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Interaction of Epstein-Barr Virus Nuclear Antigen 1 with the Viral Latent Origin of Replication

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The Epstein-Barr virus latent origin of replication (oriP) requires only one viral protein, the Epstein-Barr virus nuclear antigen 1 (EBNA-1), for activity. oriP consists of two spatially separated, essential sequence elements, regions I and II, both of which contain multiple EBNA-1-binding sites. Region II contains, or is close to, the site at which DNA synthesis initiates. The role of region I, a transcriptional enhancer in cells that express EBNA-1, in replication is not understood. To determine how the binding of EBNA-1 to sites in region II leads to the initiation of DNA synthesis and to investigate the role of region I, EBNA-1 has been overproduced in insect cells by using a baculovirus vector and purified to homogeneity, and the interaction of EBNA-1 with oriP has been examined. Footprinting experiments demonstrated that EBNA-1 binds to oriP in a sequence-specific manner and bends or untwists the DNA at two symmetry-related sites in region II. Distortion of region I by EBNA-1 was not detected, suggesting that differences in the spacing of binding sites in regions I and II and resulting protein-protein interactions underlie differences in their biological properties. $KMnO_4$ footprinting experiments did not reveal significant single-stranded structures in region II, suggesting that cellular proteins may recognize the EBNA-1–region II complex and unwind the DNA duplex. Region I did not quantitatively or qualitatively alter the interaction of EBNA-1 with region II. The contribution of an A+T-rich sequence in region II to replication was investigated by a mutational analysis. The results indicated that the overall A+T-rich nature of this sequence is not essential for replication of oriP-bearing plasmids. Nuclease protection experiments performed with these mutagenized plasmids provided additional evidence for protein-protein interactions in region II.

Infection of resting B cells with Epstein-Barr virus (EBV) in vitro results in the conversion of these quiescent cells into continuously proliferating lymphoblasts, a process termed B-cell immortalization (41). In most EBV-immortalized B cells the linear viral genome is circularized and maintained in a latent state as an extrachromosomal plasmid (41). During the first 25 to 30 cell generations following infection, the viral chromosome may be amplified, resulting in 5 to 500 EBV plasmids per cell (41). Thereafter, the replication of EBV chromosomes parallels the replication of the host cell genome. Each plasmid replicates once per cell cycle during the S phase, and the plasmid copy number remains constant for any given cell line (22, 41). These results suggest that the EBV chromosomes present in latently infected B cells respond to the same controls that regulate replication of the cellular chromosomes. Initially, synthesis occurs by the Cairns mode of replication (22). After proceeding ca. 1 kb, the leftward-moving replication fork stops and replication proceeds unidirectionally to the right (17).

Replication of latent EBV genomes initiates at a unique site termed the latent origin of plasmid replication, or oriP (61). Plasmids bearing oriP are maintained extrachromosomally in EBV genome-positive cells (38, 61) and in primate cells that express the EBV nuclear antigen 1 (EBNA-1 [38, 62]). This latter result revealed that only two components of the EBV genome, oriP and the EBNA-1 gene, are required for replication; all other gene products required for replication are provided by the host cell. Plasmids bearing oriP also replicate once per cell cycle in a semiconservative manner (64). Therefore, simple EBV replicons which may be easily manipulated provide an excellent model system for studying the regulated replication of DNA in animal cells.

oriP consists of two essential components, region I and region II, both of which contain EBNA-1-binding sites (38, 49, 51). Genetic and biochemical analyses have determined that oriP region II (also termed the dyad symmetry element [51]) contains, or is close to, the site at which DNA synthesis initiates (17, 60). Region II contains binding sites for four EBNA-1 dimers (1, 2, 49). However, all four binding sites are not required for origin activity. Plasmids that lack binding site 1 replicate, albeit less efficiently than plasmids containing an intact region II (11, 61). Deletions impinging upon binding sites 2 and 4 abrogate the ability of mutated plasmids to replicate extrachromosomally or result in abnormally large replication products of unknown structure (11). Region I, located ca. 1 kb from region II, is required for efficient transient replication in vivo (51, 60) and for the stable maintenance of plasmids in cell lines (38, 51). In addition, region I functions as a transcription enhancer element for RNA polymerase II-transcribed genes (50), allows for the nuclear retention of covalently linked sequences (34), and contains the termination site for EBV latent replication (17). All four activities of region I require EBNA-1 (16, 34, 50, 63). oriP region I is composed of 21 tandemly arrayed imperfect copies of a 30-bp repeat; 20 of these repeats contain the binding site for an EBNA-1 dimer (1, 49), and only 6 or 7 repeats are required for full replication and transcription enhancement activity (60).

To investigate how the binding of EBNA-1 to sequences in oriP leads to the initiation of DNA synthesis, we have overexpressed EBNA-1 in insect cells by using a baculovirus

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vector, purified the protein to homogeneity, and examined its interaction with oriP regions I and II. EBNA-1 produced in insect cells possessed sequence-specific DNA-binding activity and formed a nucleoprotein complex with oriP region II that is characterized by the bending or untwisting of the DNA duplex at two sites and the absence of significant single-stranded structures. Additionally, nuclease protection experiments provide evidence for protein-protein interactions between EBNA-1 dimers bound to nonadjacent sites in oriP region II. In contrast, EBNA-1 binding to the tandemly repeated sites in region I did not result in detectable distortion of the DNA duplex as measured by $KMnO_4$ footprinting. Thus, the specific alignment of EBNA-1-binding sites in region II and protein-protein interactions appear to effect alterations in the structure of this DNA. In addition, we present evidence that the binding of EBNA-1 to region I does not facilitate the binding of EBNA-1 to region II.

MATERIALS AND METHODS

Cells and plasmids. EBV-negative (Ramos [32]) and EBV-positive (Raji, IB4, and B95-8 [31, 43, 48]) lymphoblastoid cell lines were grown in RPMI 1640 containing 10% iron-supplemented calf serum (HyClone Laboratories, Inc., Logan, Utah). The human epithelial cell line D98 and the D98/Raji somatic cell hybrid (19) were grown in Dulbecco's modified Eagle's medium supplemented with 10% iron-supplemented calf serum. Insect Sf9 cells were grown in TNM-FH medium containing 10% fetal bovine serum (HyClone Laboratories, Inc.).

pHEBo-1 contains the entire EBV latent origin and the hygromycin resistance gene of *Escherichia coli* under the control of the herpes simplex virus thymidine kinase promoter (57). Sequences between the unique *Bam*HI and *Hin*dIII sites, derived from pBR322, were deleted to generate pHEBo-1.1. pRII was constructed by excision of the *Eco*RV fragment containing oriP region I from pHEBo-1. pRII(Sal) is a variant of pRII that was constructed by introducing a *Sal*I linker at EBV nucleotide 9132. pHEBo-1dlDS contains oriP region I but lacks the dyad symmetry element. It was derived from pHEBo-1 by digestion with *Sma*I and self-ligation of the largest *Sma*I fragment containing the ColE1 replication origin and oriP region I. Plasmids were propagated in *E. coli* DH1 cells (*dam*$^+$).

Expression of EBNA-1 in insect cells. A *Bam*HI-*Hin*dIII fragment containing the entire open reading frame of the EBNA-1 gene (B95-8 strain) was excised from plasmid pDF225 (12) and subcloned between the *Bam*HI and *Hin*dIII sites of pUC18 to generate pEBNA-1. pEBNA-1 was digested with *Hin*fI, and the ends were repaired with Klenow DNA polymerase. *Eco*RI linkers were ligated to the repaired ends, and the DNA was digested with *Eco*RI and *Nco*I. A 124-bp *Eco*RI-*Nco*I fragment containing the coding sequences for the amino terminus of EBNA-1 was isolated and ligated to the large *Nco*I-*Eco*RI fragment of pEBNA-1. The resulting plasmid, pEBNA-1.1, contains a unique *Eco*RI site just 5′ to the initiating ATG for the EBNA-1 open reading frame. EBNA-1 coding sequences were excised from pEBNA-1.1 with *Eco*RI and *Hin*dIII, and *Bgl*II linkers were added following repair of the ends with Klenow DNA polymerase. This fragment was introduced into the unique *Bam*HI site of the baculovirus (*Autographa californica* nuclear polyhedrosis virus [AcNPV]) transfer vector pVL941. pVL941 is a derivative of pAc311 that contains a G-to-T transversion in the initiating ATG of the polyhedrin open reading frame and a unique *Bam*HI site at +35 (relative to the former ATG [37]). The sequence of the junction between AcNPV and EBV sequences was determined by dideoxy sequence analysis (53). The relevant sequences of the resulting vector, pVL/EBNA-1, are as follows: 5′-TAAT *ATT* CCG CAT TAT TCA TAC CGT CCC ACC ATC GGG CGC GGATCTG AATTCC **AATC ATG TCT GAC GAG**.... .....3′. The underlined sequences represent nucleotides derived from the *Bam*HI-*Bgl*II junction and *Eco*RI linkers. The boldface nucleotides are derived from EBV DNA. The former ATG codon for the polyhedrin initiating methionine is indicated by italics.

The recombinant virus AcEBNA-1 was derived by in vivo recombination between purified AcNPV and pVL/EBNA-1 DNA following transfection of Sf9 cells. Occlusion-negative viruses resulting from the cotransfection were screened for their ability to induce EBNA-1 synthesis in Sf9 cells by immunoblot analysis with a polyclonal human serum containing antibodies to EBNA-1. A detailed description of the techniques used to generate and propagate AcEBNA-1 may be found in reference 58.

Purification of EBNA-1. EBNA-1 was purified from Sf9 spinner cultures 48 to 60 h postinfection with 10 PFU of AcEBNA-1 per cell by sequential immunoaffinity and sequence-specific DNA affinity chromatography. All purification steps were carried out at 4°C. Infected cells (0.5×10^8 to 5×10^8) were washed twice with phosphate-buffered saline and either reserved at −20°C for future use or lysed with 20 ml of Nonidet P-40 (NP-40) lysis buffer [0.5% NP-40, 50 mM Tris-HCl (pH 7.5), 0.2 mM phenylmethylsulfonyl fluoride, 100 mM NaCl, 5 mM EDTA, 5 mM ethylene glycol-bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid (EGTA), 0.01 mg of aprotinin per ml). The lysate was incubated on ice for 10 min, and the nuclei were broken by sonication and clarified by centrifugation for 30 min at 38,000 × *g*. The clarified extract was preadsorbed by passage through a 2-ml Sepharose CL-4B column and incubated for 16 to 18 h with gentle mixing with 2 ml of EBNA-1 immunoaffinity beads. The immunoaffinity beads were prepared with the anti-EBNA-1 monoclonal antibody Aza2 E8 (24) by one of two methods. The monoclonal antibody was purified from hybridoma cells cultured in serum-free medium (UltraDOMA; Whittaker Bioproducts, Inc., Walkersville, Md.) on Avid AL columns (BioProbe International, Inc., Tustin, Calif.) as described by the manufacturer. Sepharose CL-4B beads were activated with CNBr (23) and incubated overnight with 5 mg of Aza2 E8 per ml of beads to yield preparations with 4 mg of antibody per ml of beads. Alternatively, the Aza2 E8 antibody was coupled to protein A-Sepharose beads precoated with rabbit anti-mouse immunoglobulin G by using dimethylpimelimidate (54). The immunoaffinity beads were packed into a small column and washed with 20 to 40 ml of NP-40 lysis buffer followed by 20 to 40 ml of 20 mM *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid (HEPES)-KOH (pH 7.5). Bound protein(s) was eluted with 6.0 ml of 10 M LiCl–20 mM HEPES-KOH (pH 7.5) and dialyzed against DNA affinity column (DAC) buffer (20 mM HEPES-KOH [pH 7.5], 200 mM KCl, 5 mM $MgCl_2$, 1 mM EGTA, 0.5 mM dithiothreitol, 0.2 mM phenylmethylsulfonyl fluoride, 0.1% NP-40, 20% glycerol). Insoluble material was removed from the dialyzed eluate by centrifugation at 38,000 × *g* for 30 min.

EBNA-1 was further purified by chromatography on a 2-ml sequence-specific DAC (29). This column was prepared by ligating annealed complementary oligonucleotides containing a consensus EBNA-1-binding site (5′-GATCCA GATTAGGATAGCATATGCTACCCA-3′ and 5′-GATCTG

GGTAGCATATGCTATCCTAATCTG-3' [28]) to generate oligomers and coupling these to CNBr-activated Sepharose CL-4B (29). The immunoaffinity-purified EBNA-1 preparation was incubated for 15 min at room temperature with 0.01 mg of salmon sperm DNA per ml and subsequently incubated overnight at 4°C with 2 ml of DNA affinity resin with gentle mixing. The beads were packed into a small column, washed with 20 to 50 ml of DAC buffer and with 10 ml of DAC buffer containing 0.5 M NaCl and eluted with 6.0 ml of DAC buffer containing 3 M NaCl. The eluate was diluted with NaCl-free DAC buffer to achieve a final concentration of 100 mM NaCl, and EBNA-1 was concentrated by chromatography on a ca. 0.1-ml S-Sepharose column. EBNA-1 was eluted from the S-Sepharose with 400 μl of DAC buffer containing 0.6 M NaCl and stored at −70°C in this buffer. Some DAC eluates were dialyzed against DAC buffer containing 1 M KCl and stored frozen in that buffer.

SDS-gels, immunoblots, and silver staining. Protein concentrations were determined by the bicinchoninic acid assay (56) (Sigma Chemical Co., St. Louis, Mo.) with bovine serum albumin as the standard. Samples were prepared and electrophoresed on sodium dodecyl sulfate (SDS)–10% polyacrylamide gels as previously described (25). Proteins were transferred to nitrocellulose by using a semidry electroblotter, and the locations of molecular weight markers were determined by Ponceau S staining (23). Filters were dried under a heat lamp, rehydrated with water, and blocked with 6% nonfat dry milk in 10 mM Tris-HCl (pH 7.5)–0.9% NaCl (Tris-saline) for 30 min at room temperature. Filters were probed for 2 h at room temperature with a 0.1-ml/cm^2 volume of human serum containing anti-EBNA-1 antibodies diluted 1:100 in blocking buffer. Filters were washed once with Tris-saline, twice with Tris-saline plus 0.05% NP-40, and once with Tris-saline (10-min washes at room temperature). Bound antibodies were detected by incubating the washed filters for 1 h with 0.1 μCi of ^{125}I-protein A per ml (2 to 10 μCi/μg; ICN Radiochemicals, Irvine, Calif.). The filters were washed as described above, blotted dry, and exposed to X-ray film. SDS-gels were silver stained by the procedure of Merril et al. (42).

Nuclease protection assay. DNase I protection experiments were performed largely by the procedure of Gralla (21). Briefly, 30 μl-binding reaction mixtures contained 0.5 μg of linearized plasmid DNA (unless otherwise stated) and various amounts of purified EBNA-1 in a buffer containing 20 mM HEPES-KOH (pH 7.9), 5 mM $MgCl_2$, 160 mM NaCl (or 166 mM KCl), 1 mM dithiothreitol, 0.1% NP-40, 5.3% glycerol, and 0.05 mg of bovine serum albumin per ml. Binding was performed for 30 min at either 20 or 37°C. DNase I in 25 mM HEPES-HCl, (pH 7.5)–100 mM $MgCl_2$–100 mM $CaCl_2$ was added to each reaction to a final concentration of 0.6 U/ml (20°C assays) or 0.3 U/ml (37°C assays). Incubations were continued for 1 min at either 20 or 37°C, and the digestions were stopped by the addition of EDTA to 22 mM. The reactions were extracted with phenol, adjusted to 80 μl with distilled H_2O, and desalted by gel filtration on a 1-ml Sephadex G-50 spin column (21).

The locations of nuclease cleavage sites were detected by a primer extension assay (21) as detailed by Parsons et al. (47). The DNA products of the DNase-treated binding reactions were divided into two 35-μl aliquots, and 5'-end-labeled oligonucleotides corresponding to EBV nucleotides 8989 to 9005 and complementary to EBV nucleotides 9161 to 9175 were used to visualize nuclease cleavage sites on the bottom and top strands, respectively, of oriP region II. An oligonucleotide complementary to EBV nucleotides 8048 to 8064 was used to detect nuclease cleavage sites in oriP region I. Ethanol-precipitated reaction products were analyzed on 8% acrylamide–8 M urea sequencing gels. Dideoxy sequencing ladders were generated with the same end-labeled primers (53).

For experiments examining the effect of oriP region I upon the binding of EBNA-1 to region II, the substrate DNAs were linearized with *Hin*dIII and radiolabeled by repairing the ends with Klenow polymerase in the presence of 40 μCi of [α-^{3}H]dATP per ml (23 Ci/mmol; Amersham, Arlington Heights, Ill.). The specific activity of the labeled DNAs was determined by trichloroacetic acid precipitation and used to determine precisely the amount of plasmid DNA to be added to each reaction following gel filtration and ethanol precipitation of the DNAs.

$KMnO_4$ and DMS footprinting. The in vitro binding conditions for $KMnO_4$ footprinting were the same as for DNase I footprinting. After a 30-min incubation at either 20 or 37°C, $KMnO_4$ was added from a 300 mM stock to a final concentration of 30 mM and incubation was continued for 4 min. The reaction was stopped by the addition of 2-mercaptoethanol to 1.2 M and subsequent phenol extraction (47). The deproteinized reaction products were desalted by gel filtration chromatography, and 35-μl portions were used as substrates for primer extensions as described for DNase I footprinting above. Footprinting with dimethyl sulfate (DMS) was performed by the procedure of Borowiec et al. (7) by using a region II-containing restriction fragment from pRII(Sal) that was uniquely end labeled at the *Sal*I site.

Construction of oriP mutants. Single- and double-point mutations were introduced into oriP region II EBNA-1-binding site 2 by oligonucleotide-directed mutagenesis (35). An *Eco*RV-*Sst*II fragment containing oriP region II was isolated from pHEBo-1 and subcloned between the *Eco*RV and *Sst*II sites in the polylinker region of pBluescript KS (Stratagene, La Jolla, Calif.), and single-stranded uracil-containing phagemid DNA was isolated and used as the template for mutagenesis (35). Mutagenic oligonucleotides were 5'-CGGGTAGTAGTATATGCTATCCAGAC-3' (mutant M1; nucleotide 9092T to C), 5'-CGGGTAGTAGCATA TACTATCCAGAC-3' (mutant M2; nucleotide 9097A to G), and 5'-CGGGTAGTAGCATATGCTATCCAGAC-3' (mutant M3; nucleotide 9092T to C and nucleotide 9097A to G). Mutated *Eco*RV-*Sst*II fragments were substituted for the wild-type sequences in pHEBo-1.1, and the sequence of EBV nucleotides 8992 to 9156 was determined for each mutant to confirm that mutations were limited to the desired sequences.

Replication assays. D98 and D98/Raji cells were seeded in 10-cm dishes at 0.5×10^6 cells per dish and transfected with recombinant plasmids by a modification of the calcium phosphate coprecipitation method (20) the following day. $CaPO_4$ precipitates formed in the presence of 10 μg of plasmid DNA and 10 μg of salmon sperm DNA were added to cultures, and incubation was continued for 15 h. For transient-replication assays the cells were washed with phosphate-buffered saline containing 2 mM EGTA, trypsinized, and split into three 10-cm dishes. The cells were harvested 78 h later, at which time the plates were ca. 80% confluent, and low-molecular-weight DNA was isolated by the method of Hirt (27). One-quarter of each sample was digested with *Dpn*I and *Cla*I and separated on a 1% agarose gel. The DNAs were transferred to a nylon membrane (Hybond-N; Amersham), UV irradiated by using a Stratalinker (Stratagene) and probed with ^{32}P-labeled pHEBo-1.1 generated by

random priming (15) (specific activity, 1.2×10^9 cpm/μg) as described by Maniatis et al. (39).

Plasmid maintenance assays were performed by transfection of D98 and D98/Raji cells as described above. At 15 h after addition of the $CaPO_4$ coprecipitates, the cells were washed and 50% of the transfected cells were seeded per 10-cm dish. The cells were fed with growth medium containing 0.3 mg of hygromycin B (Sigma Chemical Co.) per ml 24 h later and maintained under selection for 20 cell generations. Hirt supernatant DNA was isolated (27) and digested with *Cla*I, and recombinant plasmid DNA was detected by Southern blot analysis as described above.

RESULTS

High-level expression of EBNA-1 in insect cells. EBNA-1 is expressed in EBV-infected B cells at very low levels (41), making studies of its biochemical properties difficult. A protein consisting of the carboxy-terminal one-third of EBNA-1 fused to a portion of the bacteriophage lambda N protein has been expressed in *E. coli* and has provided valuable information regarding the sequence requirements for DNA binding (49) and the specific contacts made between EBNA-1 and its recognition sequence (2, 30). To investigate further the interaction of EBNA-1 with sequences in oriP and components of the cellular replication machinery, it would be desirable to use full-length EBNA-1 lacking exogenous amino acids. However, an attempt to express intact EBNA-1 in bacterial cells as a nonfusion protein resulted in a series of lower-molecular-weight polypeptides that were thought to arise from proteolytic processing of the full-length polypeptide chain and/or the absence of posttranslational modifications found in lymphocyte-derived EBNA-1 (46).

To overcome problems associated with expression of EBNA-1 in bacterial cells, the entire EBNA-1 open reading frame of the B95-8 virus isolate (43) was inserted into the baculovirus transfer vector pVL941 (37) such that EBNA-1 would be expressed from the strong polyhedrin promoter as a nonfusion protein. This hybrid gene was introduced into AcNPV genome by in vivo homologous recombination, and occlusion-negative viruses that directed the synthesis of EBNA-1 were identified by immunoblot analysis of infected insect cell extracts. The expression of EBNA-1 in Sf9 cells infected with a representative recombinant virus, AcEBNA-1, was compared with that in EBV-infected B lymphocytes by immunoblotting (Fig. 1). A human serum containing anti-EBNA-1 antibodies detected a predominant polypeptide of 86 kDa in AcEBNA-1-infected Sf9 cells, as well as a number of lower-molecular-weight species. These immunoreactive polypeptides were absent from uninfected and AcNPV-infected Sf9 cells (Fig. 1). The 86-kDa polypeptide was slightly retarded in its electrophoretic mobility relative to the 80-kDa EBNA-1 present in IB4 and B95-8 cells, both of which contain the B95-8 strain of EBV. The coding sequences for EBNA-1 contain a repetitive DNA element (IR3) that specifies only two amino acids, glycine and alanine (26). EBNA-1 varies in molecular mass between cells carrying different EBV strains as a result of variation in the length of the IR3 sequence (26). This variation is illustrated in Fig. 1, where Raji cell EBNA-1 (70 kDa), which contains a shorter glycine-alanine domain than B95-8 EBNA-1 (26), was also examined. The observed difference in electrophoretic mobility between insect cell-produced and B95-8 EBNA-1 may be due to differences in the posttranslational modification of EBNA-1 in insect cells and human B cells. The steady-state levels of EBNA-1 in AcEBNA-1-infected cells was determined to be approximately 600-fold greater than in EBV-infected B cells by direct counting of bands excised from immunoblots.

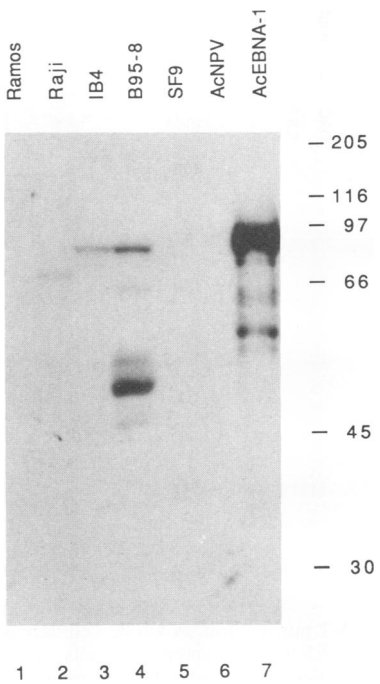

FIG. 1. EBNA-1 expression in insect cells infected with the recombinant baculovirus AcEBNA-1. The abundance and mobility of EBNA-1 in AcEBNA-1-infected Sf9 cells (lane 7) was compared with that in EBV-infected lymphocytes (lanes 2 to 4) by immunoblot analysis. An EBV-negative B-cell line (Ramos; lane 1) and uninfected and AcNPV-infected Sf9 cells (lanes 5 and 6) were also examined as negative controls. One hundred micrograms (lanes 1 to 4) or 5 μg (lanes 5 to 7) of total cell protein were separated on an SDS–10% polyacrylamide gel, transferred to nitrocellulose, and probed with a human serum containing anti-EBNA-1 antibodies. Bound antibodies were detected with ^{125}I-protein A, and the filter was autoradiographed. The positions of molecular mass markers, in kilodaltons, are indicated. The lower-molecular-mass immunoreactive species in the B95-8 cell extract are most probably lytic-cycle polypeptides (43), which are not expressed in Raji or IB4 (31) cells and react with the polyclonal human serum.

EBNA-1 purification. EBNA-1 was purified to near homogeneity from AcEBNA-1-infected Sf9 cells by sequential immunoaffinity chromatography and sequence-specific DNA affinity chromatography. Whole-cell detergent lysates were prepared from cultures 48 to 60 h postinfection, at which time EBNA-1 expression was maximal (data not shown). Eluates from the immunoaffinity column contained the 86-kDa EBNA-1 polypeptide (Fig. 2) and few contaminating polypeptides (Fig. 2B). Immunoaffinity column eluates were further fractionated by using a sequence-specific affinity matrix containing high-affinity EBNA-1-binding sites (28). EBNA-1 bound to the DNA affinity resin in 0.2 M KCl in the presence of 0.01 mg of nonspecific competitor DNA per ml remained bound during a 0.5 M NaCl wash and was eluted from the column with buffer containing 3.0 M NaCl (Fig. 2A). Lower concentrations of NaCl (e.g., 2.0 M) in the elution buffer resulted in the inefficient elution of EBNA-1 (data not shown). The DAC eluate lacked nuclease activity (see, for example, Fig. 4A and B, lanes 10) and contained

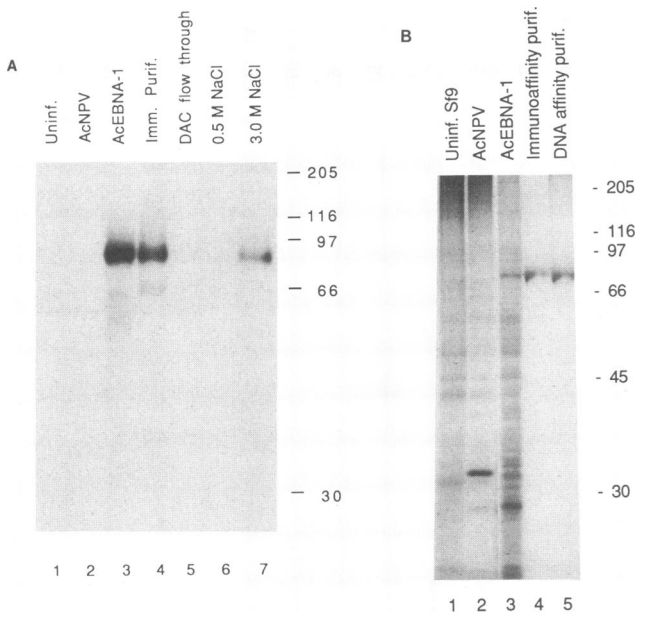

FIG. 2. EBNA-1 purification. A whole-cell detergent lysate was prepared from AcEBNA-1-infected Sf9 cells, and EBNA-1 was purified by sequential immunoaffinity chromatography and DNA affinity chromatography. (A) Five micrograms of total cell protein from uninfected (lane 1), AcNPV-infected (lane 2), and AcEBNA-1-infected (lane 3) Sf9 cells, together with a portion of immunoaffinity-purified EBNA-1 (lane 4), DAC flowthrough fraction (lane 5), 0.5 M NaCl wash (lane 6), and 3.0 M NaCl eluate were analyzed by immunoblotting with a human serum containing anti-EBNA-1 antibodies as described in the legend to Fig. 1. (B) Silver stain of 2 μg of immunoaffinity-purified (lane 4) and DNA affinity-purified (lane 5) EBNA-1. Lanes 1 to 3 contain total-cell proteins from uninfected (lane 1), AcNPV-infected (lane 2), and AcEBNA-1-infected (lane 3) insect cells. Note the absence of the 33-kDa polyhedrin polypeptide (lane 2) in AcEBNA-1-infected Sf9 cells. The positions of molecular mass markers, in kilodaltons, are indicated.

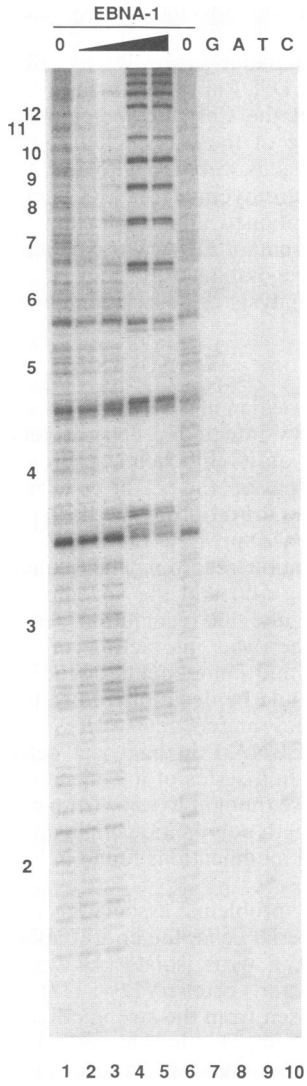

FIG. 3. Protection of sequences in oriP region I from DNase I digestion by insect cell-produced EBNA-1. DNase I protection assays were performed with 0.86 μg of pHEBo-1, 0.23 μg of pBluescript KS, and either 0 (lanes 1 and 6), 0.14 (lane 2), 0.28 (lane 3), 0.56 (lane 4), or 1.12 (lane 5) μg of purified EBNA-1. Nuclease cleavage sites on the top strand of region I were detected by primer extension with a radiolabeled oligonucleotide primer complementary to EBV nucleotides 8048 to 8064 (3). Lanes 7 to 10 are sequencing ladders generated with the same radiolabeled oligonucleotide used in the primer extension reactions. The individual EBNA-1-binding sites are numbered (through site 12) at the left of the figure, by the convention of Rawlins et al. (49).

only the 86-kDa EBNA-1 polypeptide as determined by silver-staining SDS-gels (Fig. 2B). The preparations did, however, contain minor amounts of smaller EBNA-1-related polypeptides, which could be detected by overexposure of immunoblots (data not shown). Approximately 250 μg of EBNA-1 could be rapidly purified from as few as 5×10^7 AcEBNA-1-infected Sf9 cells by using these two chromatography steps.

Sequence-specific interaction of EBNA-1 with wild-type origin sequences. The ability of EBNA-1 produced in insect cells to bind in a sequence-specific manner to oriP regions I and II was investigated by DNase I footprinting (18). The DNase I footprints of intact EBNA-1 are very similar to those obtained by using a bacterially produced carboxy-terminal DNA-binding fragment of EBNA-1 (28K EBNA [49]). EBNA-1 protected 25 bp in 20 of the 21 tandem repeats in oriP region I from DNase I digestion (Fig. 3). These protected regions differed by 1 bp at each end from the footprints generated with 28K EBNA (49). Intact EBNA-1 also protected two regions of approximately 45 bp in oriP region II from nuclease digestion (Fig. 4A and B). Each footprint spans two EBNA-1-binding sites (2, 49). Again, only minor differences were observed between the footprints generated with intact EBNA-1 and 28K EBNA (49). At low EBNA-1 concentrations, sites 1, 3, and 4 were preferentially occupied; at higher concentrations of EBNA-1, site 2 was protected (Fig. 4). Increasing the concentration of EBNA-1 16-fold over the amount required to completely protect all four binding sites (2.3 to 37.3 mg/ml) resulted in no detectable differences in the nuclease protection pattern (data not shown).

Distortion of oriP region II sequences by EBNA-1. The initiation of DNA synthesis from a Cairns-type origin may be divided into three general steps: recognition of the origin by a sequence-specific DNA-binding protein, localized melting of sequences in the origin and assembly of a presynthesis complex, and synthesis of RNA primers and daughter

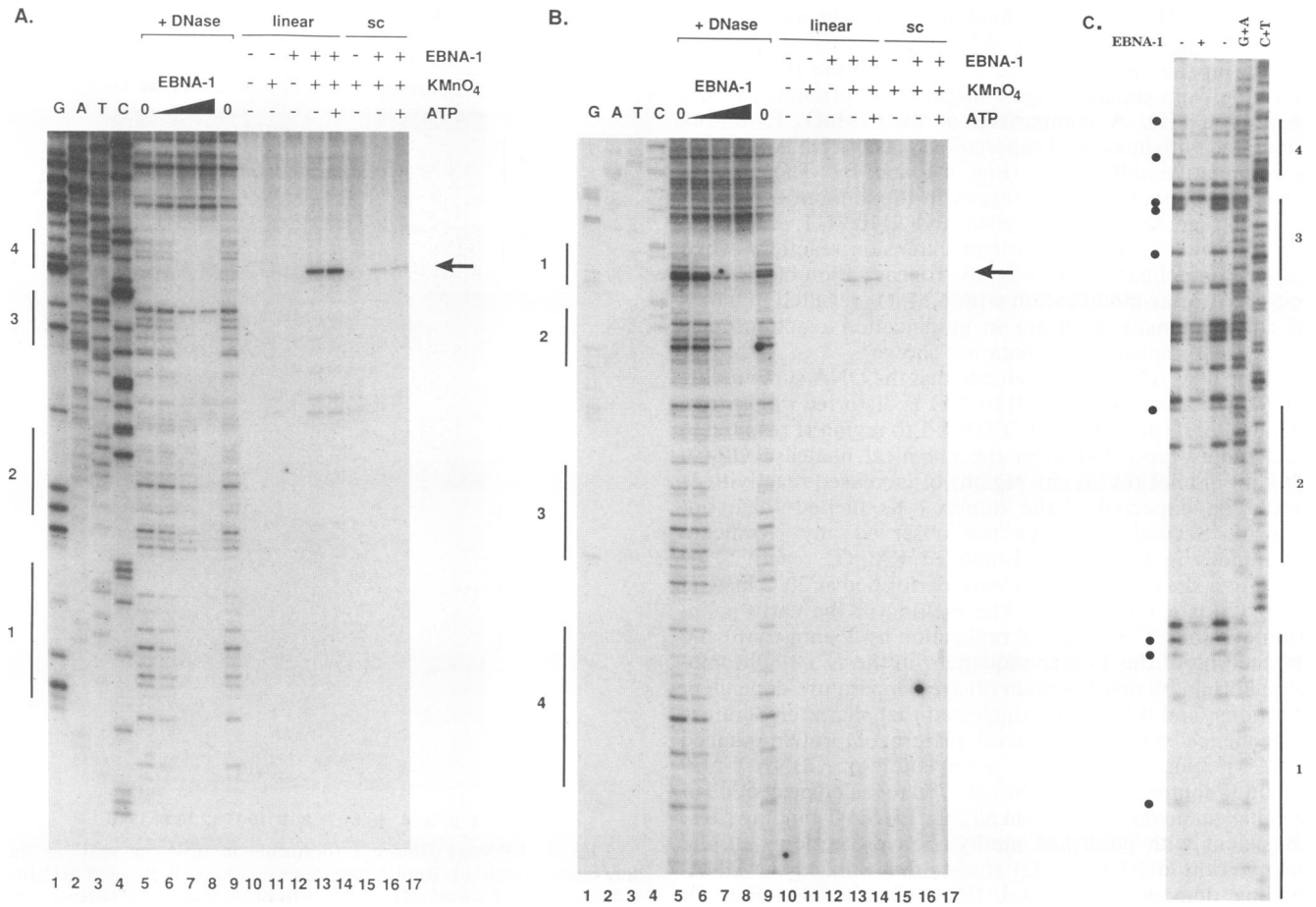

FIG. 4. DNase I, $KMnO_4$, and DMS footprints in oriP region II. (A and B) Binding reactions containing 0.5 μg of pRII and 0 (lanes 5, 9, 10, 11, 15), 0.5 (lane 6), 1.5 (lane 7), or 5.0 (lanes 8, 12, 13, 14, 16, 17) μl of EBNA-1 were carried out at 37°C for 30 min. Binding reactions analyzed in lanes 14 and 17 contained 2 mM ATP, and supercoiled plasmid DNA was substituted for linearized DNA in the reactions displayed in lanes 15 to 17. Incubations were performed at 37°C for 30 min. The reactions were treated with either DNase I (lanes 5 to 9) or $KMnO_4$ (lanes 11 and 13 to 17), and the locations of nuclease cleavage sites or oxidized thymines were detected by a primer extension assay. A radiolabeled oligonucleotide complementary to EBV nucleotides 9161 to 9175 (3) was used to monitor the top strand (panel A), and a primer corresponding to EBV nucleotides 8989 to 9005 (3) was used to monitor the bottom strand (panel B). Sequencing ladders generated with the same oligonucleotide primers are shown (lanes 1 to 4), and the four EBNA-1-binding sites defined by Ambinder et al. (2) are indicated by the bars at the left of each panel. Arrows mark nucleotides oxidized by $KMnO_4$ in the presence of EBNA-1. (C) End-labeled oriP region II DNA was incubated in either the absence or presence of saturating quantities (0.35 μg) of EBNA-1 at 37°C and then treated with DMS. The methylated DNAs were cleaved with piperidine, and the products were displayed on a denaturing acrylamide gel. Purine and pyrimidine marker ladders were generated by using chemical sequencing procedures (40). Guanosine residues protected from methylation by EBNA-1 are indicated by the solid circles. The four EBNA-1-binding sites (2) are indicated by bars at the left.

strands. Replication initiator proteins such as dnaA protein of *E. coli*, bacteriophage lambda O protein, and simian virus 40 (SV40) T antigen are able to induce localized melting of the duplex DNA upon formation of specific nucleoprotein complexes (7, 9, 55). These melted regions are enlarged by either an intrinsic helicase activity of the initiator protein (5) or a distinct helicase (4, 14) to allow the assembly of a preinitiation complex. The ability of EBNA-1 to alter the structure of the DNA duplex in oriP region II was investigated by using $KMnO_4$ footprinting (8). $KMnO_4$ oxidizes the 5,6 double bond of thymine residues in distorted (bent or untwisted) or melted duplex DNA (7, 8). Oxidized thymines prevent DNA chain elongation by *E. coli* DNA polymerase, thus allowing modified residues to be identified in a primer extension assay (52). EBNA-1 induced $KMnO_4$ reactivity at two symmetry-related sites in oriP region II (nucleotides 9045/9046 and 9110/9111 [Fig. 4A and B]). The modification of these nucleotides required both EBNA-1 and $KMnO_4$; omission of either component from the reaction resulted in the absence of DNA products terminating at these positions (Fig. 4A and B). The precise thymines oxidized by $KMnO_4$ are not known since Klenow DNA polymerase has been reported to stop both at the site of modification and 1 nucleotide 5′ to the modified base (7, 46). Each site is located within the more centrally positioned major groove of the outer two EBNA-1-binding sites (sites 1 and 4 [see Fig. 8]). The location of these modified thymines suggests that their heightened reactivity toward $KMnO_4$ may result from protein-protein interactions between EBNA-1 bound at sites 4 and 3 and at sites 2 and 1.

The melting of origin DNA by SV40 T antigen and *E. coli* dnaA protein requires ATP (7, 9). It is not known whether EBNA-1 binds ATP; however, addition of ATP to binding reactions did not alter the pattern of $KMnO_4$ reactivity (Fig.

4A and B). Bacteriophage lambda O protein melts origin sequences in the absence of ATP but requires superhelical tension in the substrate (55). Presumably, the free energy resulting from supercoiling of origin DNA promotes strand separation (55). A comparison of the $KMnO_4$ footprints generated with linear and supercoiled plasmid DNA revealed no qualitative differences (Fig. 4A and B). There were, however, quantitative differences in the amount of modification at nucleotides 9045/9046 and 9110/9111, but these were due to less efficient primer extension reactions when using supercoiled substrate DNA. Linearization of the plasmid DNA after modification with $KMnO_4$ resulted in bands of equal intensity to those in modification reactions performed with linear DNA (data not shown).

Three lines of evidence indicate that the DNA surrounding nucleotides 9045/9046 and 9110/9111 is distorted rather than melted. First, the binding of EBNA-1 to region II resulted in nucleotides protected from the chemical nuclease OP-Cu (36) but did not reveal any regions of increased reactivity as would be expected if the duplex was melted (data not shown). Second, we have not observed any significant differences in the EBNA-1-induced $KMnO_4$ reactivity of these two sites when assays were performed at 20°C instead of 37°C (data not shown). The melting of the early palindrome in the SV40 origin of replication by T antigen (6) and the melting of the 13-mer sequences in the *E. coli* chromosomal origin by dnaA protein (9) are temperature-dependent reactions, and it has been suggested that sharp temperature dependence may be a general feature of protein-induced DNA melting (6). Finally, methylation of EBNA-1–oriP region II complexes with DMS at 37°C revealed protection of specific guanosine residues in all four EBNA-1-binding sites, consistent with published methylation protection (30) and methylation interference (2) studies that utilized the DNA-binding domain of EBNA-1 (Fig. 4C; see also Fig. 8). However, methylation of cytosine residues by DMS, which occurs only when the base is not hydrogen bonded (7), was not induced in region II by EBNA-1 (Fig. 4C).

The exact sequence requirements for the EBNA-1-induced distortion of oriP region II are under investigation, but it is not a general feature of DNA containing EBNA-1-binding sites. EBNA-1-binding reactions in which regions I and II were present, either on the same plasmid DNA (pHEBo-1) or on separate plasmids (pRII and pHEBo-1dlDS), were performed. The samples were incubated at 37°C for 30 min, treated with $KMnO_4$, and divided into two equal portions. Each half of the samples was used to monitor the top strand of either region I or region II for oxidized thymines by primer extension. EBNA-1 failed to induce $KMnO_4$ reactivity in oriP region I (data not shown), although $KMnO_4$ reactivity was observed at EBV nucleotides 9045/9046 in region II. The centers of the 20 tandem EBNA-1-binding sites in region I are separated in most instances by approximately three helical turns, whereas the outer paired binding sites in region II are separated by two helical turns and sites 2 and 3 are three helical turns apart (see Fig. 8). These observations suggest that the relative spacing of EBNA-1-binding sites in oriP region II are important for the ability of EBNA-1 to alter the conformation of DNA.

Effect of oriP region I upon binding of EBNA-1 to region II. Region I is required for the efficient replication of plasmids bearing region II in vivo (38, 51). Transcriptional elements are often associated with origins of replication, and there are multiple ways that these elements may contribute to origin function (13). In the prokaryotic plasmid R6K an enhancer element, the gamma sequence, is required in *cis* for replication to initiate at the beta sequence (44). Binding of the replication initiator protein to the weaker beta site is facilitated by binding to the higher-affinity gamma sequence and the formation of a DNA loop (44). To determine whether oriP region I increases the affinity of EBNA-1 for region II, we performed DNase I footprinting experiments in which both essential elements were present in the binding reactions. In one set of reactions, regions I and II were present on separate plasmid DNAs (pHEBo-1dlDS and pRII; *trans* configuration). A second set of reactions contained regions I and II on the same plasmid DNA (pHEBo-1; *cis* configuration). The concentrations of plasmid DNAs were adjusted such that equimolar amounts of the two essential elements were present in all reactions and the total amounts of nonspecific plasmid sequences were equivalent. DNase I cleavage sites in region II were detected by the primer extension assay (top strand only). As shown in Fig. 5, there were no quantitative differences in the binding of EBNA-1 to region II in the presence of region I when the elements were

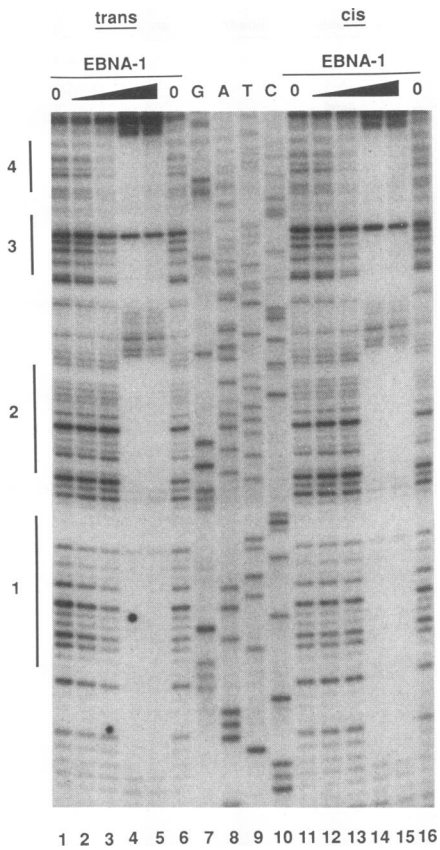

FIG. 5. EBNA-1 DNase I footprints in oriP region II in the presence of region I. Binding reactions contained 0.50 μg of pHEBo-1dlDS and 0.58 μg of pRII (lanes 1 to 6), 0.86 μg of pHEBo-1, and 0.23 μg of pBluescript KS (lanes 11 to 16) and 0 (lanes 1, 6, 11, 16), 0.14 (lanes 2 and 12), 0.28 (lanes 3 and 13), 0.56 (lanes 4 and 14), or 1.12 (lanes 5 and 15) μg of EBNA-1. Reactions were incubated for 30 min at 37°C and treated with DNase I, and nuclease cleavage sites on the top strand of region II were detected by primer extension with a radiolabeled primer complementary to EBV nucleotides 9161 to 9175 (3). Sequencing ladders generated with the same oligonucleotide primer are shown (lanes 7 to 10), and the positions of the four EBNA-1-binding sites (2) are indicated by bars at the left of the figure.

on the same DNA or on separate molecules. Thus, region I does not appear to enhance the ability of EBNA-1 to bind to region II. Additionally, the interaction of EBNA-1 with region II is not qualitatively different as determined by DNase I and $KMnO_4$ footprinting (Fig. 5; data not shown).

Effect of mutations in EBNA-1-binding site 2 in oriP region II. EBNA-1-binding site 2 in oriP region II differs at two positions from a consensus high-affinity EBNA-1-binding site (2). These base pair transitions contribute to the lower affinity of EBNA-1 for this site (2) and to the low free energy that would be predicted to be required to melt this sequence (10). It has been hypothesized that this A+T-rich sequence may be important for duplex melting during the initiation of DNA synthesis at oriP (49). The data presented in Fig. 4 suggest that EBNA-1, by itself, is unable to melt this region of the origin. This result does not exclude the possibility that EBNA-1, in conjunction with one or more components of the cellular replication machinery, utilizes this A+T-rich sequence as a site for initial duplex melting. To determine the importance of this A+T-rich sequence in replication, two single point mutations and a double point mutation were created by oligonucleotide-directed mutagenesis. Mutant M1 contains a T-to-C transition at nucleotide 9092. Mutant M2 contains an A-to-G transition at nucleotide 9097. Mutant M3 contains both of these changes. Each mutation would be predicted to raise the free energy required to melt this sequence (10) and to increase the affinity of EBNA-1 for this binding site (2).

The ability of these mutagenized plasmids to replicate transiently and to be maintained stably in cells expressing EBNA-1 was compared with that of the parental plasmid, pHEBo-1.1 (Fig. 6). Plasmids were transfected into D98 and D98/Raji cells by the calcium phosphate coprecipitation method (20). D98/Raji is a somatic cell hybrid formed between D98, a human epithelial cell line, and the EBV-positive Burkitt's lymphoma cell line Raji (19). For transient-replication assays the transfected cells were maintained as actively growing cultures for 93 h, and low-molecular-weight DNA was isolated, digested with *Cla*I and *Dpn*I, and analyzed by Southern blotting with ^{32}P-labeled pHEBo-1 DNA as the probe. *Dpn*I cleaves DNA only when its recognition site is methylated, whereas digestion of DNA by *Cla*I requires a nonmethylated recognition site. Input plasmid DNA from DNA adenosine methylase-positive *E. coli* (*dam*$^+$) is cleaved by *Dpn*I but is resistant to digestion by *Cla*I. Thus, the presence of *Dpn*I-resistant, *Cla*I-linearized bacterial plasmid DNA isolated from transfected animal cells indicates that the plasmids have replicated. To determine whether the mutagenized plasmids could be maintained stably in D98/Raji cells, we cultured transfected cells in the presence of hygromycin B to select for cells expressing the hygromycin resistance gene encoded by pHEBo-1 (57) and carried these cultures under selection for 20 cell generations. Low-molecular-weight DNA was then isolated, digested with *Cla*I, and analyzed by Southern blotting.

The parental plasmid pHEBo-1.1 differs from the well-characterized pHEBo-1 (57) by a small deletion in pBR322-derived sequences. Both plasmids replicated to the same extent in the EBNA-1-positive somatic cell hybrid D98/Raji in the transient-replication assay, as evidenced by the presence of *Dpn*I-resistant, *Cla*I-linearized plasmid DNA (Fig. 6, lanes 1 and 3). Transient replication of pHEBo-1.1 was not observed in EBNA-1-negative D98 cells (Fig. 6, lane 2), consistent with the known requirement of oriP for EBNA-1 (38, 62). All three mutated plasmids replicated as well as pHEBo-1.1 in the transient-replication assay (Fig. 6, lanes 4 to 6). Similarly, pHEBo-1.1 and the plasmids with mutations in oriP region II were present at the same level as pHEBo-1 in stably transformed D98/Raji cells (Fig. 6, lanes 8 to 12). These results indicate that the overall low free energy predicted to be required to melt nucleotides 9088 to 9103 in oriP region II is not critical for the ability of oriP to serve as a replication origin and for the replicated plasmids to be maintained in a stable manner in EBNA-1-expressing cells.

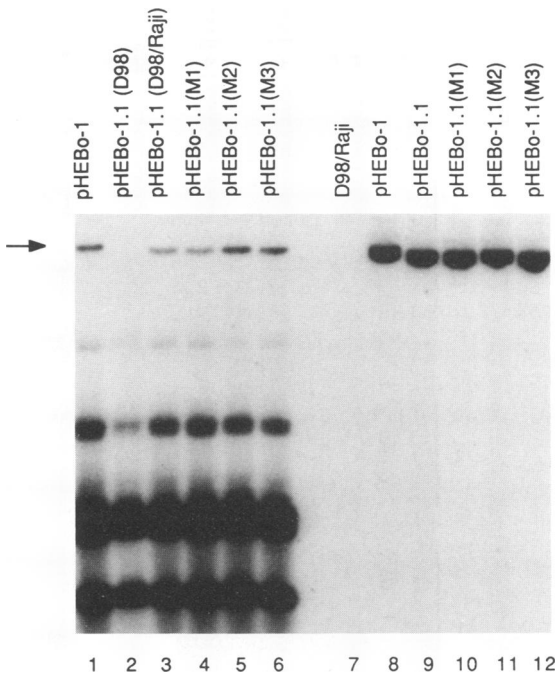

FIG. 6. In vivo replication of wild-type and mutated oriP plasmids. The ability of oriP-bearing plasmids with a wild-type or mutated region II to replicate transiently (lanes 1 to 6) or to be maintained in cells over 20 cell generations (lanes 8 to 12) was determined by Southern blot analysis of low-molecular-weight DNA isolated from transfected EBNA-1-positive (D98/Raji) or negative (D98) cells. Samples were digested with *Cla*I (both transient and stable assays) and *Cla*I plus *Dpn*I (transient assays only), electrophoresed on a 1% agarose gel, transferred to a nylon membrane, and probed with ^{32}P-labeled pHEBo-1.1 DNA. Lanes: 1 and 8, pHEBo-1; 2, 3, and 9, pHEBo-1.1; 4 and 10, pHEBo-1.1(M1); 5 and 11, pHEBo-1.1(M2); 6 and 12, pHEBo-1.1(M3). The position of linearized pHEBo-1 and pHEBo-1.1 DNA is indicated by the arrow. The lower-molecular-weight DNA fragments that hybridized to the probe represent unreplicated, *Dpn*I-digested plasmid DNA. Lane 7 contains low-molecular-weight DNA isolated from nontransfected D98/Raji cells.

Sequence-specific binding of EBNA-1 to mutated origin sequences. Interestingly, DNase I protection experiments performed with the three mutated region II DNAs revealed a new site of nuclease cleavage within EBNA-1-binding site 4 (nucleotide 9036 [Fig. 7]). In a separate experiment, mutant M3 contained an additional hypersensitive site at nucleotide 9037 (data not shown). These DNase I cleavage sites lie within an otherwise unaltered DNase I footprint over EBNA-1-binding site 4, and there were no other differences in the nuclease protection patterns spanning the remaining three EBNA-1-binding sites (Fig. 7). Because these nuclease protection assays were performed on linear DNA substrates, the presence of new DNase I cleavages in site 4 suggests either that interactions occur between EBNA-1 dimers

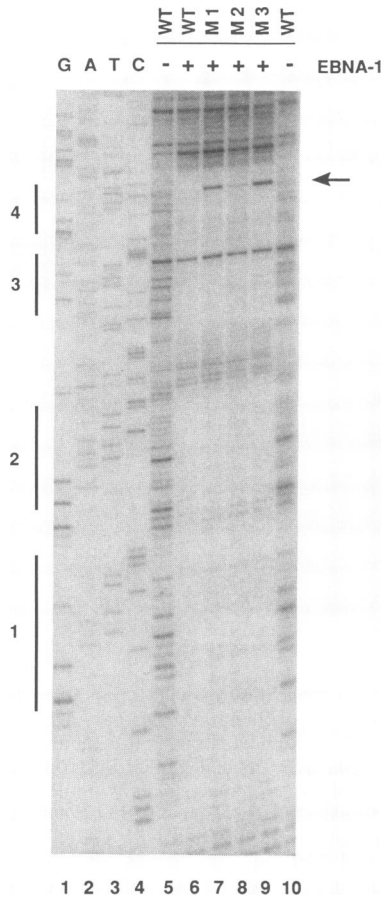

FIG. 7. Protection of wild-type and mutated oriP region II DNAs from nuclease digestion by EBNA-1. DNase I protection experiments were performed with 0.5 μg of plasmid DNA containing wild-type region II (pRII; lanes 5, 6, and 10), a T-to-C transition at nucleotide 9092 (M1; lane 7), an A-to-G transition at nucleotide 9097 (M2; lane 8), and both mutations (M3; lane 9), as described in the legend to Fig. 4A. Binding reactions contained 5 μl of EBNA-1 (lanes 6 to 9) or buffer lacking EBNA-1 (lanes 5 and 10). Sequencing ladders generated with the same primer are presented (lanes 1 to 4), and the novel DNase I cleavage observed in binding reactions containing the mutated region II DNAs is indicated by the arrow.

bound to nonadjacent sites (for example, sites 2 and 4 or sites 1 and 4) and that these interactions are subtly altered in the mutated DNAs or that the introduction of mutations in binding site 2 leads to the formation of novel interactions between dimers bound at nonadjacent sites. The presence of single and double point mutations in EBNA-1-binding site 2 did not alter the ability of EBNA-1 to induce $KMnO_4$ reactivity at nucleotides 9046/9047 and 9110/9111, nor were there additional sites of $KMnO_4$ oxidation in the mutated origin sequences (data not shown).

DISCUSSION

Intact EBNA-1 has been expressed as a nonfusion protein in recombinant baculovirus-infected insect cells and purified to apparent homogeneity. Insect cell-produced EBNA-1 binds in a sequence-specific manner to sequences in the EBV latent origin. This observation, together with the ease with which large quantities of EBNA-1 may be purified from infected insect cell spinner cultures, makes this expression system valuable for investigations of the mechanism(s) by which EBNA-1 promotes replication of latent EBV genomes and transactivates RNA polymerase II-transcribed genes physically linked to oriP region I (38, 50, 62).

A recombinant baculovirus that directs the synthesis of a deletion derivative of EBNA-1 (baculoEBNA1) was recently reported (16). This derivative lacks the six amino-terminal amino acids and most of the glycine-alanine repeat of EBNA-1. Neither of these regions are essential for the replication-promoting and transcriptional activation properties of EBNA-1 in vivo when assayed independently (63), and baculoEBNA1 has retained the ability to dimerize and bind to sequences in oriP (16). It is noteworthy, however, that intact EBNA-1 described in this report appears to bind more efficiently to sequences in oriP than does baculoEBNA1. It was found that 800 fmol of baculoEBNA1 was required to protect fully the *Ava*I site in 10 fmol of oriP region II DNA from restriction endonuclease digestion (16). In contrast, 1.25 pmol of intact EBNA-1 (assuming a calculated molecular mass of 56 kDa) was able to saturate 0.6 pmol of EBNA-1-binding sites in oriP region II (data not shown) and 10 pmol of EBNA-1 saturated 4.32 pmol of EBNA-1-binding sites present in intact oriP (Fig. 5). The ratios of EBNA-1 to binding sites determined from these data (2:1 and 2.3:1) agree well with the dimerization of EBNA-1 in solution and when bound to DNA (1). Although these results indicate that intact EBNA-1 binds oriP region II more efficiently than does EBNA-1 lacking most of the glycine-alanine repeat and six amino acids at the amino terminus, this comparison of baculoEBNA1 and intact EBNA-1 relies upon the interpretation of different assays performed under different binding conditions.

Treatment of EBNA-1–oriP region II complexes with the chemical probe $KMnO_4$ identified two symmetry-related sites (nucleotides 9045/9046 and 9110/9111) that are bent or untwisted by EBNA-1. This distortion of region II was not enhanced or altered by the addition of ATP, by the presence of superhelical tension in the substrate, or by the presence of region I. It is particularly noteworthy that, although the contacts made between EBNA-1 and sequences in oriP region II and three EBNA-1 consensus binding sites spaced 30 bp apart are not detectably different as determined by hydroxyl radical footprinting (30), EBNA-1 was unable to induce reactivity to $KMnO_4$ in region I. The spacing of the EBNA-1-binding sites in these two essential elements of oriP are distinct; the centers of the binding sites in region I are spaced 27 to 30 bp apart, whereas the outer paired EBNA-1-binding sites in region II are 21 bp apart. These data suggest that the spacing of sites 4 and 3 and sites 2 and 1, and protein-protein interactions between EBNA-1 dimers bound to these paired sites, are responsible for the induction of $KMnO_4$ reactivity at nucleotides 9045/9046 and 9110/9111. The absence of modified thymine residues between sites 2 and 3 in region II, spaced 33 bp apart, supports this conclusion. Thus, the structure of the DNA duplex in oriP region II is altered in a unique manner by EBNA-1, and this structure may be relevant to the central role of region II in the replication of plasmids bearing oriP (17, 60). A summary of EBNA-1–oriP region II interactions is shown in Fig. 8.

oriP region II is also referred to as the dyad symmetry element, reflecting the presence of a 65-bp region of dyad symmetry (62). Because of the presence of such palindromic sequences within the replication origins of both prokaryotic organisms and viruses of animal cells (see reference 33 and references therein), it has been speculated that stem-loop or hairpin structures may form in this element and play a role in

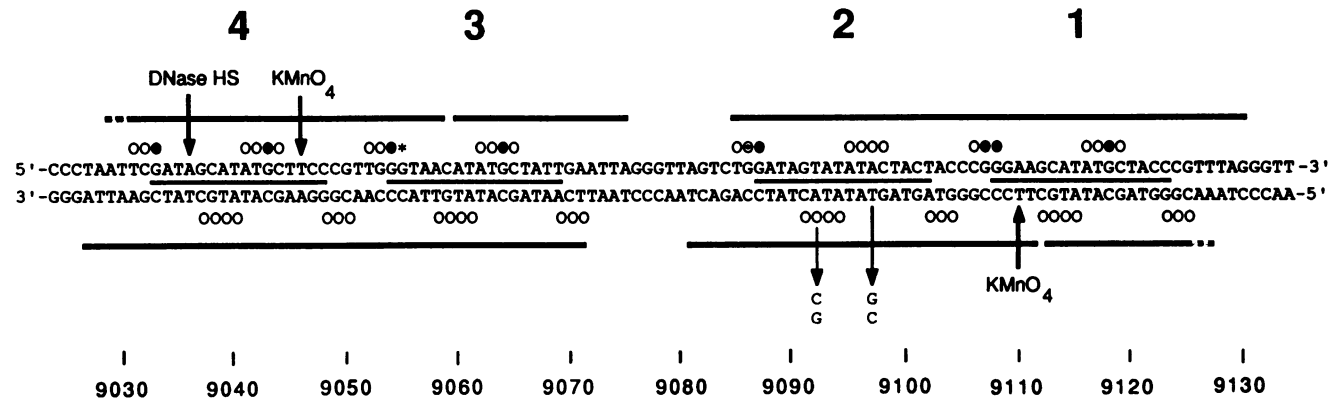

FIG. 8. Summary of EBNA-1–oriP region II interactions. The numbering of nucleotides in oriP region II is that of Baer et al. (3). The four recognition sequences for EBNA-1 (2) are indicated by bars between the DNA strands and the numbers at the top of the figure. The bars over the sequence represent regions protected by EBNA-1 from DNase I digestion. Broken ends represent ambiguities resulting from the absence of nuclease cleavage sites in naked DNA controls. The open circles indicate nucleotides protected from hydroxyl radical attack by 28K EBNA and are derived from the data of Kimball et al. (30). Filled circles represent guanosine residues protected from DMS by EBNA-1. Nucleotide 9086 was partially protected by EBNA-1. The asterisk indicates a guanosine residue protected by intact EBNA-1 that was not protected from hydroxyl radical attack by 28K EBNA-1 (30). Sites of $KMnO_4$ reactivity and the DNase I-hypersensitive site detected with the binding-site 2 mutants are indicated by arrows.

the initiation of latent EBV genome replication. Orlowski and Miller have examined the intrinsic ability of plasmids bearing oriP to extrude cruciform structures in the absence of EBNA-1 and have found that the major single-stranded regions in oriP are located in region I and that little cleavage occurs in region II (45). Experiments presented here support the absence of single-stranded structures in region II in the absence of EBNA-1 and further demonstrate that significant single-stranded structures do not form in region II in the presence of EBNA-1. Single-stranded areas were not detected within several hundred base pairs outside of region II.

The protein requirements for the initiation of DNA synthesis in the EBV latent origin are not known, but the inability of several investigators to detect helicase and ATPase activities in EBNA-1 preparations (1, 16) has indicated that EBNA-1 plays a more limited role at oriP than does the well-characterized replication initiator protein of SV40, T antigen (5). T antigen recognizes and binds specific sequences in the origin, induces the localized melting and distortion of origin sequences, further unwinds the origin in the presence of a single-stranded binding protein and a topoisomerase, and subsequently functions as a replication fork helicase (5). Experiments reported here demonstrate that EBNA-1 forms a complex with oriP region II in which the duplex is bent or untwisted at two sites but lacks significant single-stranded structures. We cannot exclude the possibility that EBNA-1 does have the ability to induce the melting of the duplex in region II but that differences in posttranslational modifications between insect cells and lymphocytes alter this activity. It is equally likely that one or more cellular proteins are required to recognize this complex and induce the localized melting of the DNA for the formation of an initiation complex. The development of an in vitro replication system for the EBV latent origin, together with unlimited quantities of purified EBNA-1, would allow such cellular factors to be identified and characterized. Studies of cellular factors that participate with EBNA-1 in the initiation of DNA synthesis from oriP may provide important insights into our understanding of the initiation of DNA synthesis from cellular origins.

Sequences which are locally melted by bound proteins may often be predicted on the basis of thermodynamic considerations. A+T-rich sequences have been implicated in the unwinding of DNA at yeast replication origins (59), the *E. coli* chromosomal origin (9), the bacteriophage lambda origin (55), the herpes simplex virus origin (33), and the SV40 origin (47). oriP region II contains a sequence that would be predicted to be thermodynamically unstable relative to the surrounding sequences (10). EBNA-1 binds to this sequence in region II but with reduced affinity than to the other binding sites in this element (Fig. 4) (2, 49). Despite the prediction that this sequence would be the most readily destabilized by bound protein, data provided here support the conclusion that the duplex in binding site 2 is not unwound by EBNA-1 and the overall A+T-rich nature of this sequence is not critical for origin function. Analysis of the interaction of EBNA-1 with origins containing either single or double point mutations in binding site 2 revealed the potential for interactions between EBNA-1 dimers bound to nonadjacent sites in region II.

oriP region I is an essential element of the EBV latency origin (38, 51) but does not appear to alter the ability of EBNA-1 to bind to region II or qualitatively alter the complex formed between EBNA-1 and region II in vitro. These data do not exclude the possibility that interactions occur between EBNA-1 molecules bound to these sequence elements that were not measured in these assays, such as the formation of a DNA loop. The role of region I in replication directed by oriP remains an interesting question.

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