Overproduction, Purification, and Characterization of EBNA1, the Origin Binding Protein of Epstein-Barr Virus*

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The baculovirus expression system was used to overproduce the Epstein-Barr virus nuclear antigen, EBNA1, in insect cells. EBNA1 overproduced via baculovirus expression (baculeBNA1) was followed during purification to homogeneity using its ability to specifically retain the family of repeats of the latent origin of replication, oriP, onto nitrocellulose filters. A two-column procedure was developed which yields more than 1 mg of homogeneous baculeBNA1 from 9 \times 10^9 insect cells (1.5 liters). Pure baculeBNA1 had no detectable ATPase or helicase activity. BaculeBNA1 was labeled with \(^{32}P\)orthophosphate in vitro, and analysis showed detectable levels of phosphoserine; no phosphothreonine or phosphotyrosine could be detected. The baculeBNA1 appeared dimeric in solution, and a stoichiometry of 56 baculeBNA1 monomers per 24 EBNA1 binding sites in oriP suggests baculeBNA1 binds its consensus site as a dimer. The binding of baculeBNA1 to the dyad symmetry element of oriP, \(K_d \approx 2 \text{ nm} \), required more baculeBNA1 and appeared less stable than the binding of baculeBNA1 to the family of repeats in oriP, \(K_d \approx 0.2 \text{ nm} \).

Epstein-Barr virus (EBV)\(^1\) is the causative agent of infectious mononucleosis and is associated with some types of cancer (reviewed in Ref. 1). During the latent infection of human lymphocytes, multiple copies of the EBV genome are maintained as 172-kilobase pair extrachromosomal DNA plasmids (2). Only one viral protein, EBV nuclear antigen 1 (EBNA1), is essential for the replication of these EBV plasmids (3). EBNA1 binds to the latent origin of replication, oriP, at multiple sites present in the two regions of oriP which were found to be necessary and sufficient for origin function (4, 5). One of these regions is composed of 20 tandem copies of a 30-bp sequence (family of repeats), each of which contains an EBNA1 binding site. The other region includes four EBNA1 binding sites (dyad symmetry element), two of which are located within a 65-bp region of dyad symmetry (5). The interaction of EBNA1 with oriP occurs mainly through the carboxyl-terminal third of the protein (6). EBNA1 activates oriP to function not only as an origin of replication but also as a plasmid maintenance element (7, 8) and a transcriptional enhancer (9, 10).

Our interest in the latent phase of EBV replication is its extensive reliance on host factors. EBNA1 being the only viral encoded protein required for latent phase replication from oriP. It is our hope that an in vitro EBV replication assay can be developed for use in isolating host factors that also participate in replication of human chromosomes. Since EBNA1 is not abundant in EBV-infected cells, we have used the baculovirus expression system (11, 12) to overproduce the EBNA1 protein. This expression system was used because other proteins overproduced with the baculovirus system, including SV40 large T antigen (13) and the E2 protein of bovine papilloma virus,\(^2\) have been shown to be functionally active. We have purified baculeBNA1 to homogeneity and characterized its phosphorylation state, aggregation state, and interaction with the EBV latent origin, oriP.

**MATERIALS AND METHODS**

**Cells and Virus**—The wild-type baculovirus, *Autographa californica* nuclear polyhedrosis virus (AcMNPV), and *Spodoptera frugiperda* (SF-9) cells used to propagate the baculoviruses were kindly provided by Dr. Ora Rosen (Sloan-Kettering Cancer Center), with permission from Dr. Max D. Summers (Texas A & M University). SF-9 cells were grown as monolayer cultures in Grace’s medium (Gibco Laboratories) with 0.33% yeastolate and 0.33% lactalbumin hydrolysate (Difco) supplemented with 10% fetal bovine serum.

Plasmids—pVL941-SW was constructed from pVL941 (14) by Dr. Susan Wente in Dr. Ora Rosen’s laboratory, by insertion of a NcoI/XbaI linker into the BamHI site of the polyhedrin gene in pVL941. Plasmid p205, containing the EBNA1 gene with a 700-bp (±20 bp) deletion in the glycline-alanine repeat region, was kindly provided by Dr. Bill Sugden (5). Plasmid pGEMoriP was constructed by ligating Real/HindIII DNA linkers to the ends of the Real fragment of p220.2 (kindly provided by Bill Sugden (Ref. 15)) containing oriP and the EBNA1 gene and inserting this DNA fragment into the HindIII site of pGEMII (Promega Biotech, Madison, WI). pGEMoriP was constructed from pGEMoriP using Accl to excise 2 kilobase pairs of DNA containing the EBNA1 gene followed by religation to give pGEMoriP, which contains the entire oriP sequence.

**Construction of the EBNA1 Recombinant Baculovirus (AcMNPV-EBNA1)**—The EBNA1 gene was excised from p205 using Real and BalIII, which remove the first seven codons. The initiating methionine was regenerated upon ligation into the baculovirus transfer vector pVL941-SW to yield pVL941/EBNA1 (Fig. 1). pVL941/EBNA1 and AcMNPV DNA were cotransfected into SF-9 cells by the calcium phosphate precipitation method as described in Summers and Smith (16). Five days post-transfection, serial dilutions of the medium from the transfected cells were plated with SF-9 cells in 96-well plates. After amplification of the virus for 4 days, the cells were screened for

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\(^2\)To whom reprint requests should be addressed.

\(^3\)The abbreviations used are: EBV, Epstein-Barr virus; EBNA1, Epstein-Barr virus nuclear antigen 1; baculeBNA1, form of EBNA1 overproduced using the baculovirus expression system; CIP, alkaline phosphatase from calf intestine; SDS, sodium dodecyl sulfate; PMSE, phenylmethylsulfonyl fluoride; bp, base pair(s); HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.

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M. Lusky, personal communication.
the presence of virus containing the EBNA1 gene by dot blot analysis. The medium from a positive well was then used in a plaque assay.

for the fragment of oriP was used as a label 5'-overhangs) to which EBNAl binds (6). An equal weight of each oligomer (220 pg each) was mixed, hybridized, phosphorylated, and ligated as described (17). The ligated oligomers were coupled to 10 nl of Sepharose CL-2B (Pharmacia LKB Biotechnology Inc.) freshly activated with cyanogen bromide (17).

Nitrocellulose Filter Binding Assays—During purification, baculoEBNA1 was followed and quantitated by its ability to specifically retain a 900-bp fragment of oriP containing 20 copies of the 30-bp repeated sequence (family of repeats fragment) onto nitrocellulose filters. The family of repeats fragment was excised from pGEMoriP with HindIII, filled in with dNTPs and [a-32P]TTP. Assays for baculoEBNA1 were prepared from these cells, digested with restriction enzymes, and purified and incubated with baculoEBNA1 as described for the family of repeats fragment. The dyad and its four associated EBNAl binding sites was incubated with baculoEBNA1 as described for the family of repeats fragment onto nitrocellulose filters (Millipore). The filters were dried and counted by liquid scintillation.

Reaction mixtures were then diluted with 900 nl of 50 mM HEPES (pH 7.5), 5 mM MgCl2, and 300 mM NaCl containing 2.5-5 pg of calf thymus DNA. Reaction mixtures were then diluted with 900 nl of 50 mM HEPES (pH 7.5), 5 mM MgCl2, and immediately filtered through 0.45-pm HA filters (Nunc). The filters were dried and counted by liquid scintillation.

Native Molecular Weight Determination—The sedimentation coefficient of baculoEBNA1 was measured by layering 40 pg of baculoEBNA1 along with 60 pg of molecular weight standards (apoferritin, IgG, bovine serum albumin, ovalbumin, and myoglobin) in 200 nl of 25 mM Tris-HCl (pH 7.5), 300 mM NaCl, 0.5 mM EDTA, 10% glycerol onto a 30-ml fast protein liquid chromatography Superose 12 gel filtration column. The column was developed in the same buffer. Fractions of 160 nl were collected. Two microfilters of each fraction contained the glycerol and gel filtration columns were assayed for the presence of baculoEBNA1 using the nitrocellulose filter binding assay described above. BaculoEBNA1 and the molecular weight standards were visualized after SDS-polyacrylamide gel electrophoresis (20) analysis by staining with Coomassie Blue.

Biochemistry of BaculoEBNA1 on OriP—Thirty-five micrograms (7.5 pmol as plasmid circles) of pGMoriP was incubated with excess 35S-labeled baculoEBNA1 (56 pg, 1.1 nmol as nonomer) for 10 min at 37 °C in 200 nl of 20 mM HEPES (pH 7.5), 5 mM MgCl2, 300 mM NaCl, 40% glycerol. The reaction was gel-filtered over a 5-ml Bio-Gel A-5m column at 4 °C in the same buffer, and 140-ml fractions were collected. [35S]BaculoEBNA1 in each fraction was assayed by counting 50 nl in a scintillation counter. The molal quantity of DNA in each fraction was measured using diluting 100 nl of fraction column with 400 nl of column buffer and measuring the absorbance at 260 nm (assuming 1 absorbance unit equals 50 pg/ml DNA). Approximately 90% of the radioactivity and absorbance at 260 nm was recovered after gel filtration.

Acid Endonuclease Protection Assay—The 300-bp HindIII to EcoRV fragment of pGMoriP containing the dyad symmetry element (see “Nitrocellulose Filter Binding Assays” above) was end-labeled using the Klenow fragment of DNA polymerase I and [32P] TTP to fill in the HindIII end of the fragment. BaculoEBNA1 was incubated with 10 fmol of the labeled dyad fragment in a 20-ml reaction containing 50 mM HEPES (pH 7.5), 300 mM NaCl, 5 mM MgCl2 for 10 min at room temperature. The reactions were then diluted to 50 ml and incubated with 30 units of Aat1 at 37 °C for 3 min. Digestions were stopped by the addition of SDS to 1%. Half of each reaction was then subjected to electrophoresis on a 6% polyacrylamide gel, which was dried prior to autoradiography.

RESULTS

Expression of EBNA1 in Baculovirus—The EBNA1 gene was excised from plasmid p205 (3) and inserted into the pVL941-SW baculovirus transfer vector as described in Fig. 1. The resulting plasmid, pVL941-EBNA1, contained the EBNA1 gene, which translates into a 50-kDa protein lacking six amino-terminal amino acids and approximately 230 contiguous glycine and alanine residues of the glycine-alanine repeat region. Neither of these regions was essential for EBNA1-dependent replication in vivo when tested separately (mutants CAT-EBNA1 and DL7 in Ref. 21). Recombination of pVL941-EBNA1 with AcMNPV wild-type baculovirus DNA resulted in a recombinant baculovirus (AcMNPV-EBNA1) containing the EBNA1 gene controlled by the strong polyhedrin gene promoter. The EBNA1 produced by AcMNPV-EBNA1 will be referred to here as baculoEBNA1. BaculoEBNA1 is not a fusion protein, as the EBNA1 gene was placed directly adjacent to the only ATG sequence present.
in the 5' region of the polyhedrin gene in pVL941-SW (Fig. 1).

In initial experiments, SF-9 monolayers were infected with AcMNPV-EBNA1 and harvested at 24-h intervals to determine the time course of baculoEBNA1 expression. Baculo-EBNA1 protein levels peaked approximately 48 h post-infection as determined by the ability of whole cell extracts to specifically retain the oriP repeat fragment on a nitrocellulose filter. The level of oriP binding activity correlated with the appearance on Coomassie-stained SDS-polyacrylamide gels of a 50-kDa protein that was not present in SF-9 cells infected with wild-type baculovirus (data not shown).

Purification of BaculoEBNA1—The purification of baculoEBNA1, from approximately 9 × 10⁸  SF-9 cells infected with AcMNPV-EBNA1 baculovirus, is summarized in Table I. The cells were harvested 46 h post-infection and separated into nuclear and cytoplasmic fractions. Origin binding activity and the 50-kDa protein produced upon infection were detected only in the nuclear fraction (Fig. 2). Dialysis against low salt buffers was avoided at all stages of purification, since baculoEBNA1 had a tendency to precipitate at under 250 mM NaCl. Hence the nuclear extract was diluted to 350 mM NaCl and loaded onto a column of heparin-agarose. The heparin-agarose column was washed with 18 ml of 350 mM NaCl and then baculoEBNA1 was eluted using buffer A containing 2 M NaCl. To concentrate baculoEBNA1, the 2 M NaCl eluate containing 33% of the oriP binding activity (50 ml) was dialyzed against 500 mM NaCl, diluted with buffer A to a conductivity equivalent to 260 mM NaCl, and loaded onto a 1-ml Mono Q column. BaculoEBNA1 was eluted with buffer A containing 500 mM NaCl at 0.27 ml/min; then baculoEBNA1 was eluted with buffer A containing 1 M NaCl. Fractions of the 1 M NaCl eluate containing oriP binding activity were pooled (26 ml), diluted to 350 mM NaCl with buffer A, then loaded onto 9 ml of the DNA oligonucleotide affinity column in three batches. The DNA affinity column was washed with 18 ml of 350 mM NaCl and then baculoEBNA1 was eluted using buffer A containing 2 M NaCl. To concentrate baculoEBNA1, the 2 M NaCl eluate containing 33% of the oriP binding activity (50 ml) was dialyzed against 500 mM NaCl, diluted with buffer A to a conductivity equivalent to 260 mM NaCl (105 ml), and loaded onto a 1-ml Mono Q column. BaculoEBNA1 was eluted with buffer A containing 500 mM NaCl. Aliquots of active fractions (20 μl/tube) were stored at −70°C.

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*One unit is defined as the amount of baculoEBNA1 required to retain 1 pmol of the oriP family of repeats onto a nitrocellulose filter (see "Materials and Methods").

![Fig. 1. Construction of the EBNA1 baculovirus transfer vector pVL941-EBNA1.](image)

![Fig. 2. Purification of baculoEBNA1.](image)
least 30% pure. The 1 M NaCl eluate from the heparin-agarose column was diluted to 350 mM NaCl and loaded onto a DNA oligonucleotide affinity column containing the oriP 30-bp repeated sequence. The oriP affinity column was eluted using 2 M NaCl to yield 1.4 mg of homogeneous 50-kDa baculoEBNA1 protein (Fig. 2). The baculoEBNA1 protein was then concentrated on a Mono Q column to 0.5 mg/ml, then frozen in 20-μl aliquots at -70 °C (see legend to Table I).

**Biochemical Assays of BaculoEBNA1**—Homogeneous baculoEBNA1 was assayed for the ability to hydrolyze ATP, GTP, CTP, UTP, dATP, dGTP, dCTP, and TTP in 1, 3, and 10 mM MgCl₂, in the absence of DNA and in the presence of either oriP-containing duplex DNA or single-stranded DNA. The τ subunit of *Escherichia coli* DNA polymerase III helicase enzyme was used as a positive control for ATP hydrolysis (22). No hydrolysis of any nucleoside triphosphate by baculoEBNA1 was detected (data not shown).

Although all known helicases are ATPases, we tested baculoEBNA1 in the standard oligonucleotide displacement type of helicase assay (23). BaculoEBNA1 was examined for an ability to displace, from single-stranded circular bacteriophage φX174 DNA, a [α-32P]-end-labeled flush DNA 30-mer, a 5'-tailed DNA 30-mer, and a 3'-tailed DNA 46-mer. The SV40 large T antigen was used as a positive control (24). Although the SV40 T antigen displaced each of these DNA oligonucleotides, no helicase activity was detected for baculoEBNA1, consistent with its lack of ATPase activity. We have also tested baculoEBNA1 for DNA polymerase, DNA ligase, endonuclease, exonuclease, and topoisomerase activities without positive results (not shown).

**Phosphoamino Acid Analysis of BaculoEBNA1**—BaculoEBNA1 was labeled in vivo with [32P]orthophosphate and purified to homogeneity. BaculoEBNA1 was the major 32P-labeled protein in the nuclear extract and was not detected in the cytoplasm (Fig. 3). Treatment of pure [32P]baculoEBNA1 with CIP resulted in loss of all detectable radioactive phosphate from baculoEBNA1 (Fig. 3). Since CIP has previously been shown to dephosphorylate serine residues only (25, 26), baculoEBNA1 is presumably phosphorylated only on serine.

Further identification of phosphorylated residues in baculoEBNA1 was performed by acid hydrolysis of [32P]baculoEBNA1 hydrolyzed for 1, 2, and 4 h were analyzed to determine any [32P]phosphothreonine, which requires longer hydrolysis times, or [32P]phosphotyrosine, which is less stable to acid hydrolysis (19). Upon electrophoresis, baculoEBNA1 was performed by acid hydrolysis of [32P]baculoEBNA1 and separation of the phosphoamino acids by high voltage paper electrophoresis (Fig. 4). Samples of [32P]baculoEBNA1 hydrolyzed for 1, 2, and 4 h were analyzed to ensure identification of any [32P]phosphothreonine, which requires longer hydrolysis times, or [32P]phosphotyrosine, which is less stable to acid hydrolysis (19). Upon electropho-
resis, all the radioactive phosphate in baculoEBNA1 migrated in the position of phosphoserine; no radioactivity was detected at positions of phosphothreonine or phosphotyrosine (Fig. 4). After 4 h of acid hydrolysis most of the radioactive phosphate was detected as free phosphate (data not shown).

Native Molecular Mass—BaculoEBNA1 was analyzed by gel permeation chromatography; an s value of 4.6 was obtained by comparison with protein markers with known s values (Fig. 5A). A Stokes radius of 50 Å for baculoEBNA1 was determined by gel filtration and gel filtration analyses, oriP binding activity coeluted with the baculoEBNA1 protein visualized in SDS-polyacrylamide gel analysis of the column fractions (data not shown). The s value and Stokes radius were combined in the equation of Siegel and Monty (27) to calculate a native molecular mass of 94 kDa for baculoEBNA1. The amino acid sequence of EBNA1 deduced from the DNA sequence of the EBNA1 gene predicts a molecular mass of 50 kDa for a baculoEBNA1 monomer (28). Hence, the native molecular mass of baculoEBNA1 indicates that baculoEBNA1 is a dimer.

Stoichiometry of BaculoEBNA1 Binding to OriP—[35S]-Labeled baculoEBNA1 protein was prepared in vivo by metabolic labeling using [35S]methionine followed by purification to homogeneity. The [35S]baculoEBNA1 was used to measure the number of baculoEBNA1 molecules bound to oriP under conditions of saturating baculoEBNA1. A plasmid containing the complete oriP sequence was incubated with increasing amounts of [35S]baculoEBNA1 then gel-filtered to separate [35S]baculoEBNA1 bound to DNA in the excluded fractions from the unbound [35S]baculoEBNA1 in the included fractions. Upon saturation of oriP with baculoEBNA1, indicated by the appearance of baculoEBNA1 in the included fractions, the molar ratio of baculoEBNA1 monomers per oriP DNA which comigrated in the excluded fractions was 56 to 1 (Fig. 6). Since there are 24 EBNA1 binding sites in oriP, the stoichiometry of 2.3 baculoEBNA1 monomers per EBNA1 binding site indicates that baculoEBNA1 bound its site as a dimer, consistent with the native molecular weight of baculoEBNA1 and the palindromic structure of the consensus EBNA1 binding site.

Effect of Salt on Binding of BaculoEBNA1 to the Family of Repeats and Dyad Symmetry Element—The effect of NaCl concentration on baculoEBNA1 binding to the family of repeats or the dyad symmetry element of oriP was studied using the nitrocellulose filter binding assay. BaculoEBNA1 (50 ng) was incubated with 40 fmol of 32P-labeled dyad fragment or 32P-labeled repeat fragment in various concentrations of NaCl and in the presence of excess (2.5 μg) calf thymus DNA (Fig. 7). The binding profile indicates that the specific interaction of baculoEBNA1 with the dyad symmetry element was maximum at 250–300 mM NaCl and dropped off sharply at higher NaCl concentrations. Binding of baculoEBNA1 to the family of repeats, however, remained stable up to 500 mM NaCl. Hence, the relative binding strength of baculoEBNA1 for the family of repeats versus the dyad symmetry element depended on the salt concentration. The apparent requirement of high salt for binding baculoEBNA1 to labeled DNA in these experiments may be attributed to efficient competition by nonspecific calf thymus DNA at low NaCl concentration.

BaculoEBNA1 Binding to the Dyad Symmetry Element—The interaction of baculoEBNA1 with the family of repeats and dyad symmetry element of oriP was also assessed by examining the amount of baculoEBNA1 required to retain each element on nitrocellulose filters. Increasing amounts of baculoEBNA1 were incubated with 10 fmol of 32P-end-labeled DNA. 

Fig. 5. Native aggregation state of baculoEBNA1. BaculoEBNA1 was combined with the protein standards apoferritin (apo; 440 kDa), IgG (158 kDa), bovine serum albumin (BSA; 66 kDa), ovalbumin (oval; 45 kDa) and myoglobin (myo; 17 kDa), then analyzed by glycerol gradient sedimentation (A) or gel filtration (B) as described under "Materials and Methods." BaculoEBNA1 was identified in column fractions by the nitrocellulose filter binding assay. The sedimentation coefficient (s) and Stokes radius of baculoEBNA1 were determined by comparison to the positions of protein standards of which the s values and Stokes radii are known.

Fig. 6. Stoichiometry of [35S]baculoEBNA1 bound to oriP DNA. [35S]baculoEBNA1 was incubated with pGEMoriP7, then gel-filtered to separate [35S]baculoEBNA1 bound to pGEMoriP7 in the excluded fractions from unbound baculoEBNA1 in the included fractions as described under "Materials and Methods." Fractions were analyzed for DNA and [35S]baculoEBNA1 as described under "Materials and Methods."
containing either the dyad symmetry element. BaculoEBNA1 (50 ng) was incubated with 40 fmol of 32P-end-labeled DNA containing either the dyad symmetry element (closed circles) or the family of repeats (open circles) in the presence of 2.5 µg of calf thymus DNA and various concentrations of NaCl. After 10 min at 23 °C, the reaction mixture was filtered through nitrocellulose, and the DNA retained on the filters was quantitated by liquid scintillation.

The DNA and various concentrations of NaCl. After 10 min at 23 °C, the reaction mixture was filtered through nitrocellulose.

FIG. 7. Salt dependence of baculoEBNA1 binding to the family of repeats and the dyad symmetry element. BaculoEBNA1 (50 ng) was incubated with 40 fmol of 32P-end-labeled DNA containing either the dyad symmetry element (closed circles) or the family of repeats (open circles) in the presence of 2.5 µg of calf thymus DNA and various concentrations of NaCl. After 10 min at 23 °C, the reaction mixture was filtered through nitrocellulose, and the DNA retained on the filters was quantitated by liquid scintillation.

FIG. 8. Effect of the family of repeats on binding of baculoEBNA1 to the dyad symmetry element. Top, diagram of orIP showing the disposition of EBNA1 binding sites (boxes). Bottom, 10 fmol of 32P-labeled DNA fragment containing either the family of repeats (open circles), the dyad symmetry element (closed circles), or the complete orIP (closed triangles) were incubated with various amounts of baculoEBNA1 (shown as fmol dimers) in 50 mM HEPES (pH 7.5), 300 mM NaCl, 5 mM MgCl2 for 10 min at 23 °C. Reactions containing the family of repeats or dyad symmetry element were then filtered through nitrocellulose. Reactions containing the complete orIP (closed triangles) were treated with 50 units of EcoRV for 3 min at 37 °C to separate the family of repeats from the end-labeled dyad symmetry element (see scheme, top) prior to filtration through nitrocellulose.

repeat or dyad DNA fragment in 20 µl of buffer containing 300 mM NaCl and no calf thymus DNA. Retention of the dyad symmetry element onto nitrocellulose appeared to have a threshold where significant retention was not observed below 20 baculoEBNA1 dimers per dyad fragment (200 ng in Fig. 8, closed circles), but full retention was achieved at 50 baculoEBNA1 dimers per dyad (500 ng in Fig. 8). It would seem from this behavior that baculoEBNA1 must reach a critical concentration before it binds the dyad symmetry element. The apparent $K_d$ for baculoEBNA1 binding to the dyad symmetry element calculated from these data is 2 nM (assuming four baculoEBNA1 dimers were bound per dyad symmetry element). The family of repeats was retained onto nitrocellulose at lower levels of baculoEBNA1 than required for binding the dyad symmetry element (Fig. 8, open circles). An apparent $K_d$ for baculoEBNA1 binding to the family of repeats was calculated to be 0.2 nM (assuming four baculoEBNA1 dimers were bound per family of repeats).

The binding of baculoEBNA1 to the dyad symmetry element was further examined by an AvoI endonuclease protection assay. An AvoI site was present at the junction of two of the four EBNA1 binding sites in the dyad symmetry element (Fig. 9). Increasing amounts of baculoEBNA1 were incubated with 10 fmol of the dyad symmetry element, end-labeled with 32P at one end only. The reaction was then treated with sufficient AvoI to completely digest the DNA within 3 min at 37 °C. Digestions were stopped with SDS and subjected to polyacrylamide gel electrophoresis to separate DNA fragments cut by AvoI from uncut (AvoI-protected) DNA (Fig. 9). As in the nitrocellulose binding assay, the AvoI protection analysis showed that a 20-fold molar excess of baculoEBNA1 dimers (200 ng in Fig. 9) was required over the dyad fragment to detect protection of the AvoI site, followed by a very sharp increase in protection against AvoI at levels above 20 baculoEBNA1 dimers per dyad symmetry element.5

The small difference between the AvoI protection assay (Fig. 9) and the nitrocellulose filter binding assay (Fig. 8) showed approximately 1.5 times more baculoEBNA1 was needed to bind the dyad symmetry element onto a nitrocellulose filter relative to the amount of baculoEBNA1 needed to protect the AvoI site. This may be due to the requirement for baculoEBNA1 to bind to only one particular site

FIG. 9. Protection of the AvoI site in the dyad symmetry element by baculoEBNA1. A, the 300-bp DNA fragment containing the dyad symmetry element, 32P-end-labeled at one end only (*), was incubated with various amounts of baculoEBNA1 (shown as fmol dimers) prior to digestion with AvoI and electrophoresis on a 6% polyacrylamide gel as described under "Materials and Methods." The DNA was visualized by autoradiography of dried gels. Scheme of DNA fragment (top) shows EBNA1 consensus binding sites (boxes). B, the AvoI-protected bands in the autoradiograph in A were quantitated by a laser densitometer (LKB Bromma Ultrascan XL).

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In vivo the dyad symmetry element is accompanied by the family of repeats within oriP which may affect the interaction of EBNA1 with the dyad symmetry element. Therefore we examined the interaction of baculoEBNA1 with the dyad symmetry element in the complete oriP sequence. BaculoEBNA1 was incubated with oriP labeled with \(^{32}P\) at the end near the dyad. Just prior to filtration through nitrocellulose, the family of repeats was separated from the dyad symmetry element by digestion with EcoRV (Fig. 8). For each assay an aliquot was removed prior to filtration, quenched with SDS, and analyzed in an agarose gel to confirm that EcoRV had completely separated the dyad from the oriP DNA. The results showed significant amounts of dyad symmetry element were retained onto nitrocellulose at lower levels of baculoEBNA1 (200 fmol and less) in the presence of the family of repeats than in their absence (Fig. 8, closed triangles). However, further along in the titration, more baculoEBNA1 was required to bind the dyad on oriP than to bind the dyad alone. Complete retention onto nitrocellulose of the isolated family of repeats and dyad symmetry fragments required 300 and 500 fmol of baculoEBNA1, respectively. Hence, it seems a paradox that even 800 fmol of baculoEBNA1 was not sufficient to retain onto nitrocellulose more than half of the dyad fragment when it was within the context of oriP. Possible explanations include the following. The presence of the family of repeats may destabilize the interaction of baculoEBNA1 with the dyad. A less stable complex of baculoEBNA1 with the dyad may assemble in the presence of the family of repeats. The nonessential region of oriP between the family of repeats and dyad symmetry element may influence the nitrocellulose binding assay, or the presence of the dyad may cause more cooperative binding of baculoEBNA1 to the family of repeats, effectively decreasing the availability of baculoEBNA1 for binding the dyad.

**DISCUSSION**

In this report we describe the overproduction of EBNA1, the viral encoded protein which binds the latent phase origin (oriP) of EBV, in the baculovirus system and its purification to homogeneity. Like EBNA1 from latently infected B cell lines (4, 29, 33), the baculoEBNA1 bound tightly to oriP, arrested replication forks within or near the oriP family of repeats,\(^6\) and was phosphorylated on serine residues. Since phosphorylation can modulate protein function (30-32), it seems likely that initiation of replication from oriP will be regulated by phosphorylation of EBNA1. The palindromic nature of each EBNA1 consensus site suggests that EBNA1 binds its DNA site as a dimer. Indeed the baculoEBNA1 appeared to be a dimer in solution and the stoichiometry of 56 baculoEBNA1 molecules per 24 EBNA1 binding sites in the oriP sequence was consistent with EBNA1 binding its site as a dimer as predicted (34).

Increasing evidence suggests replication initiates within the dyad symmetry element of oriP (33, 35). Replication initiation in the dyad is greatly stimulated by the family of repeats (35). One mechanism by which the repeats might activate the dyad is by altering the interaction of EBNA1 with the dyad symmetry element. The nitrocellulose filter binding assay suggested that the family of repeats reduced the concentration of baculoEBNA1 required to initiate binding to the dyad symmetry element. Provided the interaction of EBNA1 with the dyad symmetry element is important for the initiation of replication from oriP, then the stimulation of dyad binding by the family of repeats at low EBNA1 concentration may be one mechanism by which the repeats enhance replication from oriP.

EBNA1 is essential for latent EBV replication, yet the precise biochemical function of EBNA1 remains elusive. The baculoEBNA1 protein should prove useful in biochemical assays to analyze the mechanism by which EBNA1 activates oriP to function as an origin of replication, a plasmid maintenance element, and a transcriptional enhancer (21). We find no ATPase (or other nucleoside triphosphatase), helicase, ligase, topoisomerase, DNA polymerase, exonuclease, or endonuclease activities associated with baculoEBNA1. The absence of ATPase and helicase activity suggests EBNA1 plays a different role in replication than the large T antigen of SV40. It is always possible, however, that the true activity of EBNA1 will only be revealed upon binding other proteins or by modification at a specific site(s). Furthermore, we cannot exclude the possibility that, although the six amino-terminal amino acids and glycine-alanine repeat region of EBNA1, lacking in baculoEBNA1, are nonessential for EBNA1 function in vivo (21), they may affect the biochemical activity of EBNA1 in vitro.

Elucidation of the precise role of EBNA1 in replication and the mechanism(s) of replication control at oriP would be greatly facilitated by development of an in vitro system capable of initiating replication from oriP. We hope that the availability of EBNA1 produced in baculovirus will enable the development of such a cell-free replication system,\(^7\) in a manner analogous to that by which supplementation of cell extracts with SV40 large T antigen enabled the development of an in vitro system for replication from the SV40 origin (36-38).

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**REFERENCES**


\(^6\)The EBNA1 recombinant baculovirus (AcMNPV-EBNA1) will be provided upon request.
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