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Interaction of the Lymphocyte-Derived Epstein-Barr Virus Nuclear Antigen EBNA-1 with Its DNA-Binding Sites

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The Epstein-Barr virus (EBV) nuclear antigen EBNA-1 plays an integral role in the maintenance of latency in EBV-infected B lymphocytes. EBNA-1 binds to sequences within the plasmid origin of replication (*oriP*). It is essential for the replication of the latent episomal form of EBV DNA and may also regulate the expression of the EBNA group of latency gene products. We have used sequence-specific DNA-binding assays to purify EBNA-1 away from nonspecific DNA-binding proteins in a B-lymphocyte cell extract. The availability of this eucaryotic protein has allowed an examination of the interaction of EBNA-1 with its specific DNA-binding sites and an evaluation of possible roles for the different binding loci within the EBV genome. DNA filter binding assays and DNase I footprinting experiments showed that the intact Raji EBNA-1 protein recognized the two binding site loci in *oriP* and the *Bam*HI-Q locus and no other sites in the EBV genome. Competition filter binding experiments with monomer and multimer region I consensus binding sites indicated that cooperative interactions between binding sites have relatively little impact on EBNA-1 binding to region I. An analysis of the binding parameters of the Raji EBNA-1 to the three naturally occurring binding loci revealed that the affinity of EBNA-1 for the three loci differed. The affinity for the sites in region I of *oriP* was greater than the affinity for the dyad symmetry sites (region II) of *oriP*, while the physically distant region III locus showed the lowest affinity. This arrangement may provide a mechanism whereby EBNA-1 can mediate differing regulatory functions through differential binding to its recognition sequence.

Epstein-Barr virus (EBV) infection of B-lymphoid cells results in the establishment of a latent infection in which the EBV genomes are maintained as nucleosome-covered episomes (15) and viral gene expression is restricted to a small subset of the nuclear and membrane-associated proteins (7, 8, 14, 21, 23). EBV infection also leads to immortalization of B cells and to polyclonal B-cell proliferation. These altered B-cell growth properties, in combination with other factors, e.g., chromosomal translocations, may lead to malignant B-cell disease such as Burkitt's lymphoma or B-cell lymphoma in immunosuppressed patients (24). EBV-carrying B cells can readily be established in culture, and these cell lines provide an accessible system for the analysis of mechanisms controlling the establishment and maintenance of the latent state of EBV.

Some of the key requirements for the episomal form of EBV DNA replication have been identified. Plasmids containing a DNA element (*oriP*) from EBV are maintained in an unintegrated state in cells if the viral protein EBNA-1 is concomitantly expressed in the cells, suggesting that EBNA-1 is the only viral product required for plasmid maintenance (36). *oriP*, the minimal *cis*-acting sequence necessary for the maintenance of multicopy plasmids, is located within an 1,800-base-pair (bp) segment (bases 7,334 to 9,519 of the EBV genome) and consists of two essential domains separated by approximately 1,000 bp (33, 35). The requirement for EBNA-1 proved to be mediated via sequence-specific DNA binding to the two domains of *oriP* (25). DNase I protection studies showed that region I of *oriP* contained 20 tandemly repeated binding sites for EBNA-1, while region II (the dyad symmetry region) contained 4 overlapping binding sites. An additional binding site locus (region III) was also identified in a separate region of the genome in *Bam*HI-Q (25).

In recombinant plasmid constructions the region I locus of *oriP* acts as an EBNA-1-dependent enhancer (28) and as such may modulate the expression from the two latency promoters that direct the expression of the EBNA family of genes. EBNA-1, thus, appears to be functionally pleotropic and, in addition to the initiation of episomal DNA synthesis, may be involved in controlling the copy number of EBV plasmids, segregation of the EBV genomes after replication, and autoregulation of its own transcription. The original demonstration that EBNA-1 was a DNA-binding protein utilized a bacterially synthesized 28,000-molecular-weight fusion protein, 28K EBNA, containing the carboxy-terminal one-third of EBNA-1. Subsequently, it was reported (27) that Raji EBNA-1 bound not only to the three loci identified by 28K EBNA, but also to additional sites within the EBV genome. Since interactions between EBNA-1 and the different DNA-binding loci form an integral part of the regulatory mechanisms controlling latency, it was important to clearly define the binding properties of lymphoblastoid cell-derived EBNA-1. In this study we report the isolation of EBNA-1 from Raji lymphoblastoid cells and characterization of the sequence-specific binding interaction of this native EBNA-1 protein with elements of *oriP* and with the third EBNA-1 binding locus in *Bam*HI-Q.

MATERIALS AND METHODS

Cells and media. The lymphoblastoid cell lines Ramos (RA 1), CA-46, P3HR-1, CCRF-SB, B95-8, and Raji were obtained from the American Type Culture Collection, Rockville, Md., and were maintained at 37°C with 5% CO_2 in RPMI 1640 medium supplemented with 10% fetal bovine serum. Before preparation of the protein extracts, larger

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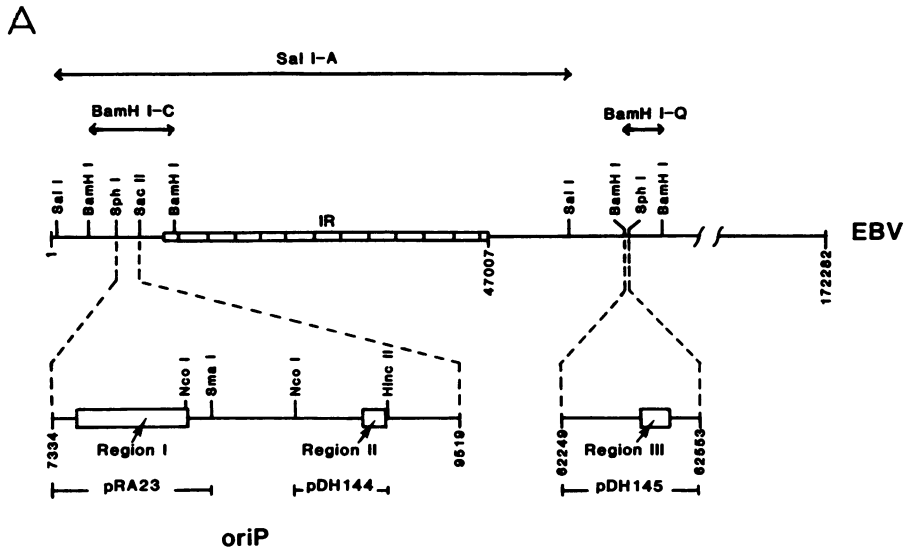

B

Consensus EBNA-1 Binding Site	Plasmid
5'GATCCAGATTAGGATAGCATATGCTACCCA	pGH66....1 Copy
GTCTAATCCTATCGTATACGATGGGTCTAG$_5$,	pGH65....2 Copies
	pBS4.....4 Copies

FIG. 1. (A) Location of the EBNA-1 binding sites in the EBV genome. The EBV sequences contained within the recombinant plasmids used in this study and the three distinct loci (regions I, II, and III) which contain EBNA-1 binding sites (25) are indicated. Base pair coordinates are from Baer et al. (1). (B) Sequence of the EBNA-1 consensus binding site oligonucleotide used in this study. The copy number in the different plasmids is also shown.

volumes (3 to 8 liters) of the cells were grown to densities of 8×10^5 to 10×10^5 cells per ml at 37°C in sealed Spinner culture bottles. The L-EBNA cells, a DNA-transfected mouse cell line constitutively expressing an integrated EBNA-1 gene (19), were grown in Eagle minimal essential medium at 37°C in 5% CO_2 with 10% fetal bovine serum. For preparative purposes the L-EBNA cells were propagated to confluence in 900-cm^2 roller bottles at 37°C.

Plasmids and DNA. Plasmids pRA23 and pDH144 (Fig. 1A) were subclones of the EBV(B95-8) *oriP* clone, pHEBO-1, which was a gift from B. Sugden (33). Plasmid pRA23, a gift from R. Ambinder, is a religation of the *Sma*I A fragment from pHEBO-1 and contains bases 7334 to 8187 of the B95-8 genome (1). Plasmid pDH144 was made by ligation of the 511-bp *Nco*I-*Hin*cII fragment (EBV bases 8622 to 9133) into pKP55, a 1,967-bp derivative of pBR322 kindly provided by K. Peden. The cosmid clone cm*Sal*-A contains the *Sal*I A fragment (EBV bases 62259 to 62553) from EBV(M-ABA) and was obtained from G. Bornkamm (22). Plasmid pDH145 was constructed by isolating a 497-bp *Sph*I fragment from a *Bam*HI-Q-containing plasmid (pSL17) and ligating this into the *Sph*I site of pGH56, a pUC18 derivative.

Synthetic oligonucleotides were made on an Applied Biosystems 380B Synthesizer by the Emory University Microchemical Facility or by the Johns Hopkins Nucleotide Synthesis Service. Complementary strands of the consensus EBNA-1 binding site (Fig. 1B) were produced with overhanging 5'-GATC ends, which facilitated the cloning of the duplexed strands and conserved the spacing of the tandem repeats of this sequence observed in region I of *oriP*. Complementary strands of the 49-base sequence TAAAA GAAGTGAGAACGCGAAGCGTTCGCACTTCGTCCCA ATATATATA were also synthesized and served as a control for the experiments in which the oligonucleotide probes were used.

Synthetic copies of the consensus EBNA-1 binding site were inserted into the plasmid vector pGH59, a 1,967-bp pBR322 derivative that had been originally designated pKP54 (from K. Peden) and was subsequently modified by the addition of a *Bgl*II linker in the multiple cloning region. Plasmids containing one (pGH66), two (pGH65), and four (pBS4) tandem copies of the binding site were constructed.

Plasmid and cosmid DNAs were isolated and purified by standard procedures, including two bandings on cesium chloride-ethidium bromide density gradients.

Preparation of protein extracts. All extraction procedures were carried out at 4°C. Harvested cells were washed with hypotonic buffer plus sucrose (25 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid [HEPES] [pH 7.5], 5 mM KCl, 1 mM dithiothreitol [DTT], 1 mM phenylmethylsulfonyl fluoride [PMSF], 1 mM EDTA, 10 mM $Na_2S_2O_5$, 10% sucrose) and then were allowed to swell in hypotonic buffer (no sucrose) for 30 min. The swollen cells were broken with 15 strokes in a tight-fitting Dounce homogenizer, and the nuclei were isolated by centrifugation for 10 min at 1,500 × *g*. The pelleted nuclei were lysed by the addition of an equal volume of high-salt extraction buffer (50 mM HEPES [pH 7.5], 2 mM DTT, 1 mM PMSF, 0.1 mM EDTA, 0.5 μg of aprotinin per ml, 0.5 μg of pepstatin A per ml, 0.5 μg of leupeptin per ml, 0.2 mM *N*-tosyl-L-phenylalanine chloromethyl ketone [TPCK], 10% sucrose, 3.6 M KCl), and the lysate was incubated for 30 min. Extraction of the nuclear

lysate was continued essentially as described by Parker and Topol (20) and modified by Heberlein et al. (10) except that the final dialysis was against heparin-agarose chromatography buffer (HAG buffer) consisting of 25 mM HEPES (pH 7.5), 1 mM DTT, 0.1 mM EDTA, 5 mM $Na_2S_2O_5$, 0.5 mM PMSF, 0.05 μg of leupeptin per ml, 0.01% Nonidet P-40, and 20% glycerol with either 100 mM KCl or 125 mM NaCl. Crude extracts prepared in this manner were then fractionated by chromatography through heparin-agarose (Bio-Rad Laboratories, Richmond, Calif.). Nuclear extracts (1 to 2 mg of protein per ml of heparin-agarose) were applied to the columns, and the bound proteins were subsequently eluted with either discontinuous or continuous linear gradients of NaCl or of KCl in HAG buffer (0.35, 0.65, and 1.0 M [discontinuous] or 0.35 to 0.75 M [continuous]). Fractions containing sequence-specific DNA-binding activity were identified by a nitrocellulose filter binding assay.

Crude extracts were also prepared from *Escherichia coli* DH1 cells induced to express the 28K EBNA polypeptide from plasmid pNAK28 as described previously (19). The 28K EBNA protein was purified by chromatography on heparin-agarose as described above.

Nitrocellulose filter binding assay. The protein-mediated retention of specific radioactively labeled DNA fragments on nitrocellulose filters was used to detect and characterize EBNA-like DNA-binding activity in extracts. Specific and nonspecific DNA probes were prepared from plasmids or from chemically synthesized oligonucleotides. DNA probes were obtained from plasmid DNAs by digesting the plasmids with the appropriate restriction enzymes, labeling by filling in the ends of the fragments with the polymerase I Klenow fragment and α-^{32}P-labeled deoxynucleoside triphosphates, and purification of the appropriate fragments by electroelution from polyacrylamide gels. Synthetic oligonucleotide probes were prepared by annealing the complementary strands of the synthesized DNAs (described above), isolating the duplex DNA molecules by chromatography over hydroxylapatite (Bio-Rad Laboratories), and labeling with T4 polynucleotide kinase and [α-^{32}P]ATP. Standard binding reactions were carried out for 30 min at 22°C and contained 25 mM HEPES (pH 7.5), 1 mM DTT, 5 mM $MgCl_2$, 1 mg of bovine serum albumin per ml, 250 mM NaCl or 125 mM KCl, 3 to 5 fmol of ^{32}P-labeled DNA probe, and various amounts of either unlabeled sonicated salmon sperm DNA or poly(dI-dC) · poly(dI-dC) (Pharmacia, Inc., Piscataway, N.J.) in a total volume of 0.05 ml. The reactions were then filtered in a Minifold apparatus (Schleicher & Schuell, Inc., Keene, N.H.) through nitrocellulose that had been treated as described by McEntee et al. (16), and each sample was washed three times with 0.3 ml of wash buffer (25 mM HEPES [pH 7.5], 1 mM DTT, 5 mM $MgCl_2$, 250 mM NaCl or 125 mM KCl). The filters were then dried and the bound ^{32}P-DNA was determined by liquid scintillation counting. For the competition assays unlabeled, uncut plasmid DNAs were added to the binding reactions.

Western immunoblot assay. Protein fractions were resolved by electrophoresis on a discontinuous 10% sodium dodecyl sulfate (SDS)–polyacrylamide gel (13) and then electrophoretically transferred to nitrocellulose in 25 mM Tris hydrochloride (pH 8.3)–12 mM glycine–0.1% SDS–10% methanol at 70 V and 300 mA for 3 h, using a Mighty Small Transphor apparatus (Hoeffer Scientific Instruments, San Francisco, Calif.). The filter was blocked for 16 h with3% gelatin in TBS (20 mM Tris hydrochloride [pH 7.5], 0.5 M NaCl, 10 U of heparin sulfate per ml) and then washed in TTBS (TBS with 0.3% Tween 20) for 15 min with three buffer changes. The filter was incubated for 2 h with nasopharyngeal carcinoma serum no. 000506 (kindly provided by Susan Spring, National Cancer Institute, Bethesda, Md.) at a 1:200 dilution in TTBS–1% gelatin and then washed with TTBS (three times, 5 min each time). The filter was developed for 16 h with gold-conjugated goat anti-human antibody (Bio-Rad Laboratories) in TBS with 0.2% bovine serum albumin–0.05% Tween 20–0.02% NaN_3–0.4% gelatin. The stained membrane was washed and enhanced by silver staining, as suggested by the manufacturer (Bio-Rad Laboratories). Prestained molecular weight standards (not shown) were used to establish the approximate molecular weights of any immunoreactive proteins.

DNase I footprinting assay. DNA fragments containing two tandem EBNA-1 consensus binding sites were isolated from pGH65 (Fig. 1B) and labeled at one end by using the large fragment of polymerase I and α-^{32}P-deoxynucleoside triphosphates. The fragments were then used for DNase I footprint analysis, performed as described previously (25), and the products of the assay were analyzed by autoradiography after electrophoresis on a denaturing polyacrylamide gel.

Immunoprecipitation of DNA-protein complexes. DNA-protein complexes were immunoprecipitated and analyzed essentially as described by McKay and DiMaio (17). Cosmid cm*Sal*-A, which contains the entire *oriP* region (Fig. 1A), was restricted with *Bam*HI and *Nco*I, and the fragments were ^{32}P labeled with the Klenow fragment of polymerase I. Standard binding reactions with 250 mM NaCl (described above) were carried out using the labeled cm*Sal*-A fragments and protein extracts from *E. coli* expressing the 28K EBNA fragment or from Raji cells. The volumes of the binding reactions were then increased to 0.2 ml by the addition of 0.15 ml of immunoprecipitation buffer (25 mM HEPES [pH 7.5], 125 mM NaCl, 0.05% Nonidet P-40, 0.1 mM PMSF, 4 μg of poly(dI-dC) per ml, 8 μg of tRNA per ml), and 1 μl of rabbit anti-28K EBNA or 1 μl of preimmunization serum from the same animal was added and incubation was continued at 4°C for 16 h. Immune complexes were precipitated by the addition of 25 μl of protein A-Sepharose (Pharmacia, Inc.) equilibrated in immunoprecipitation buffer. The mixture was incubated for 2 h at 0°C, and then the precipitate was washed five times with 0.5 ml of immunoprecipitation buffer. The washed beads were suspended in 50 μl of electrophoresis buffer (0.2% SDS, 10% glycerol, 0.025% bromphenol blue), heated for 1 min at 65°C, and then electrophoresed in an 0.8% agarose gel in 40 mM Tris acetate (pH 8.3)–2 mM EDTA–0.1% SDS. The gel was dried and autoradiographed.

RESULTS

Detection of EBNA-1 consensus site binding activity in extracts from mammalian cells. Nuclei from representative cell lines that had previously been shown by immunological methods to be either positive or negative for the EBNA-1 antigen were lysed by the addition of high molar salt solutions and the extracted proteins were precipitated with ammonium sulfate. The high-salt extracts were then fractionated by chromatography over heparin-agarose. Proteins bound to the heparin-agarose were eluted in a stepwise fashion with increasing concentrations of NaCl (0.35, 0.65, and 1.0 M), and each step was assayed for the presence of a DNA-binding activity capable of interacting specifically with the EBNA-1 consensus binding sequences. Nitrocellulose filter binding assays demonstrated that the specific binding

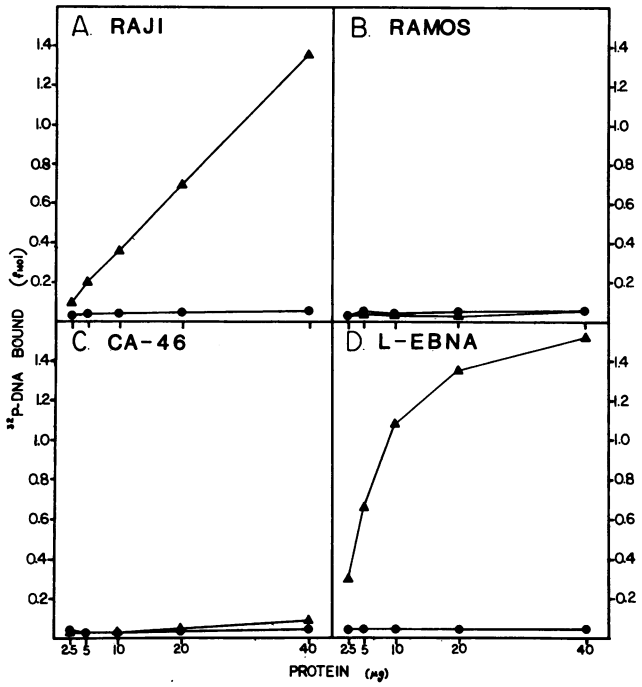

FIG. 2. Detection of specific binding to the consensus oligonucleotide in nuclear extracts of EBV-positive cells. Nitrocellulose filter binding assays were performed using identically prepared extracts from Raji (A), Ramos (B), CA-46 (C), and L-EBNA (D) cell lines. Nuclear extracts were prepared as described in Materials and Methods. The 0.65 M fraction from a heparin-agarose column was assayed for binding activity against a ^{32}P-labeled double-stranded oligonucleotide (30 bp) representing the EBNA-1 consensus binding site (▲) or a (49-bp) control DNA oligonucleotide (●). The reactions contained 25 μg of unlabeled sonicated salmon sperm DNA per ml.

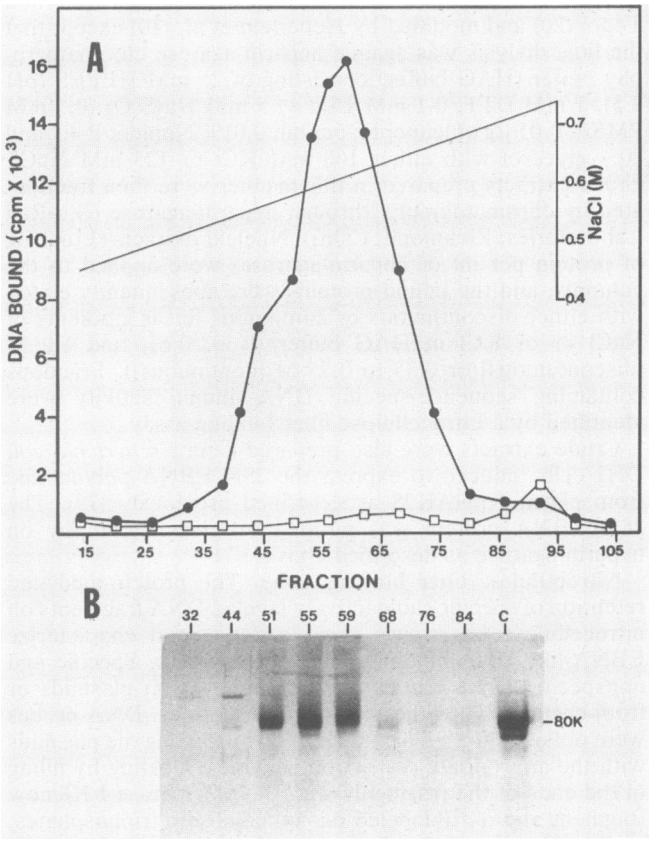

FIG. 3. Fractionation of Raji nuclear extract by heparin-agarose chromatography. Raji nuclear extract prepared as described in Materials and Methods was loaded onto a heparin-agarose column in HAG buffer containing 350 mM NaCl. The proteins retained on the column were eluted with a linear gradient between 350 and 750 mM NaCl. (A) Detection of sequence-specific DNA-binding activity. DNA-binding activity was detected by performing parallel nitrocellulose filter binding assays on the eluted fractions, using ^{32}P-labeled DNA fragments containing either two EBNA-1 consensus binding sites from pGH65 (●; specific probe, 93 bp) or sequences derived from plasmid vector pGH59 (□; nonspecific probe, 121 bp). The reactions also contained 0.8 μg of poly(dI-dC) · poly(dI-dC) per ml. (B) Immunological detection of EBNA-1. Representative fractions from the heparin-agarose gradient were analyzed by Western immunoblot analysis for the presence of proteins that were reactive with human NPC serum containing antibodies directed against EBNA-1. The numbers above the lanes indicate the gradient fraction from which the sample was taken. Lane C contained a sample of an unfractionated protein extract.

activity, when present, could be found in the 0.65 M NaCl heparin-agarose fraction (data not shown). Figure 2 shows the dose-response curves generated by four of the cell types. Specific binding was readily detected in all EBNA-1-positive cell lines tested, including the Raji and L-EBNA cells shown here. Raji is an EBV-positive Burkitt's lymphoma-derived human lymphoblastoid cell line which expresses EBV latency gene products, while the only viral protein synthesized by the L-EBNA cell line is EBNA-1. This mouse L-cell line was established by transfection with SV2-neo and a plasmid, pGD5, containing the EBV(P3HR-1) *Bam*HI K DNA fragment which encodes EBNA-1. The demonstration of binding activity directed specifically against the EBNA-1 consensus site in extracts prepared from these latter cells suggests that the protein-DNA interactions were mediated by the EBNA-1 gene product. Furthermore, the observation that L-EBNA cells contained higher levels of specific binding activity than did Raji cells (Fig. 2) is consistent with immunological data indicating a higher expression of the EBNA-1 protein in the converted mouse cell line (19). Other cell lines with detectable binding activity included P3HR-1, a Burkitt's lymphoma-derived human line; CCRF-SB, a human B-lymphoblastoid line obtained from an individual with acute lymphoblastic leukemia; and B95-8, a productively infected marmoset cell line (not shown). With the filter binding assay, detectable levels of consensus site-specific binding were not found in the EBV-negative Burkitt's lymphoma line Ramos or CA-46 (Fig. 2) or in HeLa or Vero cells (not shown).

Preparation of an EBNA-1 extract from Raji cells. To facilitate further studies of the sequence-specific binding activity detected in nuclear extracts prepared from Raji cells, the extracts were further purified by elution from heparin-agarose with linear salt gradients of either KCl (0.25 to 0.50 M) or NaCl (0.35 to 0.65 M). The elution of specific binding activity was followed by performing parallel filter binding assays on the eluted fractions. In Fig. 3A a typical activity profile for the NaCl eluant from the column is presented. When the columns were developed in KCl, the peak of binding activity appeared in fractions containing 0.30 to 0.40 M KCl. The activity was found in the 0.45 to 0.55 M fractions when NaCl was applied as the counterion. Immunologically detectable EBNA was shown to copurify with the specific DNA-binding activity by Western immunoblotting (Fig. 3B). Representative fractions from the linear salt

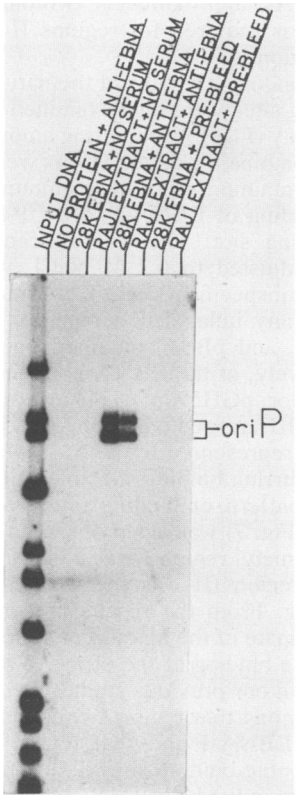

FIG. 4. Identification of DNA-binding activities by immunoassay. DNA-binding reactions were immunoprecipitated with rabbit antiserum directed against the bacterially synthesized 28K EBNA fragment or with preimmunization serum from the same animal. The probe used in the binding reactions was a ^{32}P-labeled *Bam*HI-*Nco*I digest of cm*Sal*-A (Fig. 1A). The fragments containing *oriP* are indicated.

gradient (Fig. 3A) were electrophoresed on discontinuous SDS-polyacrylamide gels, transferred to nitrocellulose, and probed with anti-EBNA-positive human serum. The dominant immunoreactive protein appeared as an 80K polypeptide (compared with prestained molecular weight standards [not shown], which is slightly larger than the size usually reported for EBNA-1 extracted from Raji cells (9, 11, 32).

Although it seemed highly unlikely that the Raji cell binding activity would not be EBNA-1, the fractionation procedures applied to the extracts thus far do not yield a homogeneous protein preparation. To further characterize the activity, we immunoprecipitated the products of the DNA-binding reactions with rabbit antiserum directed against the 28K EBNA fragment (19). Binding reactions were prepared that included the bacterial or mammalian protein extracts and a ^{32}P-labeled *Bam*HI-*Nco*I digest of cm*Sal*-A which contains regions I and II of *oriP* (Fig. 1). The binding reactions were immunoprecipitated by the addition of anti-EBNA-1 rabbit serum and removal of the immune complexes with protein A-Sepharose. The immunoprecipitated products were resolved on a 0.75% agarose gel containing 0.1% SDS. The *oriP*-specific binding activity can be precipitated from the reactions containing the bacterial or the Raji proteins with the anti-EBNA-1 antibody (Fig. 4). No DNA-binding activity was evident in the controls, including those employing preimmunization serum.

Detection of EBNA-1 binding loci in the EBV genome. It had been reported that EBV-specific DNA-binding interactions distinct from those observed with 28K EBNA could be mediated by Raji EBNA-1 (27). To address this question, an EBV DNA cosmid library containing overlapping DNA fragments representing the EBV genome (22) was assayed for the presence of Raji EBNA-1 interactive loci, using a pool of the EBNA-1-positive fractions generated by heparin-agarose chromatography. End-labeled DNA fragments from each cosmid were used as DNA probes in standard binding assays, and the binding reactions were filtered through nitrocellulose. The DNA fragments retained on the filters were then eluted from the nitrocellulose and analyzed by agarose gel electrophoresis (25).

Under standard binding conditions five DNA fragments from the EBV genome were retained on the nitrocellulose filters in the presence of the Raji extract (Fig. 5). The fragments bound by EBNA-1 were the two DNA fragments from cmSal-A and the single fragment from cmB14. The 28K EBNA protein also selectively bound these three DNA fragments from the EBV cosmid library (25). The two additional DNA fragments that were detected in this assay, EBV *Bam*HI-E (cm302-23) and EBV *Bam*HI-b (cmSal-B and cm302-21), resulted from the sequence-specific binding of cellular proteins to DNA sequence elements within those regions of the EBV genome. The two cellular proteins do not copurify with EBNA-1 or with each other, and their molecular size and sequence binding specificities have been determined (D. Rawlins, unpublished observations). The cellular proteins were detected in this assay because of the pooling of the EBNA-1-containing fractions and are not present in the peak fractions of EBNA-1 used in subsequent experiments.

Comparison of the specific DNA binding mediated by 28K EBNA and Raji EBNA-1. DNase I footprinting was used to compare the DNA-binding specificity of Raji EBNA-1 with that of a similarly prepared extract of the 28K EBNA fusion protein from bacteria (Fig. 6). A DNA fragment containing two tandem consensus EBNA-1 binding sites was labeled at a single 3′ terminus with the Klenow fragment of DNA polymerase I and served as the probe in the protection assays. In Fig. 6, protection of the tandem binding sites by the bacterially synthesized EBNA fragment and by the Raji extract is clearly evident. As indicated by the bracketed sequences listed to the left of the footprint, the protection patterns exhibited by the extracts were remarkably similar despite the expected biochemical and physical differences between the 28K EBNA and the approximately 72- to 80-kilodalton native viral protein. In this investigation and in our previous studies (25, 26), binding of the 28K EBNA to tandem binding sites led to the appearance of DNase I-hypersensitive sites both between binding sites and at the 3′ edge. However, these hypersensitive sites were not produced by the protein extracts from Raji cells, even though the extent of overall protection from DNase cleavage was equivalent.

Interaction of Raji EBNA-1 with the three binding loci from the EBV genome. As described above, a survey of an EBV DNA cosmid library identified three specific binding loci for the Raji EBNA-1 protein. The three loci included the two *cis*-acting elements of *oriP* (regions I and II) and a locus in the EBV *Bam*HI Q fragment (region III) (Fig. 1A). Since EBNA-1 interactions at the different DNA loci may form part of the regulatory mechanisms involved in the plasmid maintenance functions of EBV and possibly also in the regulation of nuclear latency gene expression, the binding of Raji EBNA-1 to regions I, II, and III was investigated.

In Fig. 7 the binding profiles obtained by the interaction of increasing amounts of Raji EBNA-1 with DNA fragments

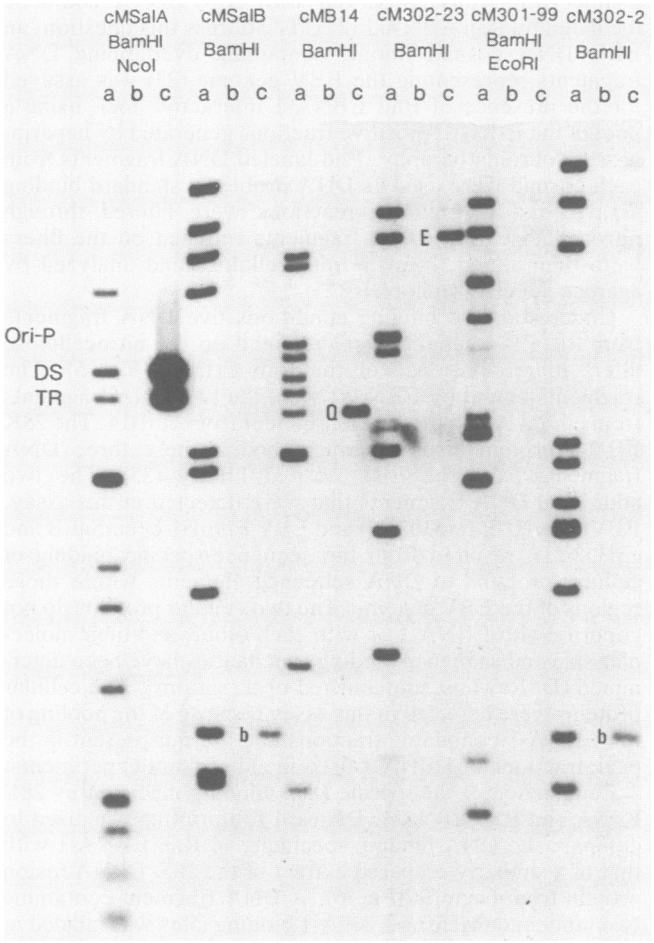

FIG. 5. Survey of an EBV cosmid library for the presence of Raji EBNA-1 binding sites by a DNA filter binding assay. A pooled fraction of heparin-agarose-separated Raji extract was incubated with radiolabeled cloned fragments of EBV(M-ABA) DNA encompassing the entire EBV genome, and binding was assayed as described previously (25). The cosmid name and the restriction enzyme used to generate the DNA fragments are listed above each set of lanes. Lanes contain a sample of the input DNA (a), the DNA retained on the nitrocellulose filter in the absence of Raji extract (b), and the DNA retained in the presence of Raji EBNA-1 extract (c). The three EBNA-1 binding loci, tandem repeats (TR), dyad symmetry (DS), and region III (Q), are indicated. The retained DNA fragments indicated by E and b were bound by cellular proteins and not by EBNA-1 (see Results).

containing either region I, region II, or region III are presented. The region I probe, pRA23 (796 bp), and the region II probe, pDH144 (540 bp), were derived from subclones of pHEBO-1 (Fig. 1A) and contain either 20 tandem binding sites (region I) or 4 partially overlapping binding sites (region II). The region III probe, pDH145, (320 bp) was derived from a subclone of the *Bam*HI Q fragment of EBV and contains two potential binding sites. The different sizes of the probes should not have significantly influenced the binding since under the conditions of this assay, the protein exhibits no affinity for nonspecific DNA sequences. Equimolar amounts of each ^{35}S-labeled DNA fragment were incubated in the binding reactions and the percentage of the DNA bound was determined after filtering the protein-DNA complexes through nitrocellulose. To achieve 50% binding of the input DNAs, approximately twofold and fourfold as much protein was required for regions II and III, respectively, as for region I.

The relative binding strengths of the natural and synthetic EBNA-1 binding sites were also examined in a competition filter binding assay (Fig. 8). Increasing amounts of unlabeled supercoiled recombinant plasmid DNAs were added to binding reactions containing a constant amount of EBNA-1 to compete for binding of EBNA-1 to a ^{32}P-labeled EBNA-1 consensus binding site. The salt concentration in these reactions was adjusted to 0.3 M NaCl, which effectively eliminated any nonspecific protein-DNA interactions even in the absence of any unlabeled competitor DNA. Plasmids pGH66, pGH65, and pBS4 contained one, two, and four copies, respectively, of the EBNA-1 consensus binding site in a plasmid vector, pGH59. pDH144 and pDH145 contained regions II and III, respectively, in pGH59. The region I competitor was represented by pRA23, which contains the 20 naturally occurring binding sites of region I (Fig. 1).

The general pattern of binding observed with the filter binding assays (Fig. 7) was again observed with the competition assays; namely, region I was a better competitor than region II, and region III was the weakest competitor for EBNA-1 binding. From the results presented in Fig. 8, a quantitative estimate of the amount of competitor DNA that it takes to reduce binding of the probe to the 50% level can be determined. In our previous studies we demonstrated by DNase I footprinting that region I contains 20 tandem sites for binding by EBNA-1 and that region II consists of 4 partially overlapping binding sites (25). If the protein-DNA interactions at the individual sites were quantitatively equivalent, a 5- to 10-fold difference in the competing strengths of regions I and II would be predicted. However, to achieve the 50% level of binding, the concentrations of regions I, II, and III in the assays were 0.013, 0.600, and >3.50 nM, respectively. In other words, in molar amounts, 45-fold less region I DNA was required than region II DNA to compete against the consensus binding site for binding to EBNA-1. These results suggest either that the individual binding sites are not equivalent or that binding of EBNA-1 to the *cis*-acting elements of *oriP* and to the *Bam*HI-Q locus involves more than simple independent additions of protein molecules to binding sites.

An indication that the primary DNA sequence of the binding site makes an important contribution to the relative affinity for EBNA-1 is provided by the observation that pGH66, which contains a single region I consensus binding site, was as effective as region II and much stronger than region III in the competition studies (Fig. 8). Cooperativity between binding sites was not readily apparent when plasmids containing two (pGH65) and four (pBS4) copies of the consensus binding site were used as competing DNAs. Compared with pGH66, their competing strengths were 2.8 and 4.8, respectively. The binding sites in these plasmids have the same spatial arrangements as those found in region I. Region I (pRA23), containing the 20 naturally occurring binding sites, competed 37 times as well as pGH66 for EBNA-1 binding. When this value is compared with 2.8 times for the synthetic dimer binding site, it again suggests that cooperative interactions between binding sites have a relatively small impact on EBNA-1 binding.

DISCUSSION

One approach to the analysis of the molecular mechanisms controlling latency is to identify and characterize protein-

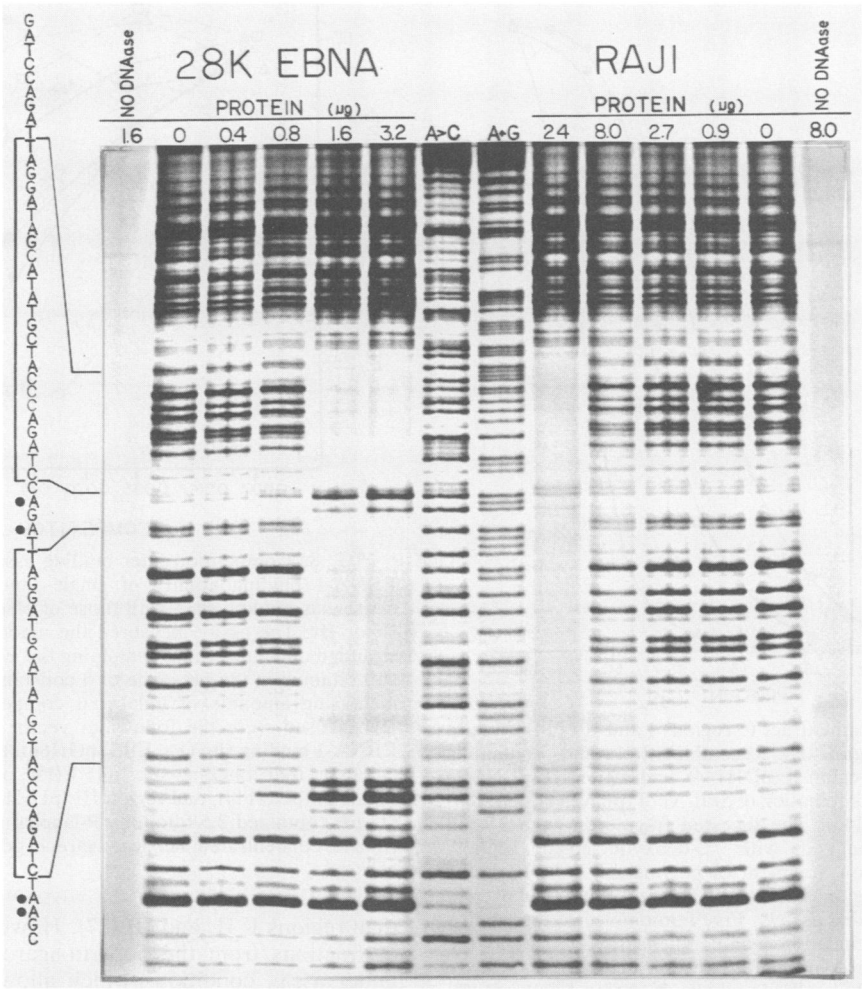

FIG. 6. DNase I footprinting analysis: protection of a dimer of the EBNA-1 consensus binding site by the bacterially synthesized 28K EBNA fragment or by a heparin-agarose gradient fraction from Raji cells. Protected regions of the DNA are indicated by the brackets around the sequence listed to the left of the footprint. DNase I-hypersensitive bases are marked by the closed circles. The DNA sequence ladders (lanes A>C and A+G) were generated by the modified chemical procedure (2).

DNA interactions that take place at DNA sequences known to have a regulatory role in gene expression or to be essential for the replication process. We have adopted this approach for studying the interaction of EBNA-1 with the plasmid maintenance system (*oriP*) that is integral to the establishment of latent infections in B lymphocytes infected by EBV. *oriP*-specific DNA binding by EBNA-1 was initially demonstrated by using a 28K fragment of EBNA-1 synthesized in bacteria. In this investigation we have characterized the interactions of the EBV EBNA-1 protein with *oriP* by using the entire EBNA-1 protein isolated from a latently infected human cell line.

Earlier attempts to purify EBNA-1 were hampered by the relatively small amounts of antigenically reactive protein present in latently infected cells (30) and by the lack of a sensitive assay with which to monitor the purification process. Our initial experiments were designed to determine whether binding to a consensus repeat unit of *oriP* could be utilized to unambiguously detect EBNA-1 in crude cellular extracts. We found that a nitrocellulose filter binding assay incorporating unlabeled nonspecific DNA and relatively high salt concentrations could readily detect EBNA-like binding activity in extracts from all EBV-carrying cells tested and also from transfected cells expressing the EBNA-1 gene. The ease of detection of the binding activity and the sensitivity of the assay most likely reflect the high specific binding affinity of the protein for its target DNA. By following the elution of this specific DNA-binding activity, a greater than 200-fold purification of EBNA-1 was achieved by repeated chromatographic separation of the nuclear extracts on heparin-agarose. Although EBNA-1 was not purified to homogeneity, no other DNA-binding activities were detected in the extract under stringent conditions and experiments directly confirmed that the activity being studied was indeed mediated by the EBNA-1 protein. The sequence-specific binding copurified with an appropriately sized polypeptide which reacted with EBNA-positive human NPC serum, and the *oriP* binding activity was immunoprecipitated by monospecific antiserum raised against purified 28K EBNA.

A qualitative comparison of the DNase I footprints generated by the 28K and Raji EBNAs revealed both similarities and differences in the protection patterns. Each of the proteins appeared to protect exactly the same bases of the consensus site, and on the DNA strand tested, the protection was directionally asymmetrical relative to the center of the core palindrome found within the binding site, with 10 bases

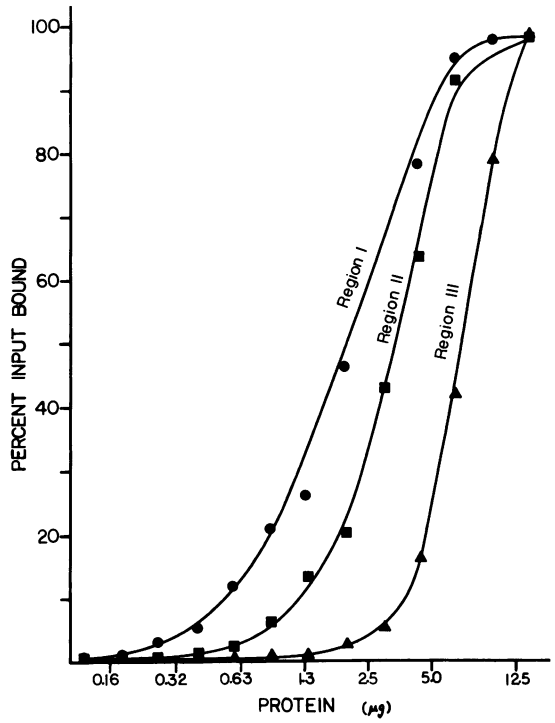

FIG. 7. Binding of Raji extract to regions I and II of *oriP* and to the *Bam*HI-Q locus (region III). Recombinant plasmids containing *oriP* region I (pRA23), region II (pDH144), and region III (pDH145) were restricted with a combination of *Sal*I-*Nco*I (pRA23 or pDH144) or *Bam*HI-*Bgl*II (pDH145). The digested fragments were 3′ end labeled by filling in the ends with ^{35}S-deoxynucleoside triphosphates, using the Klenow fragment of polymerase I. The labeled fragments containing regions I, II, and III were isolated and used in nitrocellulose filter binding assays. Five femtomoles of the labeled DNA fragments was included in each parallel assay, along with the indicated amounts of protein from a concentrated heparin-agarose gradient fraction from Raji cells. Symbols: ●, region I (796 bp); ■, region II (540 bp); ▲, region III (320 bp). The assays also included 25 μg of unlabeled sonicated salmon sperm DNA per ml.

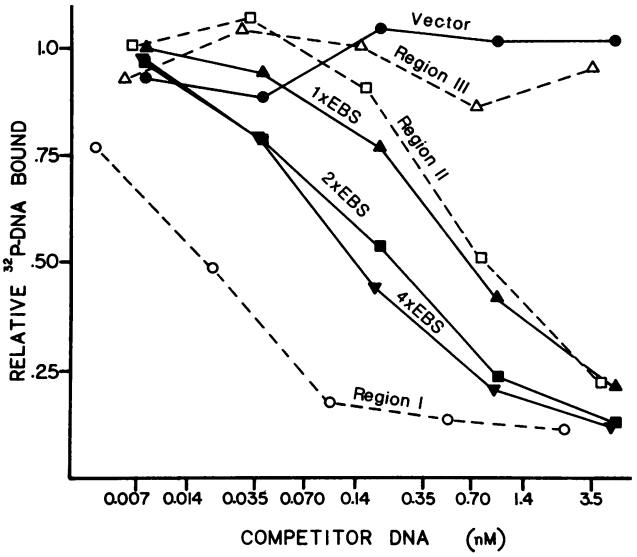

FIG. 8. Competition filter binding assay comparing the relative EBNA-1 binding affinity of single- and multiple-copy EBNA-1 consensus binding sites with those of plasmids containing region I, II, or III. The assay measured the amount of ^{32}P-labeled double-stranded oligonucleotide containing the EBNA-1 consensus binding site retained in the presence of a constant amount of EBNA-1 and increasing amounts of unlabeled competitor DNA. The plasmid DNAs used were the following: vector (pGH59), one consensus EBNA-1 binding site (1× EBS, pGH66), two binding sites (2×EBS, pGH65), four binding sites (4×EBS, pBS4), region I (pRA23), region II (pDH144), and region III (pDH145). The binding reactions (25 μl) contained 2.5 fmol of ^{32}P-labeled probe and 4 μg of protein from a concentrated heparin-agarose gradient fraction from Raji cells.

being protected 5′ to the AT center of symmetry and 14 bases being protected on the 3′ side. Asymmetrical binding is not uncommon under these conditions and may reflect the directional orientation of the individual EBNA binding sites. Although the orientation of the binding sites in the tandem repeat region appears to vary in *oriP* isolates from different strains of EBV (25), we do not know whether this has any functional significance in vivo. The presence of DNase I-hypersensitive sites 3′ to the regions protected by the 28K EBNA represented the major difference noted between the footprints produced by the bacterially synthesized and Raji EBNA-1. Although the difference in the sizes of the two polypeptides affords a reasonable explanation for the sterically limited access of the nuclease to the Raji EBNA-1-protected template, it does not explain the dramatic hypersensitivity found in the 28K EBNA-1 footprint. Again, this may indicate a characteristic of the DNA-protein interaction, in this case a change in the conformation of the bound DNA that is modified when the structure of the protein is intact. Consequently, for studies of conformation changes induced in the target sequences by EBNA-1 binding, use of the intact EBNA-1 protein may be essential. Recently, it was reported from electron microscopic studies that EBNA-1 from Raji cells bound specifically to regions of EBV other than regions I, II, and III (27). However, even when a pool of fractions from the heparin-agarose column was tested under assay conditions which allowed binding of cellular factors, the Raji EBNA-1 still bound only to the *oriP* and *Bam*HI-Q loci of the EBV genome. Earlier studies demonstrated that EBNA-1 would bind in a nonspecific manner (18, 30), and the significance of the low-stringency binding detected in the electron microscopic studies is unclear.

In both direct binding studies and competition assays we have observed a hierarchy of binding to regions I, II, and III; that is, region I has a higher relative binding affinity for EBNA-1 than does region II, and region III has the lowest affinity. The increased affinity of region I over region II is greater than would be predicted from the number of individual binding sites in each locus. In the competition assays the single synthetic binding site, which represents a consensus of the region I sites, competed more effectively than the region II locus, suggesting that the differences in affinity may reflect sequence variations found within the individual binding sites. Differences in the spatial arrangement of the sites may also contribute. The region I sites are centered 30 bp apart, while region II and region III sites are centered only 24 and 21 bp apart, respectively. Cooperativity between sites was not readily apparent when competition experiments were performed with plasmids containing one, two, and four copies of the concensus binding site.

The establishment and maintenance of latency by EBV obviously require a carefully regulated schedule of DNA metabolism and genomic segregation in vivo. Previously, we proposed a "sink and origin" model in which the initiation of plasmid replication would be controlled by the appropriate

interaction of EBNA-1 at the region II binding locus of *oriP* (25). Thus, the initiation of replication would depend upon the availability of active EBNA-1, a condition that could be modulated by sequestering the protein at the region I binding sites (the "sink") in a manner similar to that described for RepA protein-controlled replication of plasmid P1 (6). As shown here, the hierarchy of interaction of EBNA-1 with the three specific binding elements is fully compatible with this model. However, the sink and original model only addresses the regulation of replication and copy number of the latent EBV genome. The high-affinity binding of EBNA-1 to the region I binding sites may also provide a mechanism for chromosomal association and consequently segregation.

EBNA-1 appears to be functionally pleiotropic and, in addition to its role in the initiation of episomal DNA synthesis, may be involved in regulating the expression of the nuclear class of latency proteins. An analysis of cDNA clones has shown that EBNA-1 is transcribed as a highly spliced mRNA from latency promoters at position 11305 in *Bam*HI-C and position 14352 in *Bam*HI-W (5, 29, 31). The open reading frame encoding EBNA-1 lies some 90 kilobases away in the *Bam*HI K fragment (12, 34). The transcripts for EBNA-2, -3A, -3B, -3C, and -5 are similarly highly spliced and appear to be controlled from the same latency promoters as EBNA-1 (3, 4, 29, 31). Reisman and Sugden (28) have shown that in recombinant plasmid constructions, the region I locus of *oriP* acts as an EBNA-1-dependent enhancer. Although region I lies approximately 3 and 6 kilobases distant from the EBNA promoters, it would be within the operational range of an enhancer. Thus, in addition to the initiation of episomal DNA synthesis, EBNA-1 may function as an enhancer binding protein to positively regulate its own expression along with the expression of EBNA-2, -3A, -3B, and -3C and leader protein (also known as EBNA-4 or -5). Since overproduction of EBNA-1 could potentially result in an increased initiation of episomal DNA replication and loss of synchrony with cell division, it seems likely that EBNA-1 synthesis is strictly controlled. Binding of EBNA-1 to the region III sites in *Bam*HI-Q may provide a mechanism for an additional level of regulation. Our experiments show that EBNA-1 has a lower affinity for region III than for the loci in *oriP*. Thus, an EBNA-1 levels increase beyond that necessary to saturate *oriP*, binding would occur at region III. Since the primary EBNA-1 transcript passes through *Bam*HI-Q, one could postulate that an EBNA-1–region III complex might impede or block the progression of RNA polymerase, thus reducing levels of EBNA-1 mRNA. In this scenario, EBNA-1 would function as an enhancer binding protein to provide positive autoregulation through region I and as a transcriptional terminator to introduce negative autoregulation via region III. Although the exact function of the *Bam*HI-Q binding site remains to be determined, the availability of the EBNA-1 affinity data now provides a basis for testable experimental models.

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