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The Epstein-Barr Virus EBNA-1 Promoter Qp Requires an Initiator-Like Element

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Expression of the Epstein-Barr virus (EBV) EBNA-1 protein within EBV-positive tumor cells and subpopulations of latently infected B lymphocytes in vivo is mediated by the promoter Qp. Previous studies have established that Qp is a TATA-less promoter whose activation requires only proximal regulatory elements and that it is negatively autoregulated through two EBNA-1 binding sites downstream of the transcription initiation sites. The objective of this study was to better define the properties of an essential positive regulatory element (QRE-2) adjacent to a major transcription start site of Qp and to evaluate the contributions of other potential regulatory elements proximal to the Qp start site. Using DNA affinity purification and UV cross-linking, we have identified the QRE-2-binding protein as a single polypeptide of ~40 kDa. The DNA-binding properties of this protein are clearly distinct from those of the TATA-binding protein, suggesting that in the absence of a TATA box, QRE-2 may function as an initiator element to direct assembly of TFIID near the transcription start site. Mutational analysis of potential regulatory elements, furthermore, indicated that the putative E2F binding sites within the EBNA-1 binding domain can exert a positive influence on Qp that is EBNA-1 independent, suggesting that these regulatory elements play an additional if not different role in Qp regulation than previously proposed. A model for the regulation of Qp consistent with the current and previous findings which provides for a simple but efficient mechanism of ensuring the EBNA-1 expression necessary to sustain long-term latency is presented.

Epstein-Barr virus (EBV) establishes and maintains a lifelong latent infection within B lymphocytes of its host, despite the fact that most if not all EBV latency-associated genes encode proteins recognized by EBV-specific cytotoxic T lymphocytes (11, 12, 14). Within EBV-immortalized lymphoblastoid cell lines and some EBV-positive Burkitt lymphoma (BL) lines, each of the nine known viral latency-associated proteins is expressed (type III latency); these are the six EBV nuclear antigens (EBNAs) and the three integral latent membrane proteins expressed in the plasma membrane. In contrast to lymphoblastoid cell lines, viral protein expression in EBV-associated tumors is restricted to either EBNA-1 (type I latency), as in BL, or to EBNA-1 plus the latent membrane proteins (type II latency) in undifferentiated nasopharyngeal carcinoma and EBV-positive Hodgkin's and T-cell lymphomas (3, 6, 8, 18, 21, 32, 43). Moreover, it is now evident that each of these forms of latency likely occurs in healthy EBV-seropositive individuals as well (4, 37; reviewed in reference 36).

The one EBV protein expressed in all known states of EBV latency is EBNA-1. Because EBNA-1 is required for replication of the episomal EBV genome, a function mediated in part by binding to specific elements within the latent origin of DNA replication *oriP* (19, 42), expression of EBNA-1 is essential within a population of proliferating cells to prevent loss of the genome over the course of multiple cell divisions. Moreover, unlike the other latency-associated proteins, EBNA-1 is less efficiently recognized by the host immune surveillance (12, 13). Thus, the ability to exist in multiple states of latency is likely a viral adaptation to persistence within a potentially dynamic and long-lived cell (the B lymphocyte) that ensures the maintenance of a minimal pool of infected cells necessary to maintain infection of an immune host.

During type III latency, each of the six EBNA proteins is expressed from a multicistronic transcription unit greater than 100-kbp in length that is under the transcriptional control of either of two adjacent promoters, Cp and Wp (2, 25, 26, 41). Exclusive expression of EBNA-1 during type I and II latency, therefore, requires dissociation of EBNA-1 expression from that of the other EBNA proteins. This is accomplished through the inactivation of Cp/Wp-driven transcription and the activation of the EBNA-1-specific promoter Qp (formerly thought to be Fp) located ~50 kbp downstream of Cp and Wp (16, 28). Qp is a TATA-less promoter and can be negatively autoregulated through two low-affinity EBNA-1 binding sites (relative to the higher-affinity sites in *oriP*) located downstream of the sites of transcription initiation (24, 35).

We previously localized two positive regulatory elements that are essential for Qp transcriptional activity (15). One of these, downstream (of Fp) regulatory element 2 (DRE-2; referred to here as QRE-2), is immediately upstream of a major transcription initiation site. Here we report the purification of a ~40-kDa protein that binds specifically to QRE-2 and which has DNA-binding properties distinct from that of TATA-binding protein (TBP), the principal DNA-binding protein of the TFIID complex. The position of QRE-2 adjacent to a Qp transcription start site, and the unique DNA-binding properties of this cellular protein relative to TBP, suggests that QRE-2 functions as an initiator (Inr) element that directs assembly of the basal transcriptional machinery at the Qp transcription start site. Additionally, we demonstrate that the putative E2F binding sites within the EBNA-1 binding domain

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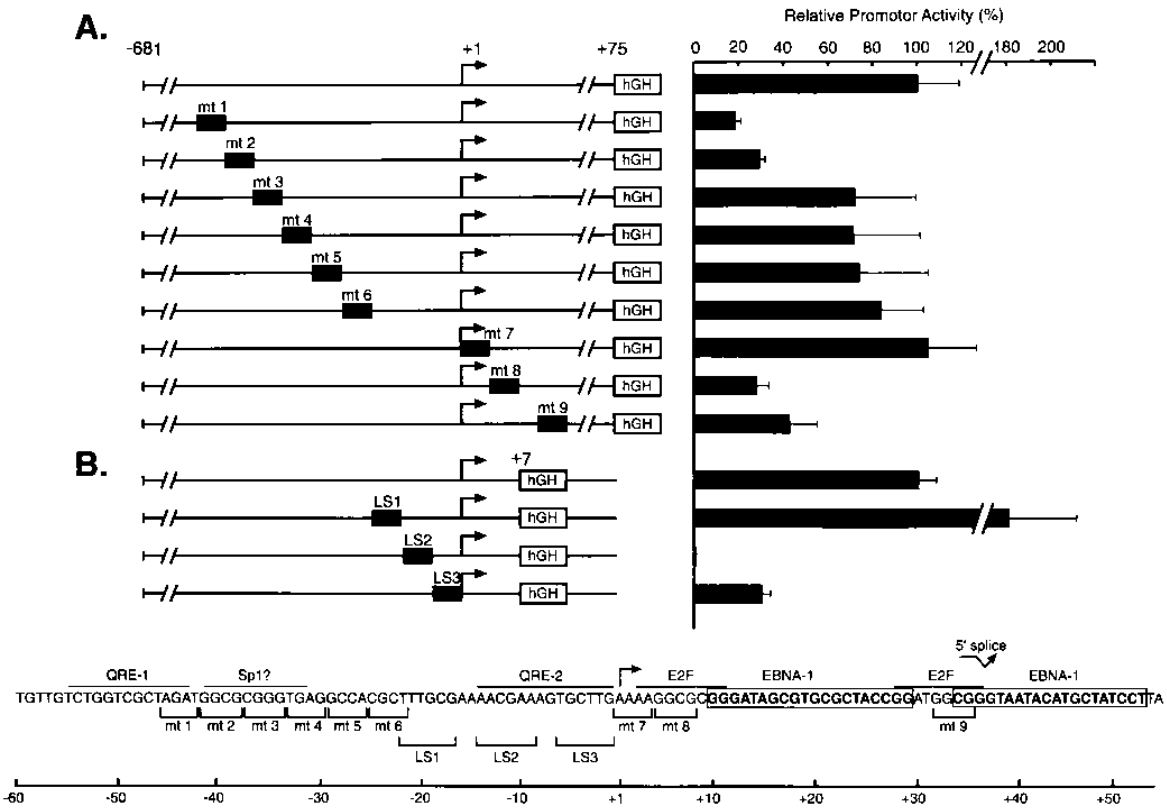

FIG. 1. Definition of Qp regulatory elements. (A) A series of four-base mutations (solid boxes) were introduced into the Qp-hGH reporter plasmid p0GH.006 (24) and analyzed for their effects on promoter activity in EBV-negative Louckes BL cells. Results are from a representative experiment, and for each construct the mean promoter activity obtained from three transfections is expressed as percentage of the activity of the nonmutated control construct (100%). (B) Three *Bam*HI linker-scanning mutations (LS1 to LS3) were introduced into the Qp-hGH reporter plasmid p0GH.015 (15) and analyzed for promoter activity as in panel A. The DNA sequence spanning the portion of Qp evaluated in this study is presented at the bottom. The positions within this sequence of mutations, known and potential regulatory elements, and the splice donor site of the 5′ exon of the EBNA-1 mRNA are indicated; the scale is in nucleotides relative to the transcription start site (bent arrow).

of Qp (35) can contribute positively to Qp function in an EBNA-1-independent manner.

MATERIALS AND METHODS

Cell culture, transfections, and reporter gene assays. EBV-negative Louckes BL cells were maintained in RPMI 1640 medium containing 2 mM L-glutamine and 10% fetal bovine serum. Cells to be used in transfection studies were maintained in roller bottle cultures. Transfection was achieved by electroporation of 8×10^6 cells as previously described (24) with 10 μg of reporter plasmid containing Qp promoter elements linked to the human growth hormone (hGH) gene and 1 μg of the β-galactosidase expression vector pCMV-βgal. Approximately 40 h posttransfection, hGH levels in the culture medium were determined in duplicate by radioimmunoassay (Nichols Institute). To correct for differences in transfection efficiency, hGH values were normalized to β-galactosidase activities (adjusted for total protein assayed) present in transfected-cell extracts.

Plasmids and site-directed mutagenesis. Reporter gene plasmids were constructed by ligating B95-8 EBV DNA fragments into the *Bam*HI site of p0GH, which contains a promoterless hGH gene downstream of the cloning site (30). Generation of the base Qp-hGH reporter plasmids p0GH.006 (−681 to +75 [Fig. 1A]) and p0GH.015 (−681 to +7 [Fig. 1B]) has been described elsewhere (15, 24). A series of four-base substitutions (A⇄C and G⇄T) in the sense strand between −55 and +35 of Qp were made in p0GH.006 by site-directed mutagenesis with the QuikChange system (Stratagene) according to the manufacturer's recommendations. Following mutagenesis, the complete DNA sequence of the EBV insert in each plasmid was determined to verify that additional mutations had not occurred. Linker-scanning mutations (LS1 to LS3) in p0GH.015, described previously (15), consist of a *Bam*HI restriction site introduced by recombinant PCR. The precise locations of all mutations are illustrated at the bottom of Fig. 1. Reporter plasmids containing EBV DNA to +5068 (Fig. 2) extend to the *Xmn*I cleavage site 14 bp downstream of the splice acceptor site of the second exon (U) of the EBNA-1 mRNA. These plasmids were engineered by inserting contiguous restriction fragments in the appropriate order downstream of a *Bgl*I-to-*Pvu*II (−681 to +75) or *Sal*I-to-*Pvu*II (−143 to +75) EBV DNA fragment previously cloned in p0GH; those lacking a functional QRE-2 element contained the LS2 mutation. Plasmid pTV104(2) (obtained from the American Type Culture Collection) was used as a source of the 700-bp hGH cDNA probe for Northern (RNA) blot analysis.

RNA analysis. Total cellular RNA was isolated from transfected cells by extraction with RNAzol B (Tel-Test, Inc.) as described previously (24). Each RNA sample was then digested with 20 U of RQ1 DNase (Promega) to remove any remaining plasmid DNA, extracted with phenol-chloroform and chloroform, and precipitated in ethanol. RNA was fractionated by electrophoresis through a 1.2% agarose–2.2 M formaldehyde gel (10 μg per lane), transferred to a GeneScreen Plus membrane (DuPont), and analyzed by Northern blot hybridization with a ^{32}P-labeled hGH cDNA probe as described previously (15).

Preparation of nuclear extracts and DNA affinity chromatography. Louckes cell nuclear extracts were prepared in batches from approximately 7×10^9 cells (10^{11} cells in total) as described previously (7), with modifications. All steps were performed on ice or at 4°C unless indicated otherwise. Cells were washed once in ice-cold phosphate-buffered saline (PBS), resuspended in 20% glycerol–0.2% Nonidet P-40 in PBS, and lysed in a Dounce homogenizer with a type B pestle (10 strokes). Nuclei were collected by centrifugation at 1,300 × *g* for 10 min, resuspended in high-salt extraction buffer consisting of 20 mM HEPES-KOH (pH 7.9), 0.42 M NaCl, 25% glycerol, 1.5 mM $MgCl_2$, 0.2 mM EDTA, and 0.5 mM DTT (dithiothreitol), and lysed in a Dounce homogenizer with a type B pestle (10 strokes). The nuclear lysate was incubated for 30 min on a rocker and then clarified by centrifugation at 90,000 × *g* for 30 min. The supernatant was dialyzed against 1 liter of buffer D (20 mM HEPES-KOH [pH 7.9], 0.1 M KCl, 20% glycerol, 0.2 mM EDTA, 0.5 mM DTT, 0.5 mM phenylmethylsulfonyl fluoride), frozen, and stored at −70°C. With the exception of buffer D, all solutions contained the following inhibitors: 1 μg each of aprotinin, pepstatin, and leupeptin per ml, 5 mM benzamidine, 10 mM β-glycerophosphate, 0.1 mM sodium orthovanadate, and 0.5 mM phenylmethylsulfonyl fluoride.

Purification of QRE-2-binding proteins was performed as follows. Pooled nuclear extract (2.16 g of total protein in 240 ml) was loaded onto a 40-ml heparin-Sepharose column (Pharmacia). The column was washed with buffer D (1 liter), and bound proteins were eluted in 1-ml fractions with a step gradient of

0.2, 0.4, 0.6, and 0.8 M KCl in buffer D (each step was monitored by A_{280} to ensure that elution was complete before proceeding to the next step). Five-microliter aliquots of peak A_{280} fractions were assayed for QRE-2-binding activity (see below), the majority of which was detected in the 0.6 M KCl eluate. These fractions were pooled, dialyzed against buffer D (1 liter), and clarified by centrifugation at 90,000 × *g* for 30 min. The supernatant (39 ml) was incubated on ice for 15 min after addition of salmon testes DNA (10 μg/ml) and then reclarified at 40,000 × *g* for 10 min. The supernatant was loaded onto a 5-ml QRE-2 DNA affinity column that had been generated as described previously (10) by conjugating concatemers of a double-stranded QRE-2-containing oligonucleotide to CNBr-activated Sepharose 4B (Pharmacia). The complementary QRE-2 oligonucleotides used to generate the column were 5′-gatcCACGCT TTGCGAAAACGAAAGTGCTTGAAAAGGCGC-3′ and 5′-gatcGCGCCTT TTCAAGCACTTTCGTTTTCGCAAAGCGTG-3′. The loaded column was washed with 50 ml of buffer D, and proteins were eluted in 1-ml fractions as above with a KCl step gradient. Fractions with peak QRE-2-binding activity (0.6 M KCl eluate) were pooled, adjusted to 0.1 M KCl by adding buffer D with no salt, and loaded onto a 1-ml QRE-2 affinity column. This column was washed with 30 ml of buffer D, and protein was eluted in 0.1-ml fractions with 0.6 M KCl (in buffer D). Fractions with peak QRE-2-binding activity were pooled, dialyzed against buffer D (100 ml), aliquoted, and stored at −70°C.

Electrophoretic mobility shift assays (EMSA). Double-stranded DNA probes were generated by filling in the recessed (four bases) ends of annealed complementary oligonucleotides with Klenow polymerase in the presence of 100 μCi of [α-^{32}P]dATP (3,000 Ci/mmol) and 1 mM each dCTP, dGTP, and dTTP. The full-length sequences of the probes used in this study were 5′-GCAGAGCATA TAAAATGAGGT-3′ (TATA) and 5′-ACGCTTTGCGAAAACGAAAGTGC TTGAAAA-3′ (QRE-2). All binding reactions were performed at 25°C in 25 μl containing either 10 ng of purified recombinant human TBP (rTBP; Promega) or various amounts of Louckes cell protein. TBP binding reactions were performed as previously described (17) in 20 mM HEPES-KOH (pH 7.9)–25 mM KCl–0.1 mM EDTA–2 mM spermidine–0.5 mM DTT–10% glycerol–0.1 mg of bovine serum albumin per ml–0.1 μg of poly(dG-dC)-poly(dG-dC). Binding conditions for Louckes cell proteins were 10 mM HEPES-KOH (pH 7.9), 60 mM KCl, 1 mM EDTA, 1 mM DTT, 10% glycerol, and 2 μg of poly(dI-dC)-poly(dI-dC). Following a 10-min incubation period, 1 ng of the ^{32}P-labeled probe was added and incubation was continued for 20 min. For competition assays, unlabeled competitor oligonucleotide was included in the 10-min incubation step. In addition to the unlabeled probe oligonucleotides, the following oligonucleotides (sense strand) were used as competitors: mTATA, 5′-GCAGAGCAgcTAAAA TGAGGT-3′; LS-2, 5′-ACGCTTTGCGAAggatccAGTGCTTGAAAA-3′; LS-3, 5′-ACGCTTTGCGAAAACGAAAGggatccAAAA-3′; and OCT1, 5′-GATCGA AATGCAAATCACTAGT-3′.

Protein-DNA complexes were resolved by electrophoresis in nondenaturing 5% polyacrylamide gels run in 0.5× TBE buffer (1× TBE is 90 mM Tris, 88 mM boric acid, and 2 mM EDTA). For analysis of TBP binding, the gels and running buffer also contained 0.05% Nonidet P-40. Following electrophoresis gels were dried and processed by autoradiography.

DNase I footprinting and methylation interference assays. The DNA fragment used in these binding assays was a *Bst*EII (−89)-to-*Pvu*II (+75) restriction fragment labeled at the *Bst*EII site (sense strand) with T4 polynucleotide kinase and [γ-^{32}P]ATP. For DNase I footprinting, the standard EMSA binding reaction mixture was scaled up to 45 μl and contained 5 × 10^4 cpm of the gel-purified (see below) end-labeled probe and up to 10 μg of unfractionated Louckes cell nuclear extract. The DNA was then digested at 25°C for 30 s after addition of 0.6 μl each of 0.1 M $MgCl_2$ and 0.5 M $CaCl_2$ and 0.5 U of RQ1 DNase. Digestion was stopped by adding 100 μl of 100 mM Tris-HCl (pH 7.6), 0.375% (wt/vol) SDS (sodium dodecyl sulfate), 100 mM NaCl, 15 mM EDTA, and 8.5 μg of herring sperm DNA. Protein was removed by digestion with proteinase K and extraction with phenol-chloroform. DNA was precipitated in ethanol, dissolved in formamide gel-loading dye, and analyzed on a 6% sequencing gel.

For methylation interference assays, the ^{32}P-end-labeled probe was methylated with dimethyl sulfate (1) and then used in a standard EMSA mixture containing 5 × 10^5 cpm of DNA fragment and 5 μl of affinity-purified protein. Following nondenaturing gel electrophoresis, the unbound probe and protein-DNA complexes were located by autoradiography, excised, and eluted from the gel overnight at 37°C in 400 μl of 0.5 M ammonium acetate–10 mM magnesium acetate–1 mM EDTA–0.1% SDS. Protein was removed by extraction with phenol-chloroform, and the DNA was precipitated in ethanol. The DNA was then cleaved in 1 M piperidine at 90°C for 30 min, lyophilized, dissolved in formamide gel-loading dye, and resolved in a 6% sequencing gel.

Labeling of protein by UV cross-linking. An oligonucleotide containing the QRE-2 element (5′-gatcCACGCTTTGCGAAAACGAAAGTGCTTGAAAAG GCGC-3′) was annealed to a 10-bp complementary oligonucleotide (5′-GCGC CTTTTC-3′) at a molar ratio of 1:5. The double-stranded probe was generated by strand extension with Klenow polymerase in the presence of dATP, dGTP, bromo-dUTP, and [α-^{32}P]dCTP (3,000 Ci/mmol). Fully annealed and extended probe was isolated by polyacrylamide gel electrophoresis, eluted from the gel (as above), and precipitated in ethanol. Binding conditions were as described for EMSA and included 3.5 × 10^5 cpm of the QRE-2-containing probe and 5 μl of protein. Competitor oligonucleotides (100-fold) were added to some reactions 10 min prior to addition of the probe. Following the 20-min incubation period, samples were transferred to a plastic 96-well microtiter plate and irradiated with UV light (312 nm) for 30 min on a UV transilluminator at room temperature. Samples were then digested with 0.05 U of RQ1 DNase in 10 mM Tris-HCl (pH 7.5)–10 mM $MgCl_2$–10 mM $CaCl_2$ for 20 min at 25°C. Proteins were fractionated by electrophoresis through SDS–10% polyacrylamide gels, which were subsequently fixed in 40% methanol–10% acetic acid, dried, and processed by autoradiography.

RESULTS

Definition of Qp regulatory elements. Transcription of the EBNA-1 gene during type I and II EBV latency was previously believed to originate from the promoter Fp (23, 29, 33). However, the subsequent demonstration that Fp is exclusively associated with the EBV lytic cycle (27) and the identification of latency-specific EBNA-1 transcription start sites downstream of Fp in the *Bam*HI Q restriction fragment (16, 28) have warranted redefinition of the type I (and most likely type II) latency-specific EBNA-1 promoter as Qp. Although multiple transcription start sites are associated with Qp, the major EBNA-1-specific start site common to all BL cell lines that maintain type I latency is located approximately 193 nucleotides downstream of the Fp start site (15, 16, 28). Therefore, we hereafter will refer to this position of Qp-specific transcription initiation as +1.

The minimal amount of DNA sequence information previously shown to be essential for Qp activity within reporter plasmids is that between −113 and +7 (15). Our initial mutational analysis identified two positive regulatory elements within this region: an element between −56 and −44 containing two potential binding sites for the cellular transcription factor known as leader binding protein 1 (LBP-1), and an apparent novel element between −16 and −1 (15). We originally designated these elements DRE-1 and DRE-2, respectively, but have renamed them QRE-1 (the putative LBP-1 binding sites) and QRE-2, since they clearly contribute to Qp function, not Fp function (15, 16). Previous mutagenesis of Qp, however, did not target potential regulatory elements between QRE-1 and QRE-2, which include a GC-rich domain that may serve as an Sp1 binding site, or elements downstream of QRE-2 that were subsequently shown to be potential binding sites for the E2F family of transcription factors (35) (Fig. 1, bottom). We therefore created a series of Qp-hGH reporter plasmids containing four-base substitutions within these and other potential regulatory domains to evaluate their contribution to Qp function (Fig. 1A). Because endogenous levels of EBNA-1 in EBV-infected cells effectively repress Qp in *trans* through the two EBNA-1 binding sites immediately downstream of the transcription start site (24, 35), Qp-mediated reporter gene expression was analyzed in the EBV-negative BL cell line Louckes.

The data presented in Fig. 1A demonstrate that of the nine four-base mutations made in this series of reporter constructs, only four mutations (mt 1, 2, 8, and 9) resulted in significantly lower promoter activities. The reduction of promoter activity due to mt 1, which overlaps QRE-1, is consistent with our previous mutational analysis of this element. Conversion of the potential Sp1 site (GGCGCGGG) to TTATCGGG (mt 2) resulted in a 70% reduction of promoter activity. However, this decrease in promoter activity associated with mt 2 is more likely due to disruption of QRE-1 rather than an inhibition of Sp1-mediated transactivation of Qp, since mt 3 converts this potential (Sp1) element to GGCGATTT without significant reduction in promoter activity. mt 8 and 9, which reduced promoter activity by approximately 70 and 60%, respectively, each target elements that can be bound by E2F factors in vitro (35). Although substantial promoter activity is observed in the

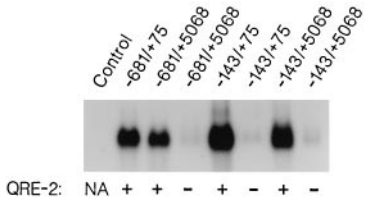

FIG. 2. QRE-2 is essential for Qp function in the context of proximal and distal DNA sequence information. The contribution of QRE-2 was evaluated in the context of downstream sequence information extending to +75 (as in Fig. 1A) and to +5068 within the second exon (U) of the EBNA-1 mRNA, as well as in the absence of Fp promoter elements in constructs extending to −143 relative to the Qp start site. Reporter gene expression was monitored by Northern blot analysis with an hGH cDNA probe to ensure that expression was consistent with Qp activation (the matched EBV splice donor site at +36 and acceptor site at +5055 in the longer constructs should result in Qp-specific mRNAs indistinguishable in size from those generated from the shorter constructs, which lack an acceptor site). Constructs lacking QRE-2 (−) contain the LS2 mutation; the plasmid used in the control transfection (p0GH) is the base reporter plasmid which contains a promoterless hGH gene. NA, not applicable.

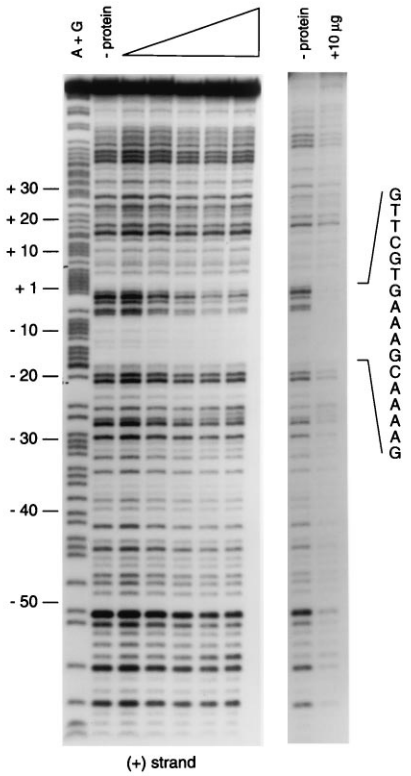

FIG. 3. DNase I footprint analysis of Qp. Analysis of protein binding to the region encompassing QRE-2 was performed by DNase I digestion of a ^{32}P-labeled DNA fragment (−89 to +75) in the absence or presence of unfractionated Louckes cell nuclear extract. Shown are two representative autoradiograms; in the gel depicted on the left, an increasing amount of protein (1 to 10 μg), indicated by the triangle, was included in the binding reaction. The product of a chemical sequencing reaction (A+G) of the purified probe fragment is presented as a reference; numbers indicate nucleotide positions relative to the transcription start site (+1). The nucleotide sequence contained within the footprint is indicated to the right.

absence of these elements (15), the current data indicate that they can contribute significantly to Qp activity.

As demonstrated in Fig. 1B (LS2 and LS3), the most dramatic reduction of Qp activity was observed when the sequence between −16 and −1 (QRE-2) was targeted by mutation, consistent with the result obtained previously in EBV-positive BL cells (15). We next determined whether QRE-2 was essential in the context of additional downstream sequence information (not present in the constructs illustrated in Fig. 1B), since this may contain regulatory elements, e.g., the E2F sites, capable of compensating for mutations in QRE-2. To do this, we evaluated the requirement for QRE-2 in the context of proximal (to +75) and distal (to +5068) sequence elements. Reporter gene expression in these experiments was also monitored by Northern blot analysis, in part to ensure that Qp was indeed the dominant promoter in the construct extending to +5068. Because the construct extending to +5068 contained the first splice donor (+36) and acceptor (+5055) sites of the EBNA-1 mRNA, Qp-mediated expression should result in an hGH mRNA indistinguishable in size from the mRNA generated from the construct extending to +75. As demonstrated in Fig. 2, this was indeed the case, and we found little difference in promoter activities, as indicated by the level of either hGH mRNA or protein (not shown) expressed, which would implicate additional positive elements downstream of +75. Most importantly, QRE-2 was essential regardless of the extent of downstream sequence information present in the constructs. Furthermore, none of the promoter activity detected could be attributed to Fp, since constructs extending upstream to −143, which lack Fp, were at least as active as those extending to −681.

Analyses of protein-DNA interactions within the QRE-2 region of Qp by DNase I footprinting with crude nuclear protein extracts of Louckes cells consistently revealed a light footprint between −17 and −1 (Fig. 3). Although DNase I cleaved DNA poorly in the absence of protein within the sequence between −15 to −7 (AAACGAAAG), making it difficult to determine if these bases are truly within the footprint, the results of additional DNA-binding studies presented below confirm that they are involved in binding. This footprint coincides precisely with our functional mapping of QRE-2 by the linker-scanning mutations LS2 and LS3.

The DNA-binding properties of the QRE-2-binding protein are distinct from those of TFIID. The absence of an apparent TATA box upstream of the Qp transcription start site (Fig. 1, bottom) suggests that QRE-2 may function as an Inr element. In general, known Inr-binding proteins recognize a rather loosely defined pyrimidine-rich sequence element ($PyPyA^{+1}$ NA/TPyPy) that overlaps or lies immediately adjacent to the transcription start site (22, 38, 44). These elements are often present in TATA-containing promoters as well as those that lack a canonical TATA box. Because such an Inr element is absent within Qp, our initial presumption was that QRE-2 acts as a low-affinity but functional binding site for TBP, which can recognize core elements of some TATA-less promoters that often bear little resemblance to a consensus TATA box (40). However, using an EMSA with purified rTBP and double-stranded oligonucleotides containing either a consensus TATA element or QRE-2, we were unable to detect a specific interaction between rTBP and QRE-2. Notably, an oligonucleotide containing QRE-2 did not compete with the consensus TATA element for rTBP binding (Fig. 4A), and although we did detect binding of rTBP to QRE-2 (Fig. 4B), this binding was nonspecific, as indicated by the inability of oligonucleotides containing either QRE-2 or the consensus TATA to compete with the QRE-2 probe for rTBP binding. We also did not observe binding of rTBP to QRE-2 in DNase I footprinting assays (data not shown). The inability to detect a specific interaction between TBP and QRE-2, in conjunction with the absence of a TATA box upstream of the Qp transcription start site, suggests that QRE-2 functions as an Inr element to enable

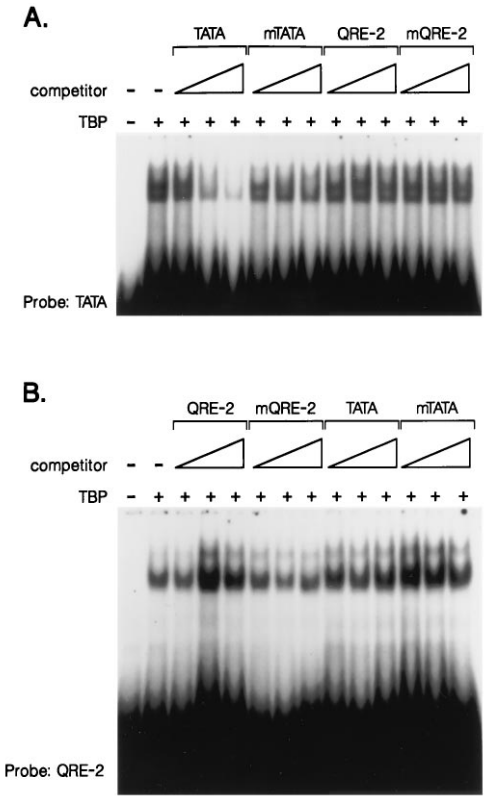

FIG. 4. QRE-2 is not a direct binding site for TBP. EMSAs were performed with rTBP (10 ng) and a double-stranded oligonucleotide probe containing either a consensus TATA box (A) or QRE-2 (B). Competitor oligonucleotides containing the TATA or QRE-2 element or mutations thereof were present in 10-, 50-, and 100-fold molar excess of the probe.

assembly of the TFIID complex at the Qp transcription start site.

Characterization of the QRE-2-binding protein(s). The absolute requirement for QRE-2 in conjunction with its position relative to the transcription start site supports a central role for QRE-2 and its binding protein(s) in the activation of Qp-mediated transcription. Furthermore, because the EBNA-1 binding sites are adjacent to QRE-2, autoregulation of Qp is also likely to be mediated by a direct effect on QRE-2 function. Addressing the mechanisms through which QRE-2 contributes to Qp activation and regulation, however, would first require the identification and a basic characterization of the protein(s) that interacts with QRE-2. Since the nucleotide sequence of QRE-2 did not directly implicate a known transcription factor, we proceeded to purify the QRE-2-binding proteins.

Analysis by EMSA of protein-DNA complexes generated in vitro with a QRE-2-containing oligonucleotide and crude nuclear extracts of Loucke cells consistently revealed three complexes (A, B, and C_1 [Fig. 5A]). Virtually identical results were obtained with extracts of EBV-positive BL cells maintaining either a type I or type III latency (not shown), indicating that neither of these complexes is dependent on EBV infection or is type I latency specific. The generation of complexes A and B, unlike that of complex C_1, however, could be readily inhibited by including non-QRE-2 competitor oligonucleotides in the binding reaction (data not shown), suggesting that formation of these complexes is not QRE-2 specific. We sometimes observe a fourth and faster-migrating complex, C_2 (Fig. 5B). Since C_1 and C_2 represent proteins binding to the same DNA

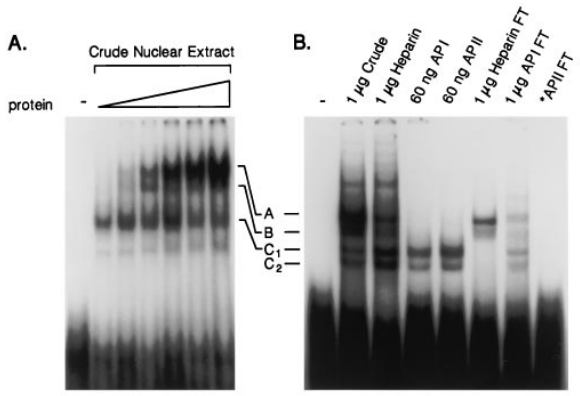

FIG. 5. Analysis of binding to QRE-2 by unfractionated and affinity-purified protein. (A) Analysis by EMSA of binding to the QRE-2 probe by proteins within a Loucke cell nuclear extract. (B) Binding to QRE-2 within the various protein fractions obtained during purification: heparin-Sepharose eluate (heparin), first affinity column eluate (API), second affinity column eluate (APII), and the various flowthrough (FT) fractions. *Insufficient protein was present in the APII FT fraction to enable quantitation.

element (demonstrated below), C_2 is most likely the result of partial proteolytic degradation of the same QRE-2-binding protein as in C_1. We therefore refer to these complexes as C_1 and C_2 to distinguish the slower- and faster-migrating complexes, respectively. Following purification of DNA-binding proteins by two passages through a QRE-2 affinity column, only the C complexes were detected by EMSA (Fig. 5B).

To confirm that the protein(s) that we had purified exhibited QRE-2-specific-binding properties, we performed EMSA analysis with various competitor oligonucleotides as shown in Fig. 6. Only two oligonucleotides competed for binding to the QRE-2 probe: the unlabeled probe itself, and a QRE-2 oligonucleotide that contains the LS3 mutation (as in Fig. 1) which targets bases downstream of the actual contact point(s) for QRE-2 binding (see below). Most notably, neither a QRE-2 oligonucleotide containing the LS2 mutation (which completely eliminates Qp activity) nor an oligonucleotide containing a consensus TATA box was capable of competing with the probe for complex formation. Identification of the protein contact points by methylation interference assay indicated that the purified protein binds to DNA within the sequence 5′-AAAC GAAA-3′ (Fig. 7). This element corresponds precisely to the sequence AACGAA, which when mutated eliminated Qp ac-

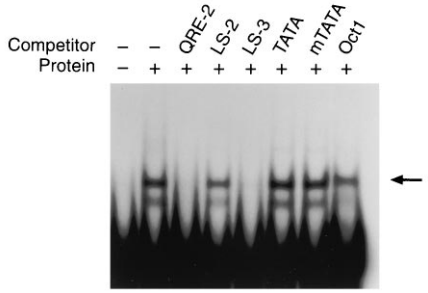

FIG. 6. EMSA analysis of the QRE-2-binding properties of affinity-purified protein. Binding reaction mixtures contained the QRE-2 oligonucleotide probe with or without affinity-purified protein (5 μl) in the absence or presence of a 100-fold excess of the indicated competitor oligonucleotide. The upper (arrow) and lower bands correspond to the C_1 and C_2 complexes, respectively, detected in Fig. 5.

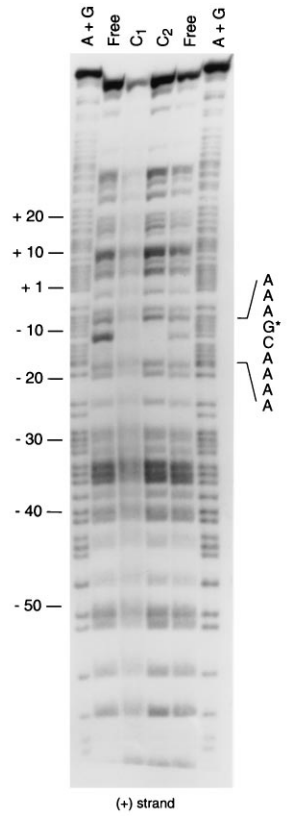

FIG. 7. Identification by methylation interference assay of the site bound by affinity-purified protein. The complexes corresponding to C_1 and C_2 (Fig. 5) were evaluated to confirm that the affinity-purified protein(s) recognized QRE-2. The G residue representing the contact point identified within both complexes is marked with an asterisk.

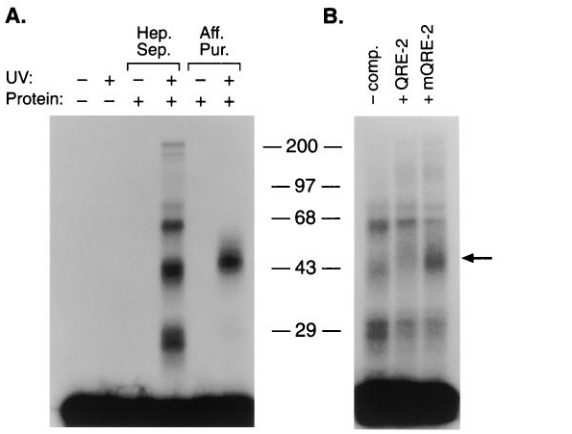

FIG. 8. Identification of the QRE-2-binding protein by UV cross-linking. (A) Protein from the heparin-Sepharose eluate or the affinity-purified fraction was labeled by UV cross-linking to a ^{32}P-labeled QRE-2 oligonucleotide followed by fractionation in an SDS–10% polyacrylamide gel. (B) Proteins from the heparin-Sepharose fraction were cross-linked in the absence of competitor or in the presence of a 100-fold excess of unlabeled specific (QRE-2) or nonspecific (mQRE-2) oligonucleotide competitor; mQRE-2 is the LS-2 oligonucleotide. The arrow indicates the protein that was specifically labeled. Numbers indicate the positions (kilodaltons) to which the molecular weight standards migrated in the gels.

tivity (LS2 [Fig. 1B]). Thus, the protein(s) that we had purified did indeed bind to the QRE-2 element as it had been functionally defined. Note also that the bases downstream of this element, which are targeted by the LS3 mutation, did not contain detectable contact points, consistent with the ability of the LS3-containing oligonucleotide to compete for QRE-2 binding (Fig. 6). Therefore, although bases targeted by the LS3 mutation contribute to Qp activation (Fig. 1B), they do not appear to be essential for protein binding to QRE-2.

QRE-2 is bound by a ~40-kDa protein. Examination of our affinity-purified protein fraction by silver staining following SDS-polyacrylamide gel electrophoresis revealed proteins with apparent molecular masses of 115, 66, and 40 kDa (not shown). To identify which protein(s) bound to QRE-2, we performed a series of UV cross-linking experiments. As demonstrated in Fig. 8A, we detected several QRE-2-bound proteins when using partially purified DNA-binding proteins (heparin-Sepharose-binding fraction). However, the only QRE-2-bound protein detected in our affinity-purified fraction was a protein that migrated slightly slower than the 43-kDa standard (Fig. 8A). Since it was unclear whether this was the same protein as a slightly faster-migrating protein detected in the partially purified fraction, we performed the cross-linking in the presence of competitor oligonucleotides. As demonstrated in Fig. 8B, the predominant protein labeled in the presence of a nonspecific competitor (mQRE-2), but whose labeling was not readily detected in the presence of the specific competitor, was a protein that comigrated with the protein detected in the affinity-purified fraction. Assuming that several kilodaltons in mass is contributed to the protein-DNA complex by the remaining cross-linked oligonucleotide, the predicted mass of this protein would be ~40 kDa, consistent with the 40-kDa protein observed by silver staining.

DISCUSSION

Qp is a relatively simple promoter. Previous analyses and work reported here suggest that the regulation of Qp is relatively simple in comparison to the majority of characterized EBV promoters (15, 35). For example, in addition to not requiring an EBV gene product for activation, Qp does not require B-cell-specific factors, consistent with its role in mediating EBNA-1 expression in both B lymphocytes and epithelial cells (and potentially other cell types), nor is its activation latency specific, since Qp reporter plasmids (lacking the EBNA-1 binding sites) are fully active in B-cell lines maintaining either a type I or type III latency (15, 24, 27, 35). Furthermore, unlike many class II promoters, Qp contains relatively few regulatory elements, and those that have been identified are proximal to the transcription start site. These include two major positive *cis*-regulatory elements, QRE-1 and QRE-2 (15), and the EBNA-1 binding sites immediately downstream of the transcription start site that function in *trans* with EBNA-1 to negatively regulate Qp function (24, 35).

The role of QRE-2 in Qp function. The absolute requirement for QRE-2 demonstrated here (Fig. 1 and 2), in conjunction with its defined position adjacent to the Qp transcription start site, indicates that QRE-2 is an essential core element of Qp. As such, QRE-2 likely plays a central role not only in the activation of Qp through cooperation with transactivating factors but also as a target for negative regulation mediated through the adjacent EBNA-1 binding sites. Several findings reported here provide insight to possible mechanisms of Qp regulation as controlled through QRE-2. Because Qp lacks a TATA box upstream of the transcription start site, assembly of the basal transcriptional machinery, most often initiated by TBP-mediated binding of the TFIID complex, may require an

alternative mechanism to direct assembly of TFIID, a function possibly mediated through QRE-2. Consistent with such a mechanism, we have demonstrated that neither QRE-2 nor surrounding DNA is specifically bound by TBP in vitro and that the QRE-2-binding protein has DNA-binding properties distinct from those of TBP. If QRE-2 does indeed compensate for the lack of a TBP binding site, this would suggest that QRE-2 functions as an Inr element (22, 44). Examination of QRE-2 and the surrounding DNA sequence, however, does not reveal a pyrimidine-rich sequence indicative of previously identified Inr elements. Thus, if QRE-2 does function as an Inr, it likely represents a novel class of these core promoter elements.

As a first step in addressing the function of QRE-2 in Qp regulation, we have begun to characterize the proteins which bind to this element. UV cross-linking assays using both partially purified and affinity-purified protein fractions suggest that this is a single protein of approximately 40 kDa. Most importantly, characterization of the DNA-binding properties of the purified protein fraction (Fig. 6 and 7) indicates that it does contain QRE-2-specific binding activity, suggesting that the 40-kDa protein is indeed the QRE-2-binding protein. The amount of QRE-2-binding protein that we obtained, unfortunately, was insufficient to permit amino acid sequence analysis to determine the identity of this protein or whether it is a novel factor as the QRE-2 sequence would suggest. Further attempts to identify this protein to support functional studies are in progress.

The contribution of E2F to Qp regulation. In addition to QRE-1 and QRE-2, two elements shown here to contribute to Qp activity are the E2F-like binding sites identified by Sung et al. (35). These sites, which overlap the EBNA-1 binding sites (Fig. 1), were previously excluded (unknowingly) from our reporter plasmids to allow mapping of positive elements in EBV-positive BL cells without interference from EBNA-1-mediated repression (15). Although lacking the three to four thymidine residues common to most previously characterized E2F binding sites (consensus, 5′-TTTTG/CG/CCGG/C-3′ [5]), these can be bound in vitro by E2F factors (35). Based on the results of in vitro DNA-binding assays suggesting that E2F-1 can exclude EBNA-1 from binding to its Qp regulatory domain, in conjunction with cotransfection studies suggesting that E2F-1 can reverse EBNA-1-mediated repression, Sung et al. concluded that E2F factors activate Qp by displacement of EBNA-1 from Qp (35). However, such a mechanism would seem unlikely, since under conditions in which the lower-affinity EBNA-1 binding sites are occupied, one would expect there to be sufficient occupation of the higher-affinity binding sites within *oriP* to enable DNA replication, and therefore no need for additional (E2F-mediated) expression of EBNA-1. Furthermore, these authors also observed the activation of Qp associated with overexpression of E2F-1 in EBV-negative keratinocyte cells (SVK), suggesting that E2F can activate independently of EBNA-1.

In an attempt to clarify the potential role of E2F factors in the regulation of Qp, we have shown, by mutation of the putative E2F response elements, that these sites can contribute positively to Qp activity, and in an EBNA-1-independent manner (Fig. 1A, mt 8 and 9). However, we caution that although our results are consistent with an E2F-mediated effect on Qp, they do not rule out the possibility that non-E2F factors are acting through these same elements. If E2F family members do contribute directly to the regulation of Qp, as the previous report suggests (35), then this raises the possibility that Qp is also subject to E2F-dependent negative regulation. This function of E2F is mediated by the E2F-associated proteins pRB and p107, which have transcriptional repressor activities when targeted to promoters by E2F/DP dimers (31, 34, 39). Such a mechanism is particularly attractive as a means to repress EBNA-1 expression in a resting B cell, for example, in which there would be no advantage to expressing this potentially immunogenic viral protein (12).

A model for the regulation of EBNA-1. Our current knowledge of the events that govern Qp function permit us to make several predictions with respect to the contribution of Qp to the EBV life cycle. The fact that Qp is functional when introduced into a variety of cell types (15, 35) suggests that it is constitutively active, much as a housekeeping gene would be, in the absence of its only known negative regulator, EBNA-1. Such a means of regulation is consistent with a fail-safe mechanism of expression to ensure that sufficient EBNA-1 is available in the cycling cell to mediate DNA replication. Most importantly, such a mechanism is compatible with the Qp activity observed, or the lack thereof, in the three known states of EBV latency. For example, in the absence of Cp- or Wp-mediated EBNA-1 expression during type I and II latency, Qp-mediated expression of EBNA-1 would automatically be activated if EBNA-1 levels were insufficient to occupy its low-affinity binding sites. During type III latency, the expression of EBNA-1 via Cp or Wp would not only supersede the need for Qp but repress it as well. This proposed repression by EBNA-1 is in fact supported by in vivo footprinting studies suggesting that most if not all Qp EBNA-1 binding sites are constitutively occupied by EBNA-1 within a cell during type III latency (9). Thus, during periods of B-cell proliferation in vivo, this simple but efficient mechanism of ensuring EBNA-1 expression via Qp would enable the virus to maintain the critical pool of infected B cells necessary to sustain the lifelong infection of its host.

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