ice for 10 min. Samples were electrophoresed on a 5% polyacrylamide gel in 0.5× Tris-borate-EDTA (TBE), after which the gel was dried and exposed to film. PU.1 and AML1 antibodies were described previously (30, 35).

Protein purification. Louckes or IB4 nuclear extracts from 18 liters of cells were diluted with 4 volumes of 20 mM morpholineethanesulfonic acid (MES; pH 6.2)–2 mM dithiothreitol–1 mM EDTA–10% glycerol–0.1 mM phenylmethylsulfonyl fluoride–10 μg of aprotinin per ml, loaded onto an S-Sepharose column, and step eluted with 100 to 1,000 mM NaCl. For LBF4 purification, peak fractions for LBF4 EMSA activity after S-Sepharose were pooled and fractionated on DNA cellulose; then peak DNA cellulose fractions (0.3 M NaCl) were subjected to sequence-specific DNA affinity chromatography with multimerized −176 to −151 oligonucleotide as described previously (16).

CAT assays. BJAB cells (10^7) were electroporated (Bio-Rad Gene Pulser) with 5 μg of pUC-β-galactosidase, 5 to 15 μg of pSG5 or pSG5-EBNA-2, and 5 μg of CAT reporter plasmid. Cells were incubated in RPMI 1640 with 10% fetal calf serum for 48 h (LMP promoter assays) or 65 h (TK promoter assays). Cell extracts were assayed for CAT and β-galactosidase activity (41), as well as for EBNA-2, by Western immunoblot with pE2 antibody (49).

Methylation interference assay. The *Eco*RI-*Sal*I fragment from plasmid pTK−236/−145 was singly end labeled with [^{32}P]CTP, and used for methylation interference assays of PU.1 and LBF4 complexes as described previously (5).

Western blotting and elution from SDS-PAGE gels. PU.1 (35), ets (Santa Cruz Biochemicals), and NFAT-p (29) antibodies used in Western blot experiments have been described previously. LBF4 was eluted and renatured from sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gel slices as described previously (4).

RESULTS

The minimal LMP-1 E2RE requires the cooperation of at least two discrete elements. The relative contribution of Jκ to the EBNA-2 responsiveness of the LMP-1 promoter was investigated with CAT reporter constructs (Fig. 1A) which contained no Jκ site (−215/LMP), the −223 to −213 site (−236/LMP), or both the −223 to −213 and −298 to −290 sites (−512/LMP). In B lymphocytes, p−236/LMP, and p−512/LMP were equally EBNA-2 responsive (10- versus 8.5-fold [Fig. 1A]). p−215/LMP, which contains no Jκ sites, was significantly less responsive (4.5-fold), and p−205/LMP, p−147/LMP, and p−55/LMP showed no responsiveness above that of a promoterless vector control (Fig. 1A). The activity of the −215/LMP construct was consistently higher than that of the vector control but significantly lower than that of the

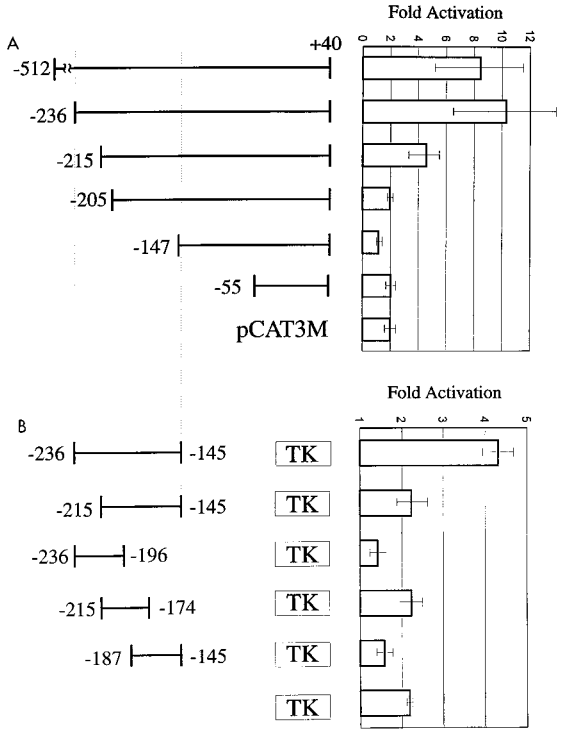

FIG. 1. EBNA-2 responsiveness in the LMP-1 upstream region. EBNA-2 induced increases in CAT activity in BJAB Burkitt's lymphoma cells transfected with an LMP1 promoter-CAT reporter plasmid (A) or with an LMP1 regulatory fragment upstream of an HSV TK promoter-CAT reporter plasmid (B). CAT activity with a cotransfected EBNA-2 expression vector is compared with CAT activity with an expression vector without EBNA-2. CAT activity was normalized for transfection efficiency by using a cotransfected simian virus 40 early promoter-driven β-galactosidase expression plasmid. Error bars indicate the standard deviation of values in independent assays.

−236/LMP construct. These data therefore show that one Jκ site is necessary for full EBNA-2 effect and confirm the previous finding that a −214/+40 LMP promoter without either Jκ site is still EBNA-2 responsive (12). Furthermore, the data suggest that a factor interacting with sequence between −215 and −205 is essential for the residual activity of the −215/LMP construct.

To confirm the 3′ boundary of the LMP-1 E2RE, a −236 to −145 LMP-1 upstream sequence was cloned 5′ to the herpes simplex virus TK minimal promoter in a CAT reporter construct. EBNA-2 induced a 4.5-fold activation of this construct (Fig. 1B), which was similar to the 4-fold induction previously demonstrated for the −234 to −92 element (41). Also consistent with previous results (41), a sequence containing just the Jκ-binding site (−236 to −196) was not sufficient to mediate EBNA-2 responsiveness (Fig. 1B). The −215 to −174, −187 to −145, and −215 to −145 constructs were similarly unresponsive (Fig. 1B). Thus, EBNA-2 transactivation of the LMP1 E2RE depends on cooperation of the Jκ site (−223 to −213) with an element(s) partially or fully within the −215 to −145 sequence. In contrast to previous experiments in which sevenfold activation was seen (12), the −215 to −145 construct did not show a significant EBNA-2 response. This may be explained by the lower sensitivity of the TK reporter system used in these experiments in which the larger −236 to −145 construct gave only 4.5-fold activation, as opposed to the previously reported 20-fold activation for this sequence (12). Nevertheless, our results with the −215/LMP native promoter

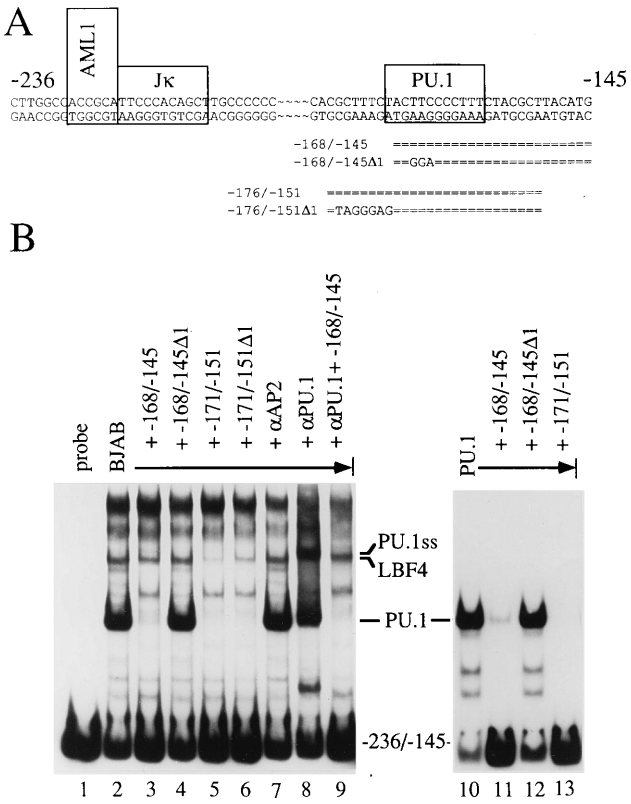

FIG. 2. PU.1 binds to the minimal LMP-1 E2RE. (A) Nucleotide sequence of the upstream region of the EBV LMP-1 gene. Numbering is relative to the mRNA cap site (B95-8 virus [3, 13]). Binding sites conforming to PU.1, AML1, and Jκ consensus sequences are boxed. Native and mutated competitor oligonucleotides used in the EMSA experiments in panel B are shown underneath the native sequence. =, native nucleotide. Mutated nucleotides are displayed. (B) EMSA with nuclear extracts from the EBV-negative BJAB cells (left) or with in vitro translated PU.1 (right). Lanes: 1 and 2, −236 to −145 LMP-1 probe without or with nuclear extract, respectively; 3 through 6, probe with nuclear extract and a 100-fold molar excess of the indicated oligonucleotides; 7 through 9, probe with nuclear extract plus an irrelevant antibody (AP2), nuclear extract plus PU.1 antibody, and nuclear extract plus PU.1 antibody plus a PU.1 competitor oligonucleotide, respectively; 10 through 13, probe incubated with a reticulocyte lysate programmed with a T3 PU.1 expression vector (22). Lanes 11–13 are with a 100-fold molar excess of the competitor oligonucleotide listed above each lane. The positions of the PU.1, LBF4, and PU.1 supershifted (PU.1ss) complexes are noted.

construct do confirm that LMP-1 promoter constructs without the Jκ site retain residual EBNA-2 responsiveness.

The minimal LMP-1 E2RE binds multiple factors including the ets protein PU.1. The DNA sequence of the −236 to −145 minimal E2RE element was examined for binding sites of known transcription factors. A perfect 6- of 6-bp match for the AML1 protein-binding site (TGYGGT [30]) was found between −229 and −224, and an 11- of 12-bp match to a PU.1-binding site (AAAGGGGAAGTa [22]) was found at −169 to −158 (Fig. 2A). The putative AML1 site is immediately 5′ to the consensus Jκ site agctGTGGGAA (16) at −223 to −213 (Fig. 2A). AML1 is the human homolog of the mouse PEA2 factor, which is important for the activity of the polyomavirus enhancer and is also the target of translocations in a number of human leukemias (2, 11, 31, 34). PU.1 is an ets family protein important for the regulation of macrophage- and B-cell-specific promoters (22).

To confirm activity for the hypothetical AML1- and PU.1-binding sites and to identify other factors that bind to the −236 to −145 E2RE sequence, EMSAs were performed with B-cell extracts and a −236/−145 probe (Fig. 2B). A predominant complex and a second, less abundant complex were noted (Fig. 2B, lane 2). Overlapping oligonucleotide competitors (Fig. 2A) were used to identify the binding sites of these factors. A −168 to −145 oligonucleotide (Fig. 2A) that contains the PU.1 site inhibited the most abundant complex (Fig. 2B, lane 3), and a −168 to −145 oligonucleotide with a mutation in the PU.1 consensus site (Fig. 2A, −168/−145Δ1) no longer inhibited it (Fig. 2B, lane 4). When antibody to PU.1 (35) was added to a mixture of nuclear extract and probe, the putative PU.1 complex disappeared and a larger, "supershifted" complex appeared, whereas control antibody had no effect (Fig. 2B, lanes 7 and 8). Addition of competitor −168 to −145 oligonucleotide specifically inhibited formation of the supershifted complex (lane 9). Moreover, in vitro translated murine PU.1 (which is only 2 amino acids longer than human PU.1) formed a complex with the −236/−145 probe which had the same mobility as the putative PU.1 complex (Fig. 2B, compare lane 2 with lane 10). The recombinant PU.1 EMSA complex was inhibited by the −168 to −145 and −176 to −151 oligonucleotides but not by the −168 to −145Δ1 oligonucleotide, which is mutated within the PU.1 site (lanes 11 to 13). These data indicate that the predominant EMSA complex observed with the −236/−145 LMP-1 probe and B-lymphoma cell extracts is due to PU.1.

The other major −236 to −145 EMSA complex, LBF4 (LMP-1 binding factor 4), was inhibited by a −176 to −151 oligonucleotide (Fig. 2A and 2B, lane 5), which also contains the PU.1 site. However, neither the smaller −168 to −145 PU.1 consensus site oligonucleotide nor a partially overlapping −180 to −158 oligonucleotide inhibited the LBF4 complex (Fig. 2B, lane 3, and data not shown). Mutation of nucleotides −175 to −169 in the −176 to −151 oligonucleotide (Fig. 2A, −176/−151Δ1) partially impaired competition for LBF4 binding, but PU.1 was still inhibited effectively (Fig. 2B, lane 6). All other mutations within this region showed similar effects on both factors (summarized in Fig. 3A). Neither PU.1 nor LBF4 bound to −236/−145 probe mutated at the PU.1 site (see Fig. 5A, lane 2). Mutation of nucleotides −166 to −164 or −160 to −158 in the −236 to −145 fragment abolished both PU.1 and LBF4 competitor activity (Fig. 3A). Mutations at either −162 to −161 or −157 to −151 within the −176 to −151 oligonucleotide had no effect on PU.1 or LBF4 binding (Fig. 3A). On the basis of these data with wild-type or mutated oligonucleotide competitors, LBF4 must bind to a sequence which includes the PU.1 consensus site and additional 5′ sequence that is not essential for PU.1 binding (Fig. 3A).

To further differentiate PU.1- and LBF4-binding sequences, guanosine contacts were evaluated by methylation interference. As shown in Fig. 3B, methylation of guanosines at positions −163 and −164 strongly interfered with the binding of both PU.1 and LBF4, while methylation of guanosine at position −162 interfered less completely with the binding of both. These interference patterns center on a GGAA core (−159 to −162) which is characteristic of ets family DNA-binding sites, and the putative PU.1 methylation intereference pattern is similar to that reported previously (22).

To determine if PU.1 is a subunit of LBF4 (as is seen with the PU.1–NF-EM5 complex [36]), LBF4 was purified from IB4 cell nuclear extracts by sequential cation exchange, DNA-cellulose, and sequence-specific DNA affinity chromatography, using EMSA to monitor LBF4 activity. LBF4 eluted from S-Sepharose in 500 mM NaCl, whereas PU.1 eluted in 900 mM NaCl (results not shown). The S-Sepharose fraction was chromatographed on a nonspecific DNA-cellulose matrix, and the

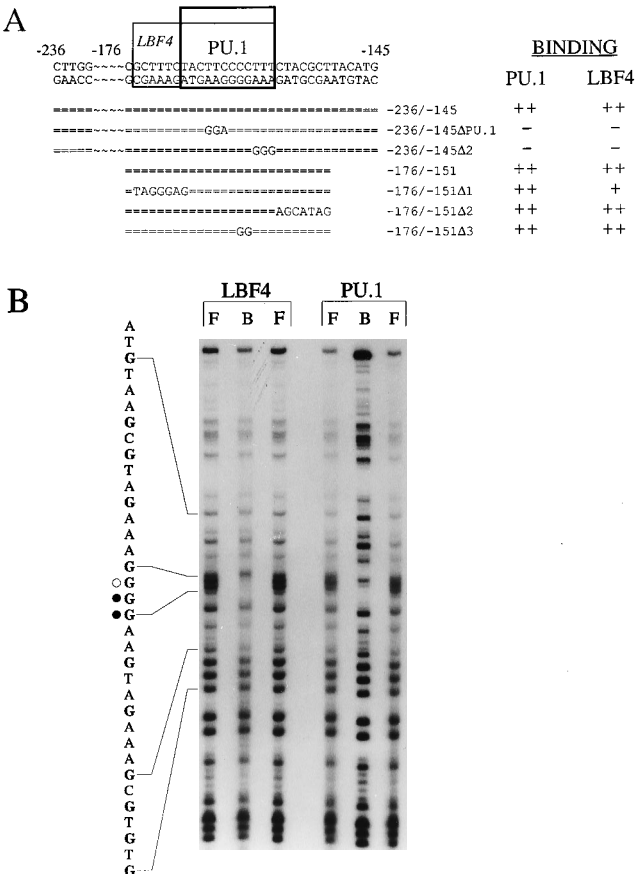

FIG. 3. LBF4 and PU.1 bind and protect the same core nucleotides. (A) EMSA competitors used to investigate PU.1- and LBF4-binding sites are shown beneath the corresponding native LMP1 −236 to −145 sequence. The PU.1- and LBF4-binding sites are boxed. For competitor oligonucleotides, native nucleotides are denoted by = and mutated oligonucleotides are indicated. The ability of each competitor to block PU.1 or LBF4 shifts is graded as noncompetition (−), weak competition (+), or competition equal to unlabeled probe (++). (B) Methylation interference with a −236 to −145 LMP-1 E2RE probe and LBF4 or PU.1 S-Sepharose column fractions of a BJAB nuclear extract. End-labeled DNA was methylated and incubated with partially purified (S-Sepharose) LBF4 or PU.1. The PU.1 and LBF4 EMSA complexes and corresponding free probe bands were transferred to DEAE paper and eluted. The DNA was cleaved with piperidine and electrophoresed in a denaturing polyacrylamide gel. F and B indicate free or bound LBF4 or PU.1 EMSA complexes, respectively. Open and solid circles indicate weak and strong interference with LBF4 and PU.1 binding, respectively. Corresponding sequence is displayed at the left, with the 5′ end at the top.

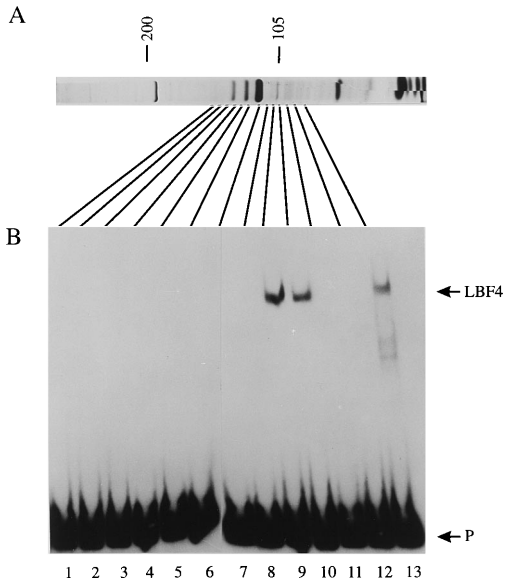

FIG. 4. Determination of the size of LBF4. The LBF4 peak activity fraction from a sequence-specific DNA affinity column containing a multimerzied −176 to −151 oligonucleotide was fractionated on an SDS–6% polyacrylamide gel. (A) A silver-stained lane of the gel. (B) A nonstained lane was cut into slices corresponding to different protein sizes, and eluted proteins from each slice were tested for EMSA activity with a labeled −176/−151 probe. A sample containing the peak activity of LBF4 from an S-Sepharose column is shown in lane 13. The positions of LBF4 and free probe (P) are indicated.

0.3 M NaCl peak LBF4 fraction was further fractionated on a sequence-specific DNA affinity column containing multimerized −176 to −151 oligonucleotide (16, 43). To identify the size of LBF4, polypeptides in the peak LBF4 fraction from the sequence-specific DNA affinity column were separated by SDS-PAGE. The gel was sliced into small pieces, each containing proteins of a defined size. The proteins were eluted from each slice, renatured, and analyzed by EMSA (4). LBF4 activity was detected in the gel slice harboring a 105-kDa protein, was not detected in slices from above, and was detected at a lower concentration in slices from below (Fig. 4). Western blotting of the purified protein with antibodies directed against the ets domain (Santa Cruz Biotechnology) or against the NF-ATp protein (29) (which is similar in size and recognizes a similar DNA sequence) showed no reactivity with p105/LBF4 (results not shown). The NF-ATp and ets antibodies did, however, react with the expected proteins in cell extracts and in other column fractions (results not shown). These data indicate that PU.1 and LBF4 are distinct polypeptides that interact with the same core sequence, although the LBF4-binding site is larger and includes sequence 5′ to the PU.1 site.

A probe mutated for PU.1 and LBF4 binding allows resolution of additional LMP-1 E2RE EMSA complexes and confirmation that AML1 is LBF1. EMSA with B-cell nuclear extract with a −236/−145 probe mutated at the PU.1- and LBF4-binding site (−236/−145ΔPU.1) allowed resolution of complexes obscured by the presence of the abundant PU.1 and LBF4 complexes. Five novel complexes were identified with this probe and termed LBF1, LBF3, LBF5, LBF6, and LBF7 (Fig. 5A, lane 2). A sixth complex was identical in size to the expected Jκ complex (lane 2). Overlapping competitor oligonucleotides spanning the probe sequence were then used to map the approximate binding site for each factor (Fig. 5D). The binding sites were further defined by mutations within competitor oligonucleotides (summarized in Fig. 5D).

The Jκ and LBF1 complexes were both inhibited by the −236 to −215 oligonucleotide (Fig. 5B, lane 3), which contains the consensus Jκ- and AML1-binding sites. Two separate trinucleotide mutations within this oligonucleotide at the Jκ site (Fig. 5D, −236/−215Δ3 and −236/−215Δ4) disrupted competition of the putative Jκ complex (Fig. 5B, lane 5, and data not shown). The putative Jκ complex was also inhibited by a Jκ-binding site derived from the EBV LMP2A E2RE (16) (data not shown). Moreover, preincubation of extract with a glutathione *S*-transferase (GST)–EBNA-2(310–376) fusion protein resin (48) efficiently depleted the putative Jκ-binding activity from nuclear extracts (data not shown), confirming that this complex is due to Jκ. Mutation of the −236 to −215 oligonucleotide within the consensus AML1 site (Fig. 5D, −236/−215Δ1 and −236/−215Δ2) disrupted competition for

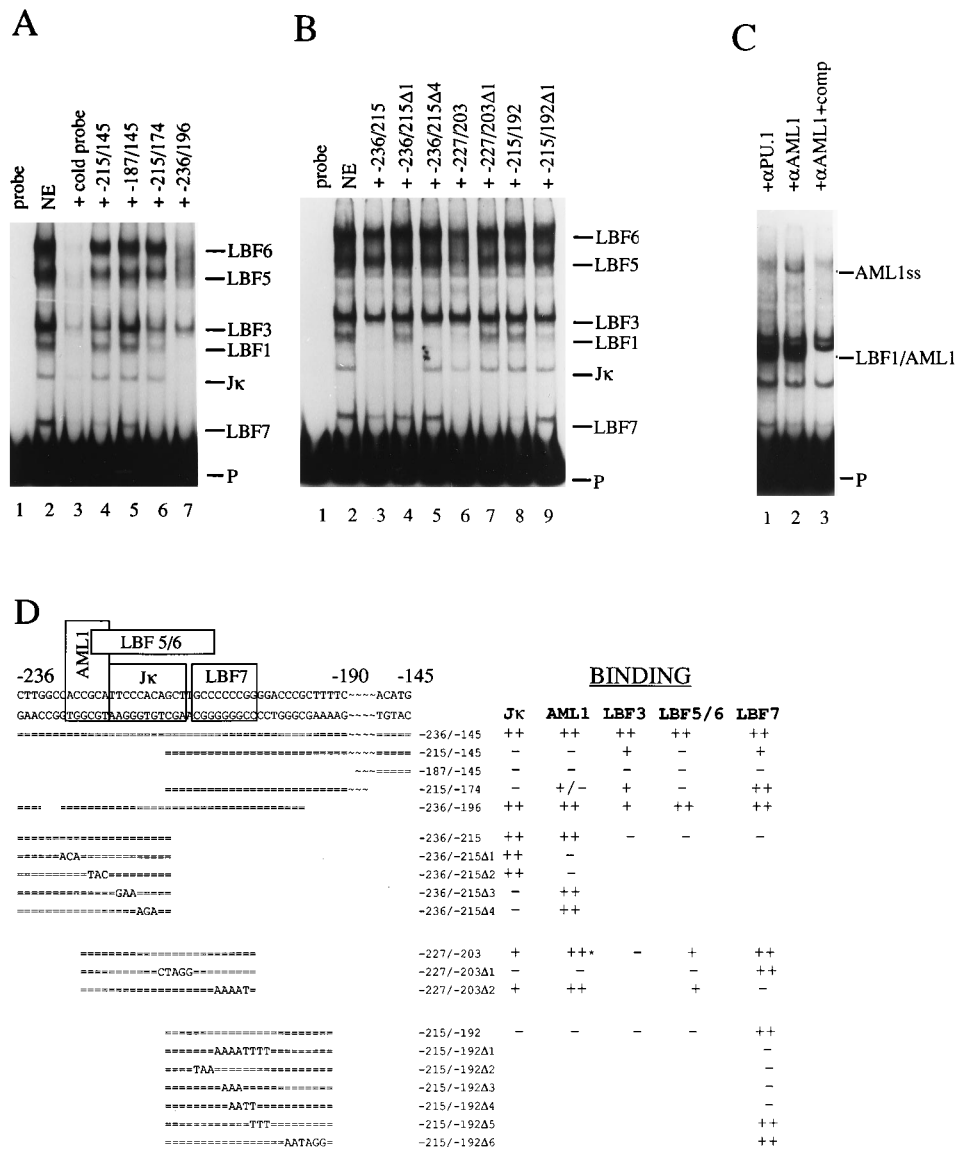

FIG. 5. Mutation of the PU.1 site facilitates detection of other factors that bind to a −236/−145 LMP-1 E2RE, including Jκ and AML1. EMSA analysis of B-cell extract with a −236/−145 LMP-1 probe mutated at the PU.1 site (same mutation as in Fig. 2A, −168/−145Δ1). (A) Six EMSA complexes are evident and are labeled LBF1, LBF3, LBF5, LBF6, LBF7, and Jκ. Overlapping competitor oligonucleotides (described below in panel D) were used to localize the binding site for each factor. (B) Binding sites for each factor were further defined by mutated competitor oligonucleotides. (C) The LBF1 complex is supershifted by AML1 antibody (lane 2) and not by control antibody to PU.1 (lane 1). The supershifted complex (ss) was inhibited by a specific AML1-binding-site oligonucleotide (lane 3). The AML1 oligonucleotide sequence was GGATATT**TGCGGT**TAGCA (47). (D) Summary of wild-type and mutated oligonucleotide competitors used to localize LBF1/AML1-, LBF3-, LBF5-, LBF6-, LBF7-, and Jκ-binding sites. Oligonucleotides are described at left. =, native sequence. Mutated bases are listed. +/−, barely detectable competition. LBF5 and LBF6 are grouped together because the results were identical for each competitor sequence. ∗, the −227 to −203 oligonucleotide inhibited the AML1 complex because of a 5- of 6-bp match to the consensus AML1 site (30) in this oligonucleotide.

the LBF1 complex but not the Jκ complex (Fig. 5B, lane 4, and data not shown).

The identity of LBF1 as AML1 was confirmed by incubation of nuclear extract with anti-AML1 antibody prior to EMSA. Anti-AML1 but not control antibody (Fig. 5C, lanes 2 and 1, respectively) resulted in an additional supershifted complex and partial depletion of the LBF1 complex, confirming that LBF1 is AML1. The supershifted AML1 complex was inhibited by an oligonucleotide containing a CBFα (murine AML1)-binding site (47) that is also recognized by human AML1 (30).

The AML1, LBF5, and LBF6 complexes were all partially inhibited by a −227 to −203 oligonucleotide (Fig. 5B, lane 6). Competition for the AML1 complex was unexpected with −227 to −203, since it does not include the entire AML1 consensus. Inspection of the −227 to −203 sequence, however, revealed a degenerate (5- of 6-bp match) AML1 site, TGT GGG, at −220 to −216 (within the Jκ consensus [Fig. 5D]). Mutation within this site (Fig. 5D, −227/−203Δ1) completely disrupted AML1 inhibition by this oligonucleotide. In the context of the native LMP-1 promoter sequence, this degenerate AML1 site within the Jκ site does not have significant binding activity, since the −236 to −215 oligonucleotide which includes both the consensus AML1 site at −224 to −229 and the degenerate site at −220 to −216 does not bind any AML1

when the consensus site is mutated (Fig. 5D). Mutation of the −227 to −203 oligonucleotide at −211 to −215 (Fig. 5D, −227/−203Δ1) also disrupted the partial inhibition of LBF5 and LBF6 (Fig. 5B, lane 7), whereas an adjacent pentanucleotide mutation at −208 to −204 (Fig. 5D, −227/−203Δ2) had no effect on competitor activity (results not shown). Since the mutation of nucleotides −208 to −204 did not ablate competition by the −227/−203Δ2 oligonucleotide, the LBF5- and LBF6-binding site(s) must lie between −227 and −209 (Fig. 5D).

The LBF7 complex was inhibited by both the −227 to −203 and −215 to −192 overlapping oligonucleotides (Fig. 5B, lanes 6 and 8), thus narrowing its binding site to within 13 bp (−215 to −203). An array of mutations placed within both of these oligonucleotides (Fig. 5D) allowed further localization of LBF7. Five separate mutations that fell within the −211 to −203 sequence GCCCCCCGG (Fig. 5D, −215/−192Δ1, −215/−192Δ2, −215/−192Δ3, −215/−192Δ4, and −227/−203Δ2) disrupted LBF7 competition (Fig. 5B, lane 9, and data not shown), whereas three mutations that bracket this sequence on either end (Fig. 5D, −227/−203Δ1, −215/−192Δ5, and −215/−192Δ6) had no effect on LBF7 competition (Fig. 5B, lane 7, and not shown). These data thus map the LBF7 recognition site to the sequence GCCCCCCGG.

The LBF3-binding site was less precisely mapped. LBF3 was inhibited by unlabeled −236/−145ΔPU.1 probe (Fig. 5A, lane 3) and partially inhibited by the −215/−145, −215/−174, and −236/−196 oligonucleotides (Fig. 5A, lanes 4, 6, and 7, respectively). The smaller overlapping 22-bp oligonucleotides derived from this region (Fig. 5D, −236/−215, −227/−203, and −215/−192) failed to compete for LBF3 binding (Fig. 5B, lanes 3, 6, and 8). This indicates that the core of the LBF3-binding site is likely to be within the sequence from −215 to −196 and that additional 5′ and 3′ sequences are required for optimal binding. Mutation of residues −208 to −201 in the context of the full-length −236 to −145 E2RE oligonucleotide (see Fig. 5D, −215/−192Δ1, for mutation), however, did not eliminate its ability to compete for LBF3. Thus, the core of the LBF3-binding site is between −215 and −196, and nucleotides −208 through −201 are not critical for sequence-specific binding.

Lymphocytes and epithelial cells express different nuclear factors that recognize the LMP-1 E2RE. Previous studies have demonstrated that EBNA-2 responsiveness of the LMP-1 E2RE is readily demonstrable in B lymphocytes but not in T lymphocytes or epithelial cells (12, 46). To determine which factors might be responsible for LMP-1 E2RE cell type specificity, EMSA was performed with nuclear extracts from various epithelial and B-lymphocyte cell lines, using the −236/−145 and −236/−145ΔPU.1 LMP-1 probes. EBV-positive cell lines were also tested to ascertain whether any LMP-1 E2RE-binding factors are modulated by EBV latent-gene expression. Figure 6A demonstrates that Jκ was present in B, T, and epithelial cells while PU.1 expression was restricted to B cells, in agreement with the previously reported distribution of these factors (17, 22). LBF3, LBF5, and LBF7 were also expressed in all cell lines tested (Fig. 6B). LBF4 was present in B and T lymphocytes but absent from epithelial cells (Fig. 6A). LBF1/AML1 was restricted to B cells and was upregulated in EBV-positive B-cell lines, except in the BL41 cell line infected with the defective P3HR1 virus (Fig. 6B). P3HR1 virus expresses only EBNA-1, EBNA-3A, EBNA-3B, and EBNA-3C in latently infected cells. A novel complex, LBF2, was noted only in epithelial cells (Fig. 6) and was localized by competition experiments to the LBF7-binding region between −215 and −192 (data not shown).

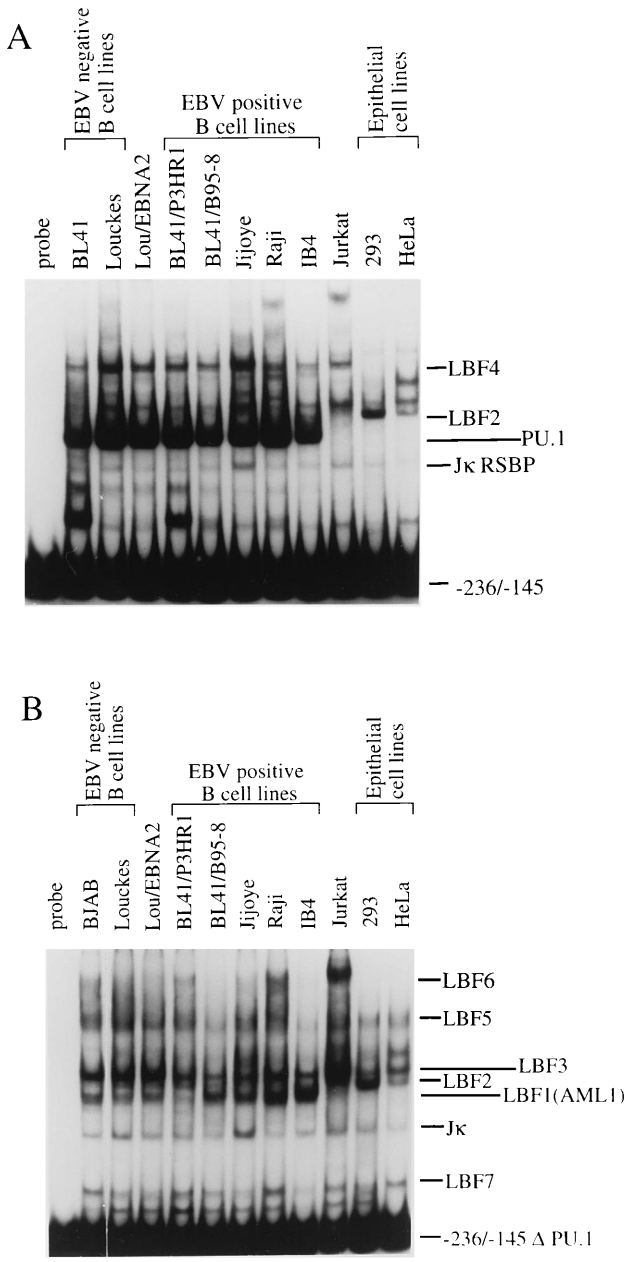

FIG. 6. B, T, and epithelial cells have distinct factors that recognize the minimal E2RE. EMSAs with −236/−145 probe (A) or −236/−145ΔPU.1 probe (B) were performed with nuclear extracts from various cell lines. BL41 and Louckes are EBV-negative Burkitt's lymphoma cell lines. Lou/EBNA2 is a Louckes cell line stably transfected with an EBNA-2 expression vector (45). BL41/P3HR1 and BL41/B95-8 are EBV-infected BL41 cell lines (6). Jijoye and Raji are EBV-infected Burkitt's lymphoma cell lines, and IB4 is an EBV-transformed cell line. Jurkat is a T-cell line. 293 and HeLa are kidney and cervical epithelial-cell lines, respectively. Complexes identified by competition experiments are listed to the right. The AML1 complex is upregulated in the EBV-positive cell lines except in the BL41 cell line infected with the defective P3HR1 virus that expresses only the EBNA-1, EBNA-3A, EBNA-3B, and EBNA-3C EBV gene products.

PU.1 is critical for EBNA-2 transactivation of the LMP-1 upstream region. To assess the contribution of LMP-1 E2RE-binding factors to EBNA-2 transactivation, trinucleotide mutations that disrupted the binding of LBF1/AML1, Jκ, PU.1+LBF4, or LBF4 alone were introduced into LMP-1

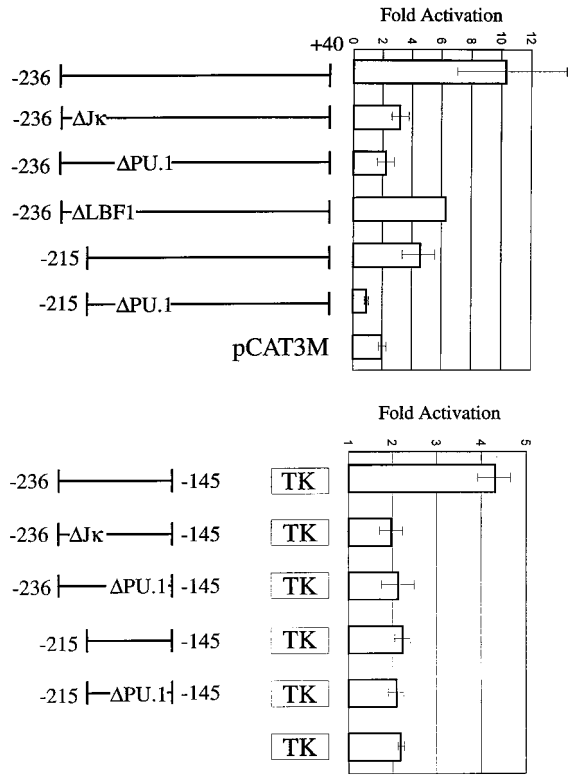

FIG. 7. Mutations in the AML1-, Jκ-, and PU.1-binding sites indicate the relative importance of these factors in EBNA-2 responsiveness. EBNA-2-induced increases in CAT activity from LMP1 promoter CAT reporter plasmids or from LMP1 promoter upstream elements positioned upstream of the HSV TK promoter and CAT reporter are indicated. Error bars indicate the standard deviation of values in independent assays.

promoter-CAT constructs. Mutation of nucleotides −211 to −213 disrupted the binding of Jκ and reduced the EBNA-2 responsiveness of the p−236/LMP (Fig. 7A) or pTK−236/−145 (Fig. 7B) constructs from 10- to 3.2-fold or from 4.7- to 2.0-fold, respectively. Mutation of nucleotides −230 to −228 disrupted LBF1/AML1 binding and had a small effect on the EBNA-2 responsiveness of p−236/LMP in the one experiment shown for this construct (Fig. 7A). In other experiments, the AML1-binding-site mutant had slightly greater EBNA-2 responsiveness than did the wild type (data not shown). This indicates that AML1 binding is not required for EBNA-2 responsiveness. The LBF4 mutation (Fig. 2A, −176/−151Δ1) also had little effect on the activity of −236/LMP (results not shown). However, this does not rule out the participation of LBF4 in E2RE activity, since this mutation only partially disrupts binding activity (Fig. 2B). Disruption of both PU.1 and LBF4 binding with the −166 to −164 mutation surprisingly abolished EBNA-2 responsiveness of the LMP-1 E2RE in the context of the p−236/LMP, p−215/LMP, and pTK−236/−145 constructs (Fig. 7). Thus, PU.1 (or, less probably, LBF4) is an essential mediator of LMP-1 EBNA-2 transactivation, while Jκ is critical for full EBNA-2 responsiveness.

GST–EBNA-2 fusion protein interacts directly with PU.1. The dramatic effect of the PU.1 mutation on EBNA-2 responsiveness could be due to a direct interaction between EBNA-2 and PU.1. To test this hypothesis, a GST–EBNA-2 fusion protein containing EBNA-2 residues 310 to 376 [GST–EBNA-2(310–376) (48)] bound to glutathione-Sepharose was incubated with either B-cell nuclear extract or ^{35}S-labeled in vitro translated PU.1 (Fig. 8). As shown in lanes 2 and 3, GST–EBNA-2(310–376) (48) adsorbed about 30% of ^{35}S-labeled recombinant PU.1 protein whereas GST absorbed little or no PU.1. The GST–EBNA-2(310–376) fusion protein also extensively depleted PU.1 EMSA activity from B-cell nuclear extracts, whereas a smaller protein, GST–EBNA-2(310–337), had little effect (lanes 4 to 6). Thus, the EBNA-2 domain from amino acids 310 to 376 can interact with recombinant PU.1 and can specifically deplete native PU.1 from nuclear extracts. As a further internal control, LBF4, which also binds to the −236 to −145 probe, was not depleted by incubation with GST–EBNA-2(310–376) protein (lane 6). An attempt to immunoprecipitate in vitro translated PU.1 with in vitro translated full-length EBNA-2 and an EBNA-2 monoclonal antibody (PE2 [49]) was unsuccessful (results not shown). This may be due to the much smaller amount of EBNA-2 used in this experiment (at least 100-fold less than in the GST–EBNA-2 experiment).

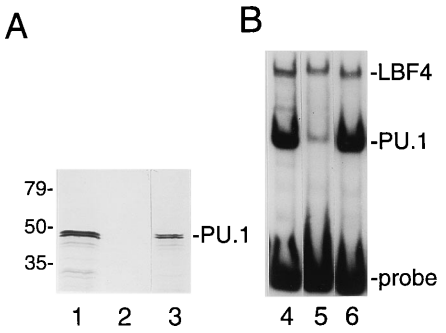

FIG. 8. EBNA-2 interacts directly with PU.1. Purified GST–EBNA-2(310–376) protein can bind in vitro translated PU.1 (A) or can deplete PU.1 from crude nuclear extract (B). ^{35}S-labeled in vitro translated PU.1 (lane 1) was incubated with glutathione Sepharose-bound GST (lane 2) or GST–EBNA-2(310–376) (lane 3) for 1 h. After being washed, the beads were eluted in sample buffer and subjected to SDS-PAGE. The amount of reticulocyte lysate in lane 1 is one-third of that used in the experiments for lanes 2 and 3. The position of the 45-kDa PU.1 polypeptide is indicated. In panel B, B-cell nuclear extract (lane 4) was incubated twice for 30 min each with GST–EBNA-2(310–376) (lane 5) or with GST–EBNA-2(310–337) (lane 6). The extracts were then analyzed by EMSA with a −236/−145 probe. The positions of PU.1 and LBF4 EMSA complexes are indicated.

DISCUSSION

These and previous data demonstrate that the EBV LMP-1 promoter is dependent for its activation on the interaction of EBNA-2 with both ubiquitous and tissue-specific host cell factors (Fig. 9). Binding-site mutational analysis showed that EBNA-2 activation of the LMP-1 promoter in B cells is partially dependent on interaction with the ubiquitous Jκ protein and is completely dependent on interaction with the B-lymphocyte-, macrophage-, and erythropoietic-cell-specific PU.1 protein. At least six other B-cell proteins bind to a −236 to −145 LMP-1 minimal E2RE and may also be involved in EBNA-2 responsiveness (Fig. 9). Of these, the AML1- and LBF4-binding sites have been specifically mutated, and these binding sites appear not to be critical for EBNA-2 responsiveness. Since a −215 LMP-1 promoter construct is partially EBNA-2 responsive, whereas a −205 construct is not (Fig. 1A) (41), a factor which interacts with −215 to −205 is likely to be important for EBNA-2 response. LBF3, LBF5, LBF6, or LBF7 map within or near this critical −215 to −205 site and may therefore mediate this effect. LBF3 and LBF7 bind to a −215/−145 probe, while LBF5 and LBF6 require additional upstream sequence, which could be sequence nonspecific and

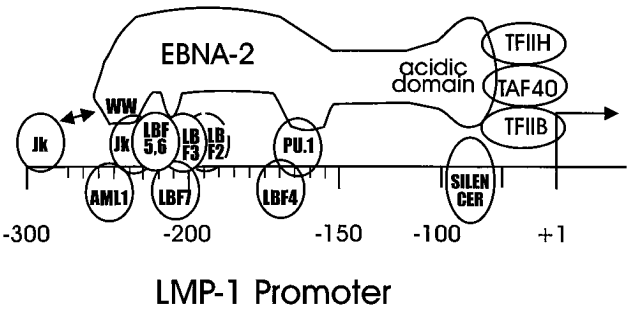

FIG. 9. Model of EBNA-2 interactions with E2RE-binding factors and basal transcription factors in B lymphocytes. EBNA-2 is an acidic transcriptional transactivator which requires sequence-specific DNA-binding proteins to direct it to response elements. The EBNA-2 acidic domain can interact with TFIIB, TAF40, and TFIIH in stimulating transcription. Whether it does so sequentially or as a complex of more than one EBNA-2 molecule interacting simultaneously with more than one factor remains to be determined. Jκ is a sequence-specific DNA-binding protein which recognizes a GTGGGAA sequence present in all E2REs and interacts with a 27-amino-acid domain of EBNA-2 that includes the sequence GPPWWPP. The LMP1 promoter has Jκ-binding sites at −298 to −290 and −223 to −213. Truncation of the −298 to −290 Jκ site has surprisingly little effect on EBNA-2 responsiveness in these and previous experiments. Truncation to −214 removes the more-proximal Jκ site as well as the AML1 site and encroaches on the LBF5 and LBF6 sites. However, the effect on EBNA-2 responsiveness is similar to that of a specific mutation in the Jκ-binding site (i.e., a 50% reduction in EBNA-2 responsiveness). EBNA-2 can also interact with PU.1, and this interaction is likely to account for the B-lymphocyte specificity of the EBNA-2 responsiveness of the LMP1 promoter. Mutation of the PU.1-binding site has a profound effect on EBNA-2 responsiveness. However, the PU.1 site is active only in the context of upstream sequence to −215, which includes the LBF3- and LBF7- and at least part of the LBF5- and LBF6-binding sites. Which of these factors acts in conjunction with PU.1 to convey EBNA-2 responsiveness is not known. An epithelial-cell-specific factor, LBF2, which recognizes a −215 to −192 site is also identified in this study. LBF2 could account for the importance of the −214 to −144 LMP-1 promoter sequence in the high basal activity of the promoter in epithelial cells (12). A previously described silencer is also shown (12).

may be provided by the −215 LMP-1 promoter CAT construct. Interaction of one of these factors with EBNA-2 may account for the activity of the −215 LMP-1 promoter construct, or a less direct interaction could be responsible. Experiments to detect a direct interaction of LBF3 or LBF7 with EBNA-2 have so far been unsuccessful (unpublished results).

Although EBNA-2 has a high specific affinity for Jκ and all EBNA-2-responsive promoters have one or more Jκ-binding sites (16, 19), the variable importance of this interaction to the regulation of different E2REs is surprising. Cp E2RE activity is absolutely dependent on Jκ, since an EBNA-2 WW to SS or SR mutation at positions 319 and 320, which disrupts Jκ interaction, or a mutation in the Jκ DNA-binding site independently abolish Cp EBNA-2 responsiveness (16, 19, 20, 25, 48). In contrast, the same EBNA-2 mutation or inactivating or deletional mutations of the LMP-1 −298 to −290 and −223 to −213 Jκ-binding sites result in only a twofold decrease in LMP-1 transactivation (Fig. 1) (12, 48). Furthermore, a −335 to −214 LMP-1 promoter DNA fragment which has both Jκ sites conveys only low-level activity to a basal TK promoter in the presence of EBNA-2, while a −214 to −144 fragment which lacks both sites showed sevenfold activation in the presence of EBNA-2 (12). Even the Cp and LMP-2 E2REs cannot be limited to a Jκ site(s) and bind other, as yet uncharacterized, factors (20, 25, 51). Thus, there is abundant evidence for cooperative functional interactions between EBNA-2 and sequence-specific DNA-binding proteins besides Jκ. Computer searches of each E2RE for common transcription factor-binding sites has, however, turned up only the Jκ-binding sequence (unpublished observations).

The central importance of the PU.1 site in LMP-1 EBNA-2 responsiveness appears to explain the restriction of EBNA-2 transactivation of the LMP1 promoter to B lymphocytes as opposed to T lymphocytes or epithelial cells (12, 46). PU.1 plays a key role in the expression of a number of lineage-specific genes in macrophages, B cells, and erythroid cells. These include the κ and λ immunoglobulin light chain, macrophage colony-stimulating factor receptor, and β-globin genes (10, 15, 35, 50). The mechanism of PU.1 activation of these genes is unknown, although, as is seen with the LMP-1 E2RE, their activity is absolutely dependent on PU.1 binding. PU.1 has only a weak activation domain in a proline-, glutamate-, serine-, and threonine-rich (PEST) region (35). In the κ and λ light-chain enhancers, PU.1 activity is dependent on an associated factor, NF-EM5, which binds to both PU.1 and an adjacent DNA sequence, even though NF-EM5 cannot itself bind DNA (10, 35). NF-EM5 interacts with PU.1 through the PEST domain (35), and its binding is dependent on phosphorylation of specific PU.1 residues (36).

Like Jκ, PU.1 alone could not convey EBNA-2 responsiveness to the LMP1 promoter. Although the PU.1 site is at −169 to −158, a −205 to +40 LMP1 promoter was not EBNA-2 responsive. Addition of sequences to −215 rendered the fragment significantly EBNA-2 responsive, indicating a requirement for LBF3, LBF5, LBF6, or LBF7 for PU.1 responsiveness. In fact, a −214 to −144 fragment is sufficient to convey EBNA-2 responsiveness to a basal TK promoter under sensitive assay conditions (12).

We have demonstrated direct high level interaction between a GST–EBNA-2 fusion protein and PU.1. The EBNA-2 domain responsible for this interaction (amino acids 310 to 376) can also interact with Jκ (48). The minimal domain for Jκ interaction consists of residues 310 to 337 (48), and that domain is not sufficient for PU.1 interaction, suggesting that sequence between amino acids 337 and 376 is important for PU.1 interaction. The physiologic importance of the in vitro PU.1–EBNA-2 interaction is indicated by the absolute dependence of the LMP-1 E2RE on the PU.1 site. Site-specific mutational analyses of the PU.1–EBNA-2 310 to 376 interactive domain and correlations of PU.1 association in vitro and in vivo with EBNA-2 activity in LMP-1 transactivation will provide further important tests of the current working model of direct EBNA-2–PU.1 interaction.

From a more formalistic perspective, PU.1 potentiation of EBNA-2 effects could be due to cooperation with other EBNA-2-binding proteins such as Jκ in assembly of an EBNA-2 complex at the LMP-1 promoter, to an effect on DNA binding so that EBNA-2 molecules are more favorably positioned relative to the promoter (as has been suggested for high-mobility group domain proteins in the human beta interferon promoter [9]), to enhanced recruitment of specific basal factors or activators, or to effects on local chromatin or nucleosome architecture. Interaction with and reversal of the negative effect of a silencer which maps between −112 and −55 is also a possibility (12). Genetic and biochemical data indicate that transcriptional transactivation by EBNA-2 is ultimately mediated by the interaction of the acidic domain with TFIIB, TAF40, and TFIIH (40). Multiple EBNA-2 molecules may be required at a promoter site for TFIIB, TAF40, and TFIIH interactions.

EMSAs of the LMP-1 promoter revealed that in addition to PU.1, a distinct but much less abundant factor, LBF4, binds to an overlapping recognition site and is found in B- or T-cell nuclear extracts. Methylation interference demonstrated that LBF4 recognized the same core guanosines as PU.1, yet it is unlikely to be an ets family protein, since it is larger than any

mammalian ets protein (105 kDa [28]) and does not react with an antibody directed at the conserved ets domain. The recently cloned NF-AT proteins NF-ATc and NF-ATp bind to a similar purine-rich sequence and have apparent molecular masses of 105 to 120 kDa, respectively (29, 33). Although LBF4 did not react with antibody directed to rat NF-ATp, LBF4 could be another NF-AT family member, such as NF-ATc (33). NF-AT is characteristically activated by B- or T-cell activation and inhibited by cyclosporin A. The effect of B- or T-cell activation and cyclosporin A on LBF4 would be of interest. Although LMP-1 promoter constructs with an LBF4-binding-site mutation were still EBNA-2 responsive, LBF4 could be important in regulating LMP1 promoter activity in latently infected cells that are stimulated by antigen or other extracellular ligands.

AML1 also binds to the LMP-1 E2RE but has little effect on EBNA-2 responsiveness in B-lymphoma cells. The AML1 gene was identified in leukemic chromosomal translocations that fuse the N-terminal portion, including its DNA-binding domain, with various other cellular genes (11, 31, 32, 34). Human AML1 may have a C-terminal activation domain exon that would be homologous to the C-terminal activation domain of the murine AML1, PEA2 (2). This exon would be removed in the translocated AML1 gene. Interestingly, EBV-transformed B cells seem to have a greater abundance of AML1 EMSA activity, raising the possibility that AML1 has a role in the establishment or maintenance of the EBV growth-transformed phenotype.

The only other cell-type-specific LMP1 E2RE-binding protein identified in this study was LBF2, which recognizes a site in the −215 to −192 sequence. The factor appears to be epithelial-cell-line specific. LBF2 may mediate the EBNA-2 independent constitutive activity of the LMP-1 promoter in epithelial cells, which has been shown to be dependent on sequence between −214 and −144 (12).

The finding that EBNA-2 transactivation is dependent on multiple cellular factors and that it may physically interact with at least two separate factors on the same promoter suggests a complex mechanism of action, as seen with other viral transactivators such as VP16 and E1A. VP16 interacts not only with Oct-1 but also with the cellular protein complex host cell factor to activate herpes simplex virus early-gene promoters (23, 26, 37, 39). E1A interacts with seemingly disparate cellular transcription factors (26), which probably explains its greater promiscuity among target promoters and host cells. Although the recent identification of the E2RE targeting factor Jκ is a major breakthrough (16, 19), the further identification of a role for PU.1 indicates that EBNA-2 transactivation involves more than simple promoter targeting by Jκ. Delineation of the full array of factor interactions essential for LMP1 expression in EBV-infected cells is of substantial importance in understanding the biology of EBV latent infection. Since LMP-1 is critical in the pathogenesis of EBV-induced cell transformations (21), factors essential for LMP-1 expression are potential targets for anti-LMP-1 pharmaceuticals.

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