The Herpes Simplex Virus 1 Origin Binding Protein: A DNA Helicase*

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The Herpes Simplex Virus type 1 is an attractive system for the study of eukaryotic chromosomal DNA replication (1). The entire 153-kilobase HSV-1 genome has been sequenced (2, 3). The virus has been well characterized genetically, and three highly homologous origins of replications, Ori, and a diploid Oriq, have been identified (4–10). Of the approximately 70 genes in the HSV-1 genome, seven have been shown to be necessary and sufficient for origin-dependent DNA replication in a transient replication assay (11–14). These genes correspond to the seven complementation groups that are temperature sensitive for HSV-1 DNA replication (15–17). Purification and characterization of the products of these genes have shown them to consist of a minimal set of enzymes required for DNA replication (14, 18). These include a DNA polymerase (19–21), (UL30) and its accessory protein (UL42) (21–24), a single-stranded DNA binding protein (UL29) (25–28), a three-subunit helicase-primase (UL5, UL8, UL52) (29–31), and an origin binding protein (UL9) (32–36).

Since only small quantities of the homogeneous origin binding protein can be obtained from HSV-1-infected mammalian cells (32, 33), we have overexpressed OBP in a mammalian cell system and obtained quantities of the nearly homogeneous protein sufficient to permit the analysis of its structure and function. We have found that the recombinant OBP has both DNA-dependent nucleoside 5'-triphosphatase and DNA helicase activities in addition to its origin binding activity. The recombinant protein is identical to the OBP isolated from HSV-1-infected mammalian cells.

EXPERIMENTAL PROCEDURES

Materials—Plasmid DNA, activated calf thymus DNA, and M13mp18 single-stranded DNA were prepared as described (37). The plasmid pON103 (32) was digested with EcoRI and PstI and 3' end labeled with [α-32P]dATP and the Escherichia coli DNA polymerase I large fragment as described (37). The labeled 32-base pair OBP-containing fragment was then separated from the vector by polyacrylamide gel electrophoresis and the DNA isolated from the gel (38). Plasmid pET3a (39) was obtained from Per Elia (Shenington University). Plasmid pOS-82 was obtained from Priscilla Schaffer and Scott Wong (Harvard University). Plasmid pDL411, containing oriL (10), was obtained from Denise Galloway (Fred Hutchinson Cancer Research Center). Oligonucleotides 3'4 and 7/9 were prepared as described (35) as were the DNA helicase substrates (3'5-tailed, 5'5-tailed, and untailed) (29). EcoRI linkers were obtained from New England Biolabs, and (da)100 and (dT)100 were from Midland Certified Reagent Co. (Midland, TX). Ribonucleoside triphosphates were obtained from Pharmacia LKB Biotechnology Inc., and [α-32P]dATP (3000 Ci/mmol) was from Amersham Corp. Restriction endonucleases were obtained from New England Biolabs and Bethesda Research Laboratories. T4 DNA polymerase and T4 DNA ligase were from New England Biolabs. DNA polymerase I large fragment, calf intestinal alkaline phosphatase, sodium persulfate, bisacrylamide, and TEMED were obtained from Boehringer Mannheim. Acetylated bovine serum albumin was obtained from Bethesda Research Laboratories. Phosphocellulose P-11 was obtained from Whatman. Heparin was from Sigma and was coupled to Sepharose CL-4 (Pharmacia) as described (40). A prepacked Superose 12 gel filtration column and molecular weight standards were obtained from Pharmacia. Sodium dodecyl sulfate was from BDH Chemicals (Poole, United Kingdom). Acrylamide was obtained from U. S. Biochemical Corp. Molecular weight standards for electrophoresis were obtained from Bio-Rad. CV-1 cells and 293 cells were from American Type Culture Collection. Sterile HEPES, glutamine, penicillin/streptomycin, and G418 were from Gibco. Fetal calf serum was obtained from Irvine Scientific, Santa Ana, CA.

Buffers—Buffer A, used during enzyme purification, contained 20 mM HEPES, pH 7.6, 10% (v/v) glycerol, 1 mM dithiothreitol, and 1 mM EDTA. 2 pg/ml each leupeptin and pepstatin A, 0.5 mM phenylmethylsulfonyl fluoride, and 10 mM sodium bisulfite. Sodium bisulfate was omitted after the phosphocellulose step. Buffer B contained 20 mM HEPES, pH 7.6, 10% glycerol (v/v), 5 mM magnesium acetate, 1 mM dithiothreitol, and 100 μg/ml bovine serum albumin. The NaCl concentration is indicated in brackets; for example, Buffer A [0.1] indicates that the buffer contains 0.1 M NaCl.

Cells and Viruses—Cells were grown at 37 °C in Dulbecco's modified Eagle's medium containing 10% (v/v) fetal calf serum and supplemented with 2 mM glutamine, 100 units/ml penicillin, and 100 μg/ml streptomycin in an atmosphere of 6.5% CO2. For large scale growth, the medium was buffered with 10 mM HEPES, pH 7.5. Active
G418 (800 μg/ml) was added during selection as described (41). Ad5.SVR3 (42) was grown using 293 cells as a host.

Electrophoresis—Agarose gel electrophoresis was performed as described (37). DNA was isolated from the gel by binding to glass beads (GeneClean, Bio 101, La Jolla, CA). SDS-polyacrylamide gel electrophoresis was performed and the gels silver-stained as described (43, 44).

DNA-dependent ATPase Assay—The reaction mixture (25 μl) in Buffer B contained 1 μg of (dT)ₙ or other DNA cofactor as indicated. After the addition of OBP the mixture was incubated at 37 °C for 30 min. Acetic molybdate containing Malachite green (45) was added, and after 10 min of color development A₅₆₀ was determined. One unit of ATPase hydrolyzes 1.0 nmol of ATP in 30 min.

Filter Binding Assay—OBP (fraction IV, 460 ng/μl) was incubated with 17 ng of labeled OriI fragment and a 100-fold molar excess of the nucleotides 3/4 or 7/8 (33) in 25 μl of Buffer B [0.1] for 5 min at 23 °C followed by 10 min at 0 °C. Ice-cold Buffer B [0.1] (950 μl) was added and the mixture immediately filtered through Millipore 0.45-μm type HA filters. The filter was washed with 1 ml of ice-cold Buffer B [0.1] and dried. Radioactivity was determined by scintillation counting.

DNA Helicase Assay—DNA helicase activity was measured as described for the HSV-1 helicase-primase (29) using 170 ng of OBP purified through Superose 12 and 4.0 μM (nucleotide) helicase substrate. ATP (3.0 mM) was added where indicated. Incubation was at 37 °C for 15 min.

Nucleic and Protein Assays—Exonuclease activity was assayed as described (29). Protein concentrations were determined by the method of Bradford (46), using bovine serum albumin as a standard.

Insertion of UL9 Gene into Expression Vector p91023B—The plasmid pET3α is an E. coli expression vector that contains the HSV-1 origin binding protein sufficient for the study of OBP through the phosphocellulose step was modified from the method of Elias et al. (32). All steps were carried out at 4 °C. Nucleic acid prepared from approximately 4 g of CV-1 UL9:38 cells was thawed and centrifuged for 15 min at 750 × g. The nuclear pellet was resuspended to a volume of 7.5 ml in Buffer A [0]. The suspension was stirred vigorously, and 7.5 ml of Buffer A [4.0] was added quickly and stirred continuously for 5 min. The resulting viscous extract was centrifuged in a Beckman TL100 centrifuge at 98,000 rpm for 15 min using a TLA 100.3 rotor. The supernatant was dialyzed against 250 ml of Buffer A [0.15] for 5 h, with one change of buffer. The dialysate was cleared by centrifugation at 750 × g for 15 min and loaded onto a 4-ml phosphocellulose column equilibrated in Buffer A [0]. The column was washed with 2 ml of Buffer A [0.1] and eluted with a 40-ml gradient from 100 to 800 mM NaCl in Buffer A. OBP eluted between 370 and 420 mM NaCl. Active fractions were pooled and dialyzed against 500 ml of Buffer A [0.15] for 5 h. The protein was loaded onto a 1-ml heparin-Sepharose column equilibrated with Buffer A [0.1]. The column was washed with 1.5 ml of Buffer A [0.15] and the protein eluted with an 18-ml gradient from 100 mM to 1 M NaCl in Buffer A. OBP eluted between 390 and 450 mM NaCl. OBP-containing fractions were frozen in liquid nitrogen and stored at −80 °C. OBP at this stage of purification was greater than 95% pure and was free of detectable exo- and endonuclease activity.

RESULTS

Purification of Recombinant OBP—To obtain quantities of the HSV-1 origin binding protein sufficient for the study of its structure and associated enzymatic activities, UL9 was cloned into the SV40 origin-containing expression vector p91023B (47) (Fig. 1). After cotransfection of CV-1 cells with pRSVneo, G418-resistant cell lines capable of expressing high levels of OBP upon infection with the T-antigen expressing adenovirus Ad5.SVR3 were selected (41, 42).

Nitrocellulose filter binding had been used initially to measure OBP activity (32, 33). However, we observed that addition
of ATP significantly increased filter retention, suggesting that OBP may have an ATP binding site and hence an associated ATPase activity. Examination of OBP preparations purified from HSV-1-infected CV-1 cells showed that they did indeed possess a single-stranded DNA-dependent ATPase. DNA-dependent hydrolysis of ATP was therefore used to quantitate the recombinant OBP during purification. As shown in Table I the level of OBP expression was sufficient to allow purification to near homogeneity after only two chromatographic steps. Although UL9 encodes a protein with a predicted molecular mass of 92 kDa, OBP from both HSV-1-infected CV-1 cells and the mammalian expression system migrated as an 83-kDa protein during SDS-polyacrylamide gel electrophoresis (Fig. 2).

The DNA-dependent ATPase activity of the OBP was coincident with origin binding activity at each step in the purification (data not shown), suggesting that it is an intrinsic property of OBP. To provide further evidence for this association, fraction IV was subjected to gel filtration on Superose 12 (Fig. 2). The proteolysis products (65-70 kDa) that often represent a major fraction of OBP purified from HSV-1-infected CV-1 cells are only a minor contaminant in the recombinant OBP preparation.

The DNA-dependent ATPase activity of the OBP was coincident with origin binding activity at each step in the purification (data not shown), suggesting that it is an intrinsic property of OBP. To provide further evidence for this association, fraction IV was subjected to gel filtration on Superose 12. A single peak of protein was observed which coincided perfectly with the DNA-dependent ATPase activity and the 83-kDa OBP (Fig. 3, A and B). The peak of protein and ATPase eluted just after aldolase (160 kDa), suggesting that OBP may exist as a homodimer. However, the shoulder on the trailing edge of the protein peak, eluting with bovine serum albumin (68 kDa), indicates that some of the OBP may also be in a monomeric form.

Recombinant OBP Binds Specifically to OriS—The minimal

![Figure 2](image-url)

**FIG. 2. Purification of OBP.** The indicated fractions (see Table I) were subjected to 10% SDS-polyacrylamide gel electrophoresis and then silver stained. Lane 1, nuclear lysate; lane 2, dialyzed nuclear lysate; lane 3, phosphocellulose pool; lane 4, heparin-Sepharose pool. HSV-1, OBP purified from HSV-1-infected CV-1 cells. The positions of molecular weight standards (M, × 10²) are indicated.

![Figure 3](image-url)

**FIG. 3. Superose 12 gel filtration of OBP.** A, 80 µg of fraction IV in 0.2 ml was applied to a Superose 12 column equilibrated and eluted with Buffer A [0.2] and eluted at the rate of 0.2 ml/min. Column fractions were assayed for DNA-dependent ATPase activity as described under "Experimental Procedures." The indicated fractions were assayed for ATPase activity in the presence (●—●) or absence (○—○) of 1 µg of (dT)₉₀₀₀, and protein (□—□). The position of elution of molecular weight standards (M, × 10²) is indicated: ferritin, 440; aldolase, 150; bovine serum albumin, 68; and ovalbumin, 43. B, SDS-polyacrylamide gel electrophoresis of Superose 12 fractions. After electrophoresis, proteins were visualized by silver staining. The position of molecular weight standards (M, × 10²) and the position of the protein peak (●) are indicated.

OriS sequence contains two sequences, designated Box I and Box II, that have been shown to bind OBP (Fig. 4B) (33-36). A third sequence that shares significant homology with Boxes I and II but has not been shown to bind OBP is contained within the minimal OriS sequence and has been designated Box III (39, 48). An A-T spacer sequence separates Boxes I and II.

To investigate the interaction of the recombinant OBP with OriS, OBP was added to reaction mixtures that included a labeled restriction fragment containing OriS and the unlabelled competing (7/8) or noncompeting (3/4) oligonucleotide (33). The two unlabelled oligonucleotides share 17 base pairs of homology, but only 7/8 contains a complete OBP binding site (box I). Interaction of OBP with the OriS sequence in the presence of the two oligonucleotides was determined by nitrocellulose filter binding. As shown in Fig. 4A, the binding curve obtained with the noncompeting oligonucleotide 3/4 was hyperbolic and reached binding saturation when 94% of the label was retained on the filter. The binding curve in the presence of the competing oligonucleotide 7/8 was sigmoidal, and 50% retention required a 6.5-fold greater amount of OBP.

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*Table I*

Purification of OBP from a mammalian expression system

| Fraction       | Volume | Protein | Units* | Specific activity |
|----------------|--------|---------|--------|------------------|
| I Lysate       | 11     | 31      | 18,700 | 603              |
| II Dialysate   | 11     | 20      | 17,600 | 880              |
| III Phosphocellulose | 4    | 1.3     | 12,600 | 9,700            |
| IV Heparin-Sepharose | 2   | 0.78    | 11,000 | 14,100           |

*Unit = 1 nmol of ATP hydrolyzed in 30 min.

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2 M. S. Dodson and I. R. Lehman, unpublished results.
Fig. 4. A. Specific binding of OBP to Oris. OBP (fraction IV, 460 ng/ml) was added to reaction mixtures that included 17 ng of a labeled Oris-containing restriction fragment in Buffer B [0.1]. A 100-fold molar excess of the noncompeting oligonucleotide 3/4 (○—○) or the competing oligonucleotide 7/8 (■—■) was added. Measurement of binding to Millipore HA filters was performed as described under “Experimental Procedures.” B, sequence of OBP binding sites in Oris. The sequences of oligonucleotides 3/4 and 7/8 are shown below the Oris sequence.

Table II

Hydrolysis of nucleoside 5'-triphosphates by OBP
Recombinant (0.3 μg) OBP or OBP from HSV-1-infected CV-1 cells (0.73 μg) was incubated with the indicated nucleosides 5'-triphosphates each at 2 mM and 1 μg (dT)₆₀₀ for 30 min at 37°C. Formation of inorganic phosphate was determined as described under “Experimental Procedures.”

| NTP* | Recombinant OBP | HSV-1 OBP |
|------|-----------------|-----------|
| NTP hydrolyzed | NTP/ATP ratio | NTP hydrolyzed | NTP/ATP ratio |
| nmol | nmol           | nmol      | nmol         |
| ATP  | 16.3           | 1.0       | 6.5          | 1.0           |
| GTP  | 2.0            | 0.12      | 0.4          | 0.06          |
| UTP  | 6.1            | 0.37      | 2.0          | 0.32          |
| CTP  | 12.3           | 0.75      | 4.8          | 0.76          |
| dATP | 13.1           | 0.80      | 4.3          | 0.68          |
| dGTP | 0.4            | 0.02      | 0.3          | 0.05          |
| dTTP | 2.4            | 0.15      | 0.6          | 0.10          |
| dCTP | 10.0           | 0.61      | 3.6          | 0.57          |

*NTP, nucleoside triphosphate.
was a relatively poor substrate (15% of that observed with ATP). The efficiency of hydrolysis of the various nucleoside 5'-triphosphates by the recombinant OBP was not significantly different from the enzyme isolated from HSV-1-infected CV-1 cells (Table II).

**OBP Is a DNA Helicase**—Inasmuch as the SV40 large T-antigen that binds specifically to the SV40 origin is both a DNA-dependent ATPase and helicase (49, 50), we examined the ability of the HSV-1 OBP to act as a DNA helicase. Three different substrates were used. The first is a 22-residue oligonucleotide annealed to M13mp18 single-stranded DNA. The other two substrates share the same 22 base pairs of nucleotide annealed to M13mp18 single-stranded DNA. (lanes 1–4), a 5' tail (lanes 5–8), or a 3' tail (lanes 9–12) were incubated with OBP for 15 min at 37°C. Displacement of the oligonucleotide from the M13 single-stranded DNA was measured by gel electrophoresis through a 15% polyacrylamide gel. Lanes 1, 5, and 9, helicase substrates; lanes 2, 6, and 10, helicase substrates boiled for 2 min; lanes 3, 7, and 11, OBP added (170 ng, Superose 12 fraction); lanes 4, 8, and 12, OBP (170 ng) and 3.0 mM ATP added.

The recombinant OBP is indistinguishable from the protein purified from HSV-1-infected CV-1 cells. Like the latter, the recombinant enzyme binds specifically to Oris and has the same DNA-dependent nucleoside triphosphatase. The nucleoside triphosphatase activity of OBP shows some specificity for its DNA cofactor. Duplex DNA, even with an HSV-1 origin of replication, is inactive. Surprisingly, (dT)GW shows a strong stimulation whereas (dA)GW is inactive. Thus, either the sequence or structure of the single-stranded DNA plays a role in its ability to serve as a cofactor.

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