Reciprocal Regulation of the Epstein-Barr Virus *Bam*HI-F Promoter by EBNA-1 and an E2F Transcription Factor

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The Epstein-Barr virus *Bam*HI-F promoter (Fp) is one of three used to transcribe the EBNA latency proteins, in particular, EBNA-1, the only viral gene product needed for episomal replication. Fp is distinguished by possession of the only EBNA-1 binding sites (the Q locus) in the Epstein-Barr virus genome outside *oriP*. Activity of Fp is negatively autoregulated by interaction of EBNA-1 at two sites in the Q locus, which is situated downstream of the RNA start site. We demonstrate in transient assays that this EBNA-1-mediated repression of Fp can be overcome by an E2F transcription factor which interacts with the DNA at a site centered between the two EBNA-1 binding sites within the Q locus. An E2F-1 fusion protein protects the sequence 5′-GGATGGCGGGTAATA-3′ from DNase I digestion, and a DNA probe containing this sequence binds an E2F-specific protein complex from cell extracts, although this region is only loosely homologous with known consensus binding sites for E2F transcription factors. In mobility shift assays, E2F can displace the binding of EBNA-1 from the Q locus but not from *oriP*, where the E2F binding site is not present. E2F also activates expression of Fp in epithelial cells. These findings identify a potentially new binding site for members of the E2F family of transcription factors and suggest that such a factor is important for expression of EBNA-1 in lymphoid and epithelial cells by displacing EBNA-1 from the Q locus. In addition, the possibility that Fp activity is under cell cycle control is raised. Since the supply of functional E2F varies during the cell cycle and since in these assays overexpression of E2F can overcome repression of Fp by EBNA-1, control of transcription of EBNA-1 mRNA by cell cycle regulatory factors may help to bring about ordered replication of episomes.

Epstein-Barr virus (EBV) is associated with the development of malignancies (lymphomas and carcinomas of the nasopharynx) in both of the cell types it infects (10, 45, 59). In each of these tumors, the viral genome exists as a circular episome, characteristic of EBV latency (42). The key gene required for initiation and maintenance of latent infection encodes Epstein-Barr nuclear antigen 1 (EBNA-1). By binding to specific repeated DNA sequence motifs at the origin of plasmid replication (*oriP*) (38), EBNA-1 activates both episomal replication and the *oriP* transcriptional enhancer (46, 47, 60, 65) and thus acts as a positive regulatory protein.

In type 1 Burkitt's lymphoma cells, as well as in nasopharyngeal carcinoma, the EBNA-1 transcript arises from Fp, a promoter in the *Bam*HI-F fragment of the EBV genome (31, 49, 50, 52, 58). The major RNA start site for Fp lies 200 bp upstream of two imperfect yet functional binding sites for the EBNA-1 protein (the Q locus), the only other location for EBNA-1 binding sites in the EBV genome (1, 29, 46). Expression of Fp is negatively autoregulated by EBNA-1 acting through the Q locus (51, 62). This EBNA-1-mediated repression can be transferred to a heterologous promoter and only requires one EBNA-1 binding site (62). The strong autorepression of Fp by EBNA-1 raises the question of how Fp can be activated in EBV-infected cells, all of which express EBNA-1.

In this paper, we show that while Fp is repressed by EBNA-1, it can also be activated by an E2F-like transcription factor which binds to DNA within the Q locus and that the activity of the promoter is reciprocally regulated by these cellular and viral proteins.

MATERIALS AND METHODS

Cell lines. Raji (44) is a latently infected EBV-positive Burkitt's lymphoma B-cell line, and Jurkat is an EBV-negative human T-cell line. SVK is a human keratinocyte cell line (35). All cells were maintained at 37°C in a 5% CO_2 environment. Lymphoid cells were grown in RPMI medium supplemented with 10% fetal calf serum.

Recombinant phagemid constructs. A series of phagemid constructs, pF1, pF2, pF3, pF4, pF2x, pF12, pF13d, and pF13, containing various amounts of sequence flanking the Fp RNA start site, were generated by PCR amplification of genomic DNA from the B95-8 cell line, using primers specific for the Fp region. 5′ and 3′ primers contained *Hin*dIII and *Xba*I adapters, respectively. The resulting PCR products were then inserted 5′ of the chloramphenicol acetyltransferase (CAT) gene in the phagemid vector pBSCAT (16) (Fig. 1A). The sequences included in constructs pF1, pF2, pF3, pF4, and pF2x, relative to the RNA start site (50, 52), are shown in Fig. 1A, and those for constructs pF12, pF13d, and pF13 are shown in Fig. 2.

Construct pQEco was generated by two-step PCR of a pF13 DNA template. Degenerate oligonucleotide primers were used to mutate 3 bp within the region of the Q locus protected from DNase I digestion by the glutathione *S*-transferase (GST)–RBP-3 fusion protein. The footprinted sequence was changed

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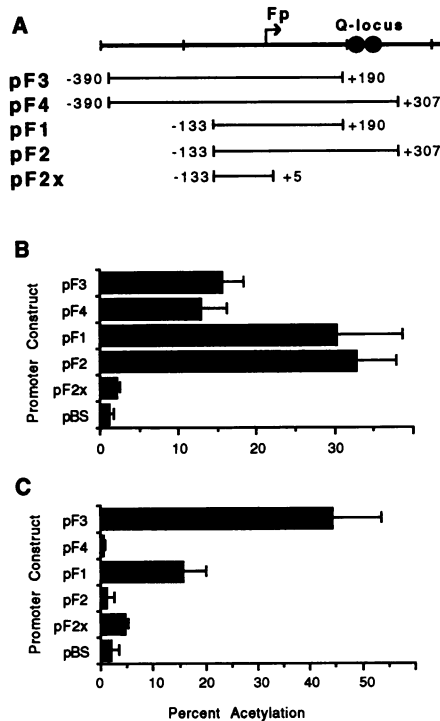
FIG. 1. Schematic representation of F promoter constructs used in transient-transfection assays. (A) RNA start site for Fp, as determined by Sample et al. (50) and Schaefer et al. (52), represented as a bent arrow. Q-locus (filled circles), the two low-affinity binding sites for the EBNA-1 protein. Promoter constructs are shown below, with coordinates relative to the RNA start site. Each was linked to the CAT gene in phagemid vector pBSCAT (16). (B) Constitutive activity of Fp constructs in EBV-negative Jurkat cells. Promoter activity is shown as average percent acetylation. (C) Constitutive activity of Fp constructs in EBV-positive Raji cells.

FIG. 2. E2F-1 activates Fp in transient promoter assays. Five micrograms of each promoter construct was cotransfected with 5 μg of either CMV–RBP-3 or pHD101-3, a negative control vector, into the EBV-positive cell line Raji (A) and into the EBV-negative human keratinocyte line SVK (B). Schematic representations of constructs pF12, pF13d, and pF13 are shown below. pE2CAT, a positive control for E2F responsiveness, contains the adenovirus E2 promoter linked to the CAT gene. Results are expressed as the relative promoter activity in the presence and absence of CMV–E2F-1.

to 5'-CTACCGAATTCCGGGTAATA-3'; mutated bases are underlined.

The constructs pRSV Ela Neo and pRSV Ela$_{928}$ Neo were generous gifts from David Johnson of Joseph Nevins' laboratory. The former consists of a pRC Rous sarcoma virus vector (Invitrogen) which expresses wild-type E1A protein, and the latter has the same vector backbone but expresses mutant E1A which cannot bind to Rb and therefore cannot release E2F (27a).

The CMV–RBP-3 construct, kindly provided by K. Helin, contains the cDNA encoding amino acids 88 through 437 of the E2F-1 transcription factor under the control of the cytomegalovirus (CMV) immediate-early (IE) promoter (22). The pHD101-3 vector, containing the CMV-IE promoter in a pGem-2 plasmid, was used as a negative control (13).

DNA transfections. Plasmid DNA was amplified in *Escherichia coli* and purified through Qiagen columns. For each sample, 10 μg of plasmid DNA was electroporated (40) into 1 × 10⁷ lymphoid or 0.5 × 10⁷ epithelial cells at 1,500 V, using the University of Wisconsin Zapper electroporation unit. Cells were then suspended in 5 ml of RPMI 1640 medium supplemented with 10% fetal calf serum and incubated for 48 h at 37°C in 5% CO_2.

CAT assays. Extracts were prepared by washing cell pellets twice in phosphate-buffered saline solution (PBS), resuspension in 200 μl of 0.25 M Tris-HCl (pH 7.5), and four cycles of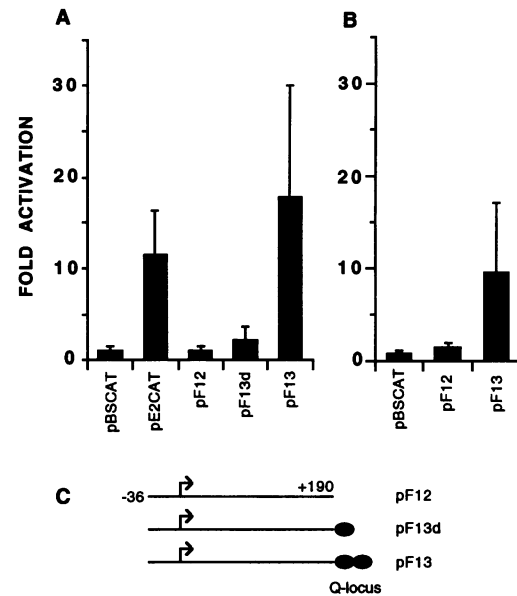 freeze-thaw. Reactions were carried out, as described previously (18), by incubating each sample with acetyl coenzyme A and [¹⁴C]chloramphenicol at 37°C for 2 h. Acetylated reaction products were separated by thin-layer chromatography, visualized by autoradiography, and quantitated by scintillation counting or by scanning on a Molecular Dynamics PhosphorImager. Percent acetylation is calculated as the ratio of counts per minute in the acetylated reaction products to counts per minute in the entire sample, and fold activation is calculated as the percent acetylation relative to a negative or positive control value.

Preparation of ³²P-labelled probes for mobility shift assays. DNA probes were generated by annealing oligonucleotides spanning the regions of interest and filling in any single-stranded overhang with the Klenow enzyme. Probes were end labelled with polynucleotide kinase and are diagrammed in Fig. 3A, 5A, and 7A.

Preparation of whole-cell extracts. Approximately 10⁸ cells were washed with PBS and resuspended in extraction buffer containing 20 mM N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid (HEPES), 450 mM NaCl, 0.2 mM EDTA, 25% glycerol, 0.5 μg of leupeptin per ml, 1 μg of aprotinin per ml, 0.5 μg of pepstatin per ml, and 0.5 μM dithiothreitol (DTT). Extracts were then sonicated on ice for 10 short bursts, and debris was removed by centrifugation at 12,000 × g for 20 min at 4°C. Aliquots of the supernatant fluids were stored at −70°C. Protein determinations were done with the Bradford protein assay.

Preparation of GST fusion proteins. The GST–RBP-3 construct, containing amino acids 88 through 437 of the E2F-1 transcription factor, was provided by K. Helin (22). The full-length, wild-type GST–E2F-1 construct and the GST–E2F-1 DNA-binding mutant, pGEX2T-E2F-1 Eco138, were kindly provided by D. Cress of Joseph Nevins' laboratory (11).

The GST–EBNA-1 construct was made by inserting an *Rsa*I-*Msc*I DNA fragment, which contains the EBNA-1 open reading frame minus the first seven amino acids and the Gly-Ala repeats, into the *Sma*I site of pGEX 1N (57). Overnight cultures of *E. coli* transformed with this plasmid were induced with 0.1 mM isopropyl-β-D-thiogalactopyranoside (IPTG) for 3 h. Induced cultures were pelleted, lysed in a 1% Triton X-100 solution, and sonicated. GST–EBNA-1 fusion protein was purified by incubating the lysate with glutathione-agarose beads. Following three washings with PBS, purified fusion protein was eluted with 5 mM reduced glutathione in 50 mM Tris-HCl (pH 8.0).

Gel mobility shift assays. Binding reactions with whole-cell extracts contained 25 mM Tris (pH 7.5), 125 mM NaCl, 1.25 mM DTT, 12.5% glycerol, 0.05 mg of sonicated salmon sperm DNA per ml, 0.06% Nonidet (NP-40), 6.25 mM $MgCl_2$, and 0.06 mg of bovine serum albumin (BSA) per ml. After preincubation for 5 min at 22°C, 25,000 cpm of radiolabelled probe was added, and the mixture was incubated for 20 min at 22°C. Samples were then electrophoresed at 4°C through nondenaturing polyacrylamide gels in 1× Tris-borate-EDTA buffer. Protein-DNA complexes were visualized by autoradiography.

For the supershift reactions, binding reaction mixtures containing whole-cell Jurkat extracts were incubated on ice with 0.5 µl of either monoclonal antibody to E2F (KH95; contributed by Nick Dyson and Ed Harlow) (23) or EBNA-1 (EBNA.OTx1; contributed by J. Middeldorp) (7) for 1 h before the addition of radiolabelled probe.

Binding mixtures for the displacement mobility shift assays were solutions of 20 mM Tris-HCl (pH 7.5), 100 mM NaCl, 1 mM DTT, 0.05 mg of sonicated salmon sperm DNA per ml, 10% glycerol, 0.5% NP-40, and 5 mM $MgCl_2$. One microgram of purified GST–EBNA-1 was incubated with 5,000 cpm of labelled Qd for 15 min to allow binding. Subsequently, purified GST–E2F-1, GST–E2F-1 DNA-binding mutant, GST–Z, or GST was added and incubated for an additional 20 min.

Gel shift competitors. All competitors for the gel mobility shift assays were annealed oligonucleotides that were purified by phenol-chloroform extractions and passed through a G-25 column (Boehringer-Mannheim), except for the thymidine kinase (TK) competitor (a gift from David Johnson in Joseph Nevins' laboratory). The sequences for the oligonucleotides are as follows: human dihydrofolate reductase promoter (DHFR), 5'-CCACAATTTCGCGCCAAACTTGAC-3', adenovirus type 5 E2 promoter (E2), 5'-CGTAGTTTTCGCGCTTAAA TTTGAGAAAGGGCGCGAAACTAGTC-3'; human c-*myc* promoter (c-*myc*), 5'-CAGAGGCTTGGCGGGAAAAAGA ACGGAGGGAGGGATCGCGCTGAGTA-3', human TK promoter, 5'-TCGACGGGGCGGGCTGCGGCAAATCTC CCGCCAGTCAGCGGCCGGGCGCTGATTGGCCC-3'; one binding site from the adenovirus E2 promoter (E2F), 5'-AATTTGAGAAAGGGCGCGAAACTAGTC-3'; and the mutant control for E2F (E2Fm), 5'-AATTTGAGAAACTA GAGTCTGCTAGTC-3' (reviewed in reference 41).

DNase I footprinting. A DNA probe, radiolabelled on the noncoding strand, was prepared by digesting the pQa plasmid construct, which contains Fp sequences from +134 to +307 cloned into the *Xba*I site of the pBSCAT vector, with *Hin*dIII and *Pvu*II. The resulting fragment, which contained Fp sequences from +134 to +260, was gel purified and filled in with radiolabelled nucleotides, using the Klenow enzyme. Binding reaction mixtures contained 7 mM $MgCl_2$, 1 µg of sonicated calf thymus DNA per ml, 0.14 µg of BSA, 0.75% NP-40, 4 mM HEPES (pH 7.6), 8 mM KCl, 0.02 mM ethylene glycol-bis(β-aminoethyl ether)-*N,N,N',N'*-tetracetic acid (EGTA), 0.5 mM EDTA, 0.4 mM DTT, and 8.3% glycerol. Following incubation

at 22°C for 20 min, 0.15 U of DNase I (Promega) was added for 1 min. Digestion was stopped by the addition of a solution containing 100 mM NaCl, 100 mM Tris-Cl (pH 8.0), 20 mM EDTA (pH 8.0), 0.1% SDS, 100 µg of proteinase K per ml, and 100 µg of glycogen per ml. Reaction products were extracted with phenol-chloroform, ethanol precipitated, electrophoresed on a 6% polyacrylamide-urea denaturing gel, and visualized by autoradiography.

RESULTS

Constitutive activity of Fp in infected and uninfected lymphoid cells. To study promoter activity, a set of phagemid constructs containing various amounts of DNA sequence flanking the Fp RNA start site was made. Promoter inserts were generated by PCR amplification of genomic DNA from the B95-8 strain of EBV and linked to the CAT gene in the phagemid vector pBSCAT (16). Constructs shown in Fig. 1A were transfected by electroporation into different cell lines, and promoter activity was gauged by CAT assay.

When constructs pF1 through pF4 were transfected into the EBV-negative Jurkat cell line, Fp was constitutively active (Fig. 1B). When the constructs were transfected into the EBV-positive Raji cell line, however, promoter activity was completely abolished in those constructs which contained the downstream Q locus, to which EBNA-1 binds with a low affinity (Fig. 1C). This result confirms that a factor present in EBV-infected cells, presumably EBNA-1, acts to repress Fp activity through interaction with the consensus binding sites downstream of the Fp transcriptional start site (51).

Promoter construct pF2x, which contained just 5 bp of downstream sequence, was far less active than constructs pF1 and pF3, which contained 190 bp of downstream sequence (Fig. 1B and C), in both infected and uninfected cells, suggesting the existence of a downstream positive regulatory element located between +5 and +190.

E2F-1 activates Fp in transient promoter assays. To determine whether a known cellular factor might serve to activate Fp, the DNA sequence of the downstream region was scanned for known transcription factor binding sites. No perfect match was found; however, a sequence resembling the consensus binding site for the E2F-1 transcription factor was identified within the downstream positive regulatory region, between nucleotides +172 and +181 relative to the RNA start site (TTTGCGAAAA). As shown in Fig. 2A, the pF13 construct normally under EBNA-1 repression in EBV-infected Raji cells was activated an average of 26-fold when cotransfected into Raji cells with the CMV–RBP-3 plasmid, which expresses the E2F-1 protein under the control of the CMV IE promoter. In addition, in the human keratinocyte cell line SVK, pF13 showed an average of 8.6-fold activation. Interestingly, CMV–RBP-3 did not transactivate the shorter constructs, pF12 or pF13d, both of which contained the suspected positive regulatory region. Unexpectedly, therefore, the E2F-responsive region of Fp appears to lie within the Q locus (+190 to +307) rather than in the region from +5 to +190. Moreover, the E2F-responsive region is functional in epithelial as well as lymphoid cells.

A fusion protein expressing the E2F-1 transcription factor binds specifically to the F promoter. To determine whether the E2F-1 protein interacted directly with DNA sequence in the F promoter region (Fig. 3A),, mobility shift assays were performed with purified GST–RBP-3 fusion protein, kindly provided by K. Helin (22). This fusion protein formed three complexes with the Qa probe, which extends from +134 to +307, and encompasses both the positive regulatory element
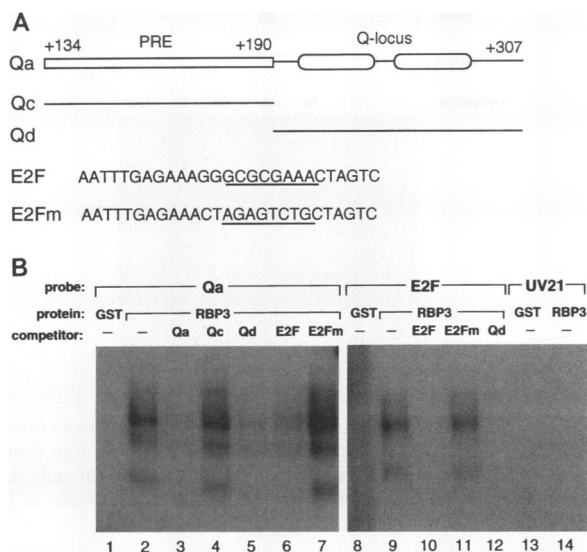
FIG. 3. Interaction of an E2F-1 fusion protein with the Fp down-stream regulatory region. (A) DNA sequence of probes and competitors used in mobility shift assays. (B) Labelled DNA probes shown were incubated with either GST protein (lanes 1, 8, and 13) or with GST–RBP-3 protein (lanes 2-7, 9-12, and 14). UV-21 is a DNA probe derived from the mouse major histocompatibility complex class II regulatory region and contains an NF-κB site (53).
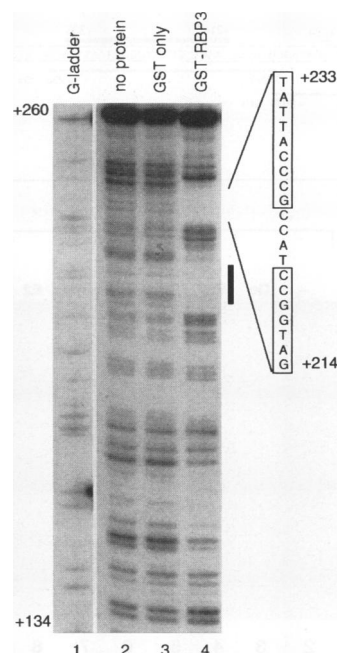

FIG. 4. DNase I footprinting of E2F binding in the Fp downstream regulatory region. An end-labelled probe extending from +134 to +260, relative to the Fp RNA start site, was digested with DNase I following incubation with no protein (lane 2), GST protein (lane 3), or GST–RBP-3 (lane 4). Lane 1 contains a G ladder of the same probe. Protected sequence is shown at right. Nucleotides included in the EBNA-1 binding sites are boxed. Heavy line indicates an additional minor footprint.

identified earlier and the Q locus (Fig. 3B, lane 2). Formation of these complexes was specifically inhibited with a 100-fold excess of unlabelled E2F-binding probe (Fig. 3B, lane 6) but not by a 100-fold excess of E2Fm (Fig. 3B, lane 7), which contains a mutated E2F-binding site that abolishes binding of E2F. These complexes were also specifically inhibited with a 100-fold excess of unlabelled Qa and Qd probes (Fig. 3B, lanes 3 and 5; probes diagrammed in Fig. 3A) but not by the Qc probe (lane 4).

As expected, the GST–E2F-1 fusion protein formed complexes with a labelled probe (E2F) containing a consensus E2F-1-binding site (Fig. 3B, lane 9); a 100-fold excess of unlabelled E2F and Qd probes competed with complex formation (Fig. 3B, lanes 10 and 12), but E2Fm did not (Fig. 3B, lane 11). The GST–E2F-1 fusion protein did not interact with a nonspecific DNA probe, UV21, containing an NF-κB binding site (Fig. 3B, lanes 13 and 14) (53).

Although the Qd probe does not contain a perfect consensus E2F-1 binding site, these results indicate that it is able to interact directly and specifically with the E2F-1 fusion protein generated by GST–RBP-3. In addition, E2F-Qa complex formation is not inhibited with the Qc oligonucleotides, a result that correlates with the functional data in Fig. 2 and suggests that the E2F-responsive region of Fp is within the Q locus.

An E2F-1 fusion protein interacts at a site centered between the EBNA-1 binding sites in the Q locus. The interaction of E2F-1 with the Qa probe was localized by DNase I footprinting. As shown in Fig. 4, binding of GST–RBP-3 to the Qa probe specifically protected a region extending from +214 to +235 relative to the RNA start site of Fp (50, 52). Interestingly, this region is only loosely homologous to known consensus binding sites for E2F-1. Furthermore, the E2F-1 footprint overlaps with parts of the two low-affinity binding sites for the EBNA-1 protein that define the Q locus. An area of incomplete protection extending from +191 to +204 was also

detected. As expected from the functional data, the E2F fusion protein did not protect sequence in the downstream positive regulatory element between nucleotides +134 and +190.

The Q locus binds an E2F-like protein complex in whole-cell extracts. Two specific protein complexes were generated when the Qd probe was incubated with whole-cell extracts from Jurkat cells (data not shown). Two probes that spanned the footprinted portions of the Qd probe, Qe (+209 to +235) and Qf (+186 to +209) (Fig. 5A), were incubated with Jurkat cell extracts in mobility shift assays (Fig. 5B). The Qe probe formed one specific band (Fig. 5B, lane 2) that could be inhibited well with a 200-fold excess of self (Fig. 5B, lane 3), and cold competitors from the E2F sites in the human c-myc promoter and the adenovirus E2 promoter (Fig. 5B, lanes 7 and 8, respectively). It could be competed to some extent by the E2F sites from the human TK promoter (Fig. 5B, lane 5), but not by the E2F sites from the DHFR promoter (Fig. 5B, lane 6). Interestingly, when only one E2F site from the adenovirus E2 promoter was used (Fig. 5B, lane 4), it competed poorly. Oct-1 and SP-1 oligonucleotides were used as negative controls (Fig. 5B, lanes 9 and 10, respectively). As an additional control, a labelled probe containing one E2F site from the adenovirus E2 promoter was inhibited by all of the E2F sites described above as well as partially by Qe (data not shown). Titrations with E2, TK, and E2F oligonucleotides suggested that a 100-fold excess of E2 was sufficient to compete with Qe (data not shown).

In order to confirm the presence of E2F in the complex with Qe, a specific monoclonal antibody to E2F, KH95 (23), was used. The antibody supershifted the Qe complex as well as the complex on the E2F probe (Fig. 5C, lanes 1 and 3, respective-

A

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        +191      +204            +214              +233
Qd: GTGCTTGAAAAGGCGC GGGATAGCGTGCGCTACCGG ATGG CGGGTAATACATGCTATCCT TACATTTTGGCATTTTGG
        +186              +209                +235  Qe
                          +209  Qf
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B

probe: Qe

extract: − Jurkat

competitor: − − Qe E2F TK DHFR c-myc E2 SP-1 Oct-1

1 2 3 4 5 6 7 8 9 10

C

probe: Qe E2F
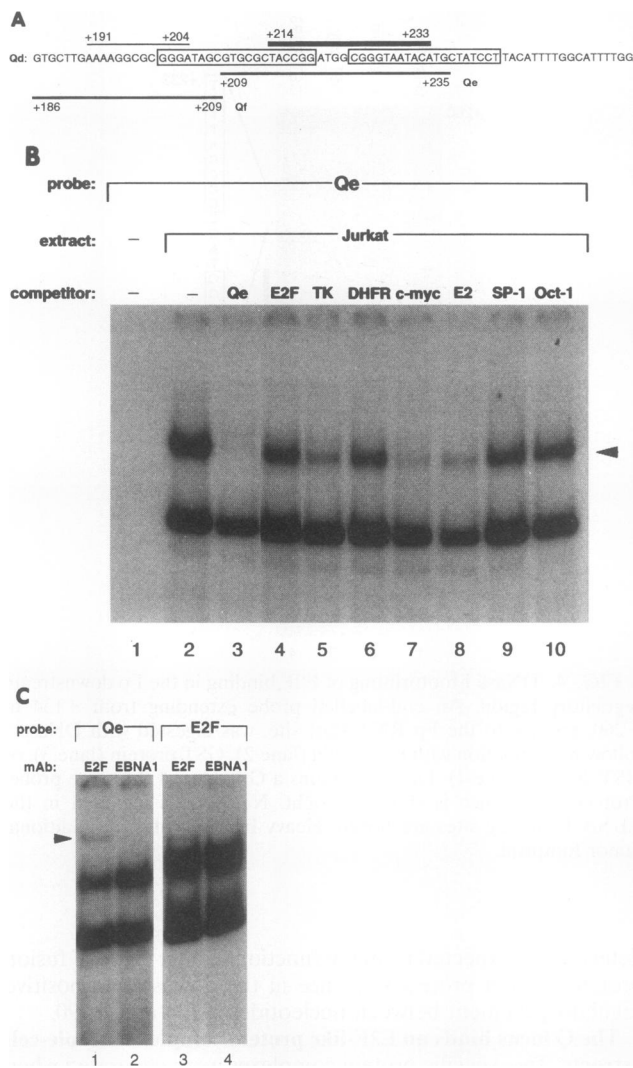
mAb: E2F EBNA1 E2F EBNA1

1 2 3 4

FIG. 5. An E2F-1-like complex in whole-cell extracts binds to the footprinted region of Fp. (A) Footprinted portion of the larger Qd probe is indicated by the bars above the sequence. Bars on the bottom indicate the region used for probes in mobility shift assays with whole-cell extracts. (B) The minimal Qe probe was incubated with either no protein (lane 1) or 10 μg of Jurkat whole-cell extracts (lanes 2 to 10) and inhibited with 200-fold excess E2F sites from various promoters. E2F complex is indicated by the arrow. (C) Labelled Qe probe (left panel) or E2F probe (right panel) was incubated with 10 μg of whole-cell extracts from Jurkat cells and 0.5 μl of a monoclonal antibody to either E2F (lanes 1 and 3) or EBNA-1 (lanes 2 and 4). Arrowhead indicates the supershifted band.

ly). The complexes on the Qe or the E2F probe were not supershifted by EBNA-1 monoclonal antibody EBNA.OTx1 (7).

The footprinting studies revealed another region of interaction with E2F (+191 to +204). A probe, Qf (Fig. 5A), containing this site also formed a specific band when incubated with Jurkat extracts (Fig. 6, lane 2). This complex bound with reduced affinity, as suggested by footprinting data, and was inhibited with Qe and E2F sites from the E2 promoter (Fig. 6, lanes 5 and 6, respectively). Therefore, our results indicate that there are two distinct sites in Fp which bind E2F. The DNA sequences of these regions had hitherto not been described as conventional E2F-binding sites.

probe: Qf

extract: − Jurkat

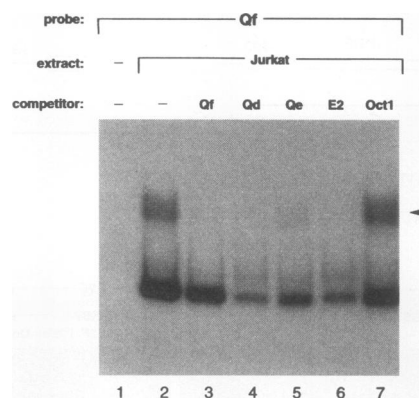competitor: − − Qf Qd Qe E2 Oct1

1 2 3 4 5 6 7

FIG. 6. An E2F transcription factor in whole-cell extracts binds to Qf. Qf probe (Fig. 5A) was incubated with either no protein (lane 1) or with 20 μg of whole-cell extract from uninfected Jurkat cells (lanes 2 to 7). Oligonucleotide competitors (200-fold excess) were as indicated. E2F complex is indicated by the arrowhead.

An E2F-1 fusion protein displaces EBNA-1 binding from the Q locus but not from *oriP*. As demonstrated, E2F-1 activates Fp in transient promoter assays. Furthermore, the major E2F-1 DNase I footprint lies between, and partially overlaps, the two EBNA-1 sites within the Q locus. Therefore, the ability of E2F to displace EBNA-1 binding from the Q locus was investigated by mobility shift assays. A purified GST–EBNA-1 fusion protein formed a specific complex with the Qd probe (Fig. 7B, lane 5). A purified, full-length GST–E2F-1 fusion protein also formed a specific complex with a mobility different from that of the GST–EBNA-1 complex (Fig. 7B, lane 4). The addition of increasing concentrations of the GST–E2F-1 fusion protein to prebound GST–EBNA-1 caused a displacement of GST–EBNA-1 from the Qd probe and the formation of the E2F complex (Fig. 7, lanes 6 to 10). As expected, a GST–E2F-1 mutant that is unable to bind DNA (lane 3) did not displace the EBNA-1 complex (lane 11). GST and a GST-Z fusion protein, used as negative controls, were also unable to displace prebound GST–EBNA-1 (lanes 12 and 13, respectively). These results suggest that an E2F transcription factor displaces EBNA-1 by binding to sequences centered between the two EBNA-1 binding sites in the Q locus.

As shown in Fig. 7A, within the E2F-footprinted region is a 15-bp sequence that is unique to the Q-locus probe (boldface line above sequence); partly protected sequence is indicated by the thin line. This 15-bp element is found nowhere among any of the 24 EBNA-1 binding sites within *oriP*. This was confirmed by the inability of GST–E2F-1 to bind to *oriP* (Fig. 7B, lane 18). The ability of E2F-1 to displace EBNA-1 from *oriP* was examined as a further control. GST–EBNA-1 formed a specific complex with the *oriP* probe (Fig. 7B, lane 17) and was not displaced by the addition of GST–E2F-1 (Fig. 7B, lane 15). This result indicates that E2F recognizes sequences specific to the Q locus. Furthermore, the displacement of EBNA-1 from the Qd probe by E2F is specific to sequences contained in the Q locus.

Point mutations within the E2F-1 binding element abolish E2F responsiveness. To confirm that E2F responsiveness could be mediated by DNA sequence within the Q locus, three point mutations were introduced into the DNase I-footprinted region (5'-CTACCGAATTCCGGGTAATA-3'; mutated bases are underlined). This mutated construct, pQEco, which did not form an E2F complex with Jurkat cell extracts (data not shown), displayed constitutive activity comparable to that of
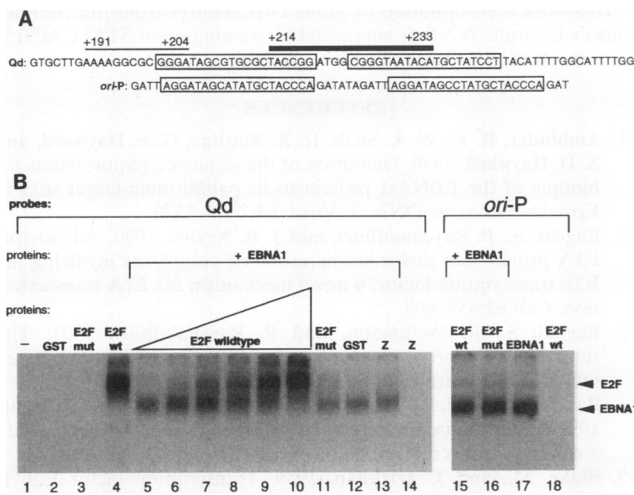
A

B

FIG. 7. E2F displaces EBNA1 binding from the Q locus but not from *oriP*. (A) Sequences of probes used for mobility shift assays. Bars indicate the two E2F footprinted regions (+191 to +204 and +214 to +235) of the Qd probe. Boxed regions indicate EBNA-1 binding sites. (B) Qd was used as a probe in the left-hand panel (lanes 1 to 14). A total of 1 μg of GST–EBNA-1 was allowed to bind to Qd (lanes 5 to 13) for 15 min. GST–E2F-1 (0, 1, 2.5, 5, 10, and 25 μg) was then added to lanes 5, 6, 7, 8, 9, and 10, respectively, and allowed to bind for 20 min. As controls, 25 μg of GST–E2F-1 DNA-binding mutant (lane 11), GST (lane 12), or GST-Z (lane 13) was added and allowed to bind for 20 min. As further controls, 5 μg of GST (lane 2), 25 μg of a GST–E2F-1 DNA-binding mutant (lane 3), 5 μg of GST–E2F-1 (lane 4), and 25 μg of GST-Z (lane 14) were incubated with Qd. A probe containing two EBNA-1 binding sites from *oriP* was used in the right-hand panel (lanes 15 to 18). GST–EBNA-1 (1 μg) was allowed to bind to *oriP* (lanes 15 to 17) for 15 min, after which 25 μg of GST–E2F-1 (lane 15) or GST–E2F-1 DNA-binding mutant (lane 16) was added and allowed to bind for 20 min. As a control, 25 μg of GST–E2F-1 was incubated with *oriP* (lane 18).

the wild-type construct, pF13, in EBV-negative Jurkat cells (data not shown), and in EBV-positive Raji cells it was completely repressed, as expected. Unlike the wild-type pF13 construct, however, the mutant pQEco construct was not significantly activated by cotransfected CMV-RPB-3 (Table 1), confirming that the nucleotides within the DNase I footprint are indeed important for E2F responsiveness of Fp.

E1A activates Fp in transient transfections. E2F is inactive, existing as part of a heteromeric complex that includes Rb, until it is dissociated from the complex (41). The adenovirus protein E1A can disrupt the interaction between E2F and Rb, thereby allowing E2F to activate responsive promoters. To show that endogenous levels of E2F were sufficient to upregulate Fp, an expression construct of E1A was cotransfected with pF13 and pCMV–EBNA-1 into Jurkat cells. Overexpression of E1A repeatedly resulted in 3.9-fold (±1.0-fold)-greater acetylation than pF13 plus EBNA-1 alone (Table 1). Activation by E1A was specific because a mutant construct of E1A, which is incapable of disrupting the Rb-E2F complex, repeatedly showed only 1.3-fold (±0.6-fold) activation.

DISCUSSION

The EBNA proteins are transcription factors essential for latent infection and cell immortalization. The recently discovered F promoter, one of three now known to govern transcription of the EBNA proteins, is unique in possessing EBNA-1 binding sites. Promoter activity arising from the *Bam*HI-F

TABLE 1. Effects of mutation of the E2F binding site on activation of Fp and activation of repressed Fp by endogenous E2F

Promoter construct	Activation (fold) of following cotransfected expression construct[a]:		
	pCMV-RBP3	pRSV E1a wt	pRSV E1a$_{928}$
pQEco	2.25 ± 0.43	ND	ND
pF13	30.3 ± 14.52	3.9 ± 1.0	1.3 ± 0.6

[a] In the first column, 5 μg of each promoter construct, pF13, containing wild-type (wt) sequences, and pQEco, containing three point mutations in the DNase I-footprinted region of Fp, was cotransfected in Raji cells with 5 μg of pCMV-RPB-3 (wild-type E2F-1). Results are presented as fold activation above that resulting from cotransfection with pHD101-3 (negative control vector). In the next two columns, Jurkat cells were transfected with 3 μg of pF13, 0.5 μg of pCMV-EBNA-1 and 6 μg of pRSV E1a (wild-type E1A) or pRSV E1a$_{928}$ (E1A Rb binding mutant). Results are presented as fold activation above that resulting from cotransfection with pCMV-EBNA-1 and pHD101-3, the negative control vector.

region of EBV is negatively autoregulated when the EBNA-1 protein interacts with its low-affinity binding sites in the Q locus (51, 62). This result raises the questions of when and how Fp is activated in EBV-infected cells, all of which express EBNA-1, a protein essential for the persistence of latent EBV infection. Recently it has become increasingly clear that EBV gene expression is often regulated by a composite of interactive viral and cellular factors (19, 30, 33, 36, 48, 55, 56, 61, 66). In this work, we demonstrate that a member of the E2F family of transcription factors can overcome the EBNA-1-mediated repression by interacting directly with DNA within the Q locus. Furthermore, a GST–E2F-1 fusion protein can displace GST–EBNA-1 bound to the Q locus in mobility shift assays.

Autoregulation of Fp during type 1 latency would permit the virus to maintain a minimum threshold level of EBNA-1. Low levels of EBNA-1 would presumably be sufficient to activate the *oriP*, to which it binds more abundantly, cooperatively, and with a higher affinity (1), before binding to the lower-affinity Q sites and shutting down expression of EBNA-1 from Fp. With the development of type III latency, in which all EBNAs are expressed, Fp would remain inactive and promoter usage for the EBNAs would switch to Cp or Wp (31, 49). EBNA-1 autoregulation could therefore account for the inactivity of Fp in type III latency.

Adding to this model is our finding that a member of the E2F transcription factor family interacts with the Q locus and can overcome the EBNA-1-mediated repression of Fp in infected cells. The E2F transcription factor, first identified by its abilities to bind to and to activate the adenovirus E2 promoter (32), is important in the regulation of cellular genes required for DNA synthesis and replication, such as DHFR, TK and DNA polymerase α (5, 14, 24, 43), as well as genes involved in cell growth, such as c-*myc*, Cdc2, and cyclin A (12, 26, 39, 41, 63). During the G$_1$ phase of the cell cycle, E2F-like factors are inactive, existing as a part of heteromeric protein complexes that include the retinoblastoma (RB) protein (3, 4, 6, 9, 15, 21, 25). Upon entry into S phase, E2F dissociates from the RB protein and is free to activate its target promoters (2, 8, 37).

Because the EBV episome is maintained during latency at a constant copy number and is replicated coordinately early in S phase in the cell cycle, and since EBNA-1 is the only viral protein required for episomal replication, it is likely that expression or function of EBNA-1 is under cell cycle control. As more activated E2F becomes available in the cell at the G$_1$/S boundary, it could activate Fp by displacing EBNA-1 from the Q locus. This mechanism must be unique in EBV to

the Q locus, since in our assays E2F-1 clearly does not interact with the higher-affinity EBNA-1 binding sites within *oriP*. The newly expressed EBNA-1 could then activate latent episomal replication and coordinately populate the progeny cells with episomes. This type of regulation, which would allow the virus to maintain the episome without expressing all of its latent genes, is consistent with current models for type 1 latency (31, 49).

It has also been suggested that Fp is activated following induction to the cytolytic phase of EBV infection (34). While our current results do not directly address this issue, it has been known for years that induction of lytic viral replication follows entry into S phase (20). If Fp is indeed active during the lytic phase of EBV infection, its activity may be triggered, at least in part, by the higher levels of active E2F available in the cell at the start of S phase (28, 54). Clearly, the potential cell cycle control of this promoter deserves further investigation. The E2F activity we have characterized is of considerable interest because of the potentially two new binding sites we have identified. Possibly, both sites work cooperatively to activate Fp. E2F exists as a family of transcription factors (8, 17, 27), but only narrow variations on a single consensus binding site have been tolerated in binding assays (41). Thus, it has not been possible to identify other promoters that E2F may activate. Although the exact contact points of the E2F factor binding in the Q locus have not yet been precisely identified, the 15-bp sequence we have mapped by DNase footprinting (5'-GGATGGCGGGTAATA-3') should prove useful in a number of cell biologic contexts. Our results suggest that an E2F-1-like protein is involved in the regulation of Fp. In mobility shift assays, the complex bound to Qe is inhibited by some E2F binding sites but not others, whereas a complex formed with the previously described E2F site from the E2 promoter (E2F) is inhibited by all sites. On the basis of mobility shift assays, it appears that the repression of Fp is overcome by displacement of EBNA-1 by E2F. In additional work, an E2F mutant, impaired in its transactivation domain (28), could only partially overcome EBNA-1 repression in transient-transfection assays (data not shown). This result suggests that the transactivation domain may contribute to overcoming Fp repression.

The discovery (51, 52, 58) and characterization of this new promoter illustrate the complexity of EBV gene expression and demonstrate a new regulatory mechanism that invokes downstream activator and repressor elements not previously reported in EBV genes. The coordinate regulation of Fp by both EBNA-1 and E2F suggests a new way in which the virus may subvert use of cell cycle regulatory factors to bring about ordered replication of episomes. It now becomes of considerable interest to determine whether EBNA-1 action in *oriP* uses a parallel mechanism that also invokes cellular proteins such as the OBP-1 protein, recently described by Wen et al. (64), or others not yet identified.

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