Interaction of origin binding protein with an origin of replication of herpes simplex virus 1

(viral DNA replication/affinity chromatography)

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ABSTRACT  Herpes simplex virus 1 encodes a protein that binds specifically to a viral DNA replication origin (oriS). This origin-binding protein has been purified to homogeneity by means of sequence-specific DNA affinity chromatography. The purified origin-binding protein, which has a molecular weight of 83,000, interacts with both parts of the oriS dyad; however, the affinity for the two sites differs by an order of magnitude. The sequence TTTCGCA occurs as a nearly perfect direct repeat within the two sites and may constitute the recognition sequence for the origin-binding protein.

We have been studying the replication of herpes simplex virus 1 (HSV-1) DNA as a model for the replication of a eukaryotic chromosome. The 150-kilobase genome of HSV-1 contains two structurally related origins of replication, oriS and oriI (1–8). The virus apparently encodes much of its own replication machinery including a highly processive Mr 136,000 DNA polymerase and an Mr 126,000 protein, ICP8, that binds tightly and cooperatively to single-stranded DNA (9–12).

In an attempt to establish conditions for the origin-dependent replication of DNA in vitro, we identified a factor that specifically recognizes the HSV-1 oriS sequence. This factor, which we have termed origin-binding protein (OBP), is synthesized at the same time as the HSV-1-induced DNA polymerase and ICP8 (13). It is our presumption that this protein is one of the components involved in the origin-dependent initiation of HSV-1 DNA replication. Here we report the purification of OBP to apparent homogeneity and describe its interaction with the oriS sequence.

MATERIALS AND METHODS

Cells and Virus. The HSV-1[FJR]A305 strain was used to infect Vero cells as described previously (13).

Enzymes. Restriction enzymes, T4 polynucleotide kinase, T4 DNA ligase, and the large fragment of DNA polymerase I were from United States Biochemical (Cleveland, OH) or Boehringer Mannheim.

Plasmids, Restriction Fragments, and Oligonucleotides. Plasmid pON103 was a source of a 360-base-pair HindIII–EcoRI restriction fragment containing the oriS sequence from HSV-1 (13). Oligonucleotides were synthesized using a model 380A DNA synthesizer (Applied Biosystems). They were deblocked in 30% (vol/vol) NH4OH, extracted three times in diethyl ether, and purified by passage through Sephadex G-25 in 10 mM triethylammonium bicarbonate. The purity of the oligonucleotides was analyzed on sequen- cing gels, and the oligonucleotides were further purified by polyacrylamide gel electrophoresis as required. Oligonucleotide concentrations were determined spectrophotometri- cally assuming that 1 absorbance unit at 260 nm is equivalent to an oligonucleotide concentration of 36 μg/ml. Duplex oligonucleotides were formed by briefly mixing equimolar amounts of complementary single strands in 0.1 M NaCl/10 mM Tris-HCl, pH 7.5, at 95°C and allowing the mixture to cool slowly to 4°C. The sequences of oligonucleotides (written 5' → 3') used are designated as follows: 3', TCCAGCTTCGGCACCATATATA; 4, TATATATTGGGA CGAAGTGCGA; 7, GCGAGCGTGGACCTGCATCCTGGCAAT; 8, ATTTGGGAAGTGGAGCAGTCTGCGA; 17, GATCTGCAGCGCTCGACATTGCAGGAC; 18, GATCCTATTGGGAAAGCTGCGAGCACTGGCGC; 20, GATCTATTAGGCGAAGTGGAGACGTCGGCA; and 21, GATCGCGCAAGTGGAGCCATGGCGACCTGCGC. A duplex oligonucleotide designated 3/4 was formed by annealing the complementary single-stranded oligonucleotides 3 and 4; the other duplex oligonucleotides are designated in a similar manner. The relationship of these oligonucleotides to the palindromic portion of the oriS sequence is shown in Fig. 1.

Labeling of Oligonucleotides and Restriction Fragments. Duplex oligonucleotides were labeled to a specific activity of 1600 Ci/mmoll (1 Ci = 37 GBq) with [α-32P]dCTP (800 Ci/ mmoll), and the HindIII–EcoRI fragment containing the oriS sequence was labeled with [α-32P]dCTP (3000 Ci/mmoll) using the large fragment of DNA polymerase I (14).

Assay for OBP. During purification, OBP was assayed by nitrocellulose filter binding. The reaction mixture (25 μl) contained 100 mM NaCl and 25 fmol of 32P-labeled oligonucleotide 17/18 (containing the entire box I sequence; see Fig. 1) in buffer A [50 mM Hepes (sodium salt), pH 7.6/5 mM MgCl2/0.1 mM EDTA/0.5 mM dithiothreitol/10% (wt/vol) glycerol]. Two separate assays were set up; one used a 100-fold excess of the unlabeled oligonucleotide 3/4, and the other used a 100-fold excess of unlabeled oligonucleotide 7/8. (When phosphocellulose fractions were assayed, sonicated calf thymus DNA at a concentration of 1.5 mg/ml was also included in the reaction mixture.) The samples were incubated at room temperature for 10 min unless otherwise indicated. They were then diluted with 1 ml of buffer A and immediately passed through nitrocellulose filters (Sartorius) that had previously been soaked in buffer A. Filtration was completed within 5–10 sec. The radioactivity of the filters was determined by scintillation counting. The difference in radioactivity between the two assays was taken as a measure of the amount of oligonucleotide specifically retained on the filter.

The concentration of homogeneous OBP was determined by filter-binding measurements. The reaction mixture (25 μl) contained 1 nM 32P-labeled duplex oligonucleotide 17/18, bovine serum albumin (0.1 mg/ml), and 100 mM NaCl in buffer A. Incubation was for 10 min at 0°C. The reaction was

Abbreviations: HSV-1, herpes simplex virus 1; OBP, origin-binding protein.
diluted with 1 ml of ice-cold buffer A and immediately passed through nitrocellulose filters. The amount of oligonucleotide 17/18 bound to the filter was estimated by assuming that 50% of the complexes formed were retained (see Fig. 4). One unit of OBP retains 1 fmol of duplex oligonucleotide 17/18.

The rate of dissociation of OBP-DNA complexes was also measured by nitrocellulose filter binding. The OBP-DNA complexes were formed using labeled duplex oligonucleotide 17/18 or 20/21 in the reaction mixture described above. At the time indicated, the reaction mixture was diluted with 0.5 ml of buffer A containing 100 mM NaCl, bovine serum albumin (0.1 mg/ml), and 2.5 pmol of duplex oligonucleotide 7/8 and was filtered immediately.

**Protein Determination.** Protein concentration was measured by the Bradford method (15) or by the BCA (bicinchoninic acid) protein assay (Pierce).

**Purification of OBP.** All procedures were carried out on ice or at 4°C as indicated. A 1.7 M NaCl extract of nuclei from HSV-1-infected cells was chromatographed on phosphocellulose as described (13). The fractions containing OBP were pooled and precipitated with solid (NH₄)₂SO₄ (50 g/100 ml). The precipitate was collected by centrifugation, resuspended in 20 mM Heps (sodium salt), pH 7.6/0.5 M NaCl/0.5 mM dithiothreitol/0.5 mM EDTA/10% (wt/vol) glycerol, and dialyzed overnight against the same buffer. The dialyzed OBP fraction (approximately 3 ml containing 5 mg of protein per ml) was frozen in liquid nitrogen and stored at −80°C. The phosphocellulose fraction was purified further by affinity chromatography as described by Kadonaga and Tjian (16). The duplex oligonucleotide 17/18 was polymerized using T4 DNA ligase and coupled to CNBr-activated Sepharose CL-2B. OBP (50 μl; 5 mg/ml) was mixed with 120 μl of sonicated calf thymus DNA (10 mg/ml) and 330 μl of buffer B [20 mM Heps (sodium salt), pH 7.6/0.5 mM dithiothreitol/0.5 mM EDTA/0.1% Nonidet P-40/0.5 mM phenylmethylsulfonate/10% (wt/vol) glycerol]. The mixture was applied to a column containing 2 ml of the affinity matrix that had been equilibrated with buffer B containing 0.1 M NaCl. The column was washed first with 5 ml of 0.1 M NaCl and then with 10 ml of 0.4 M NaCl (both solutions were prepared in buffer B). OBP was eluted with 1.0 M NaCl in buffer B. The fractions containing OBP were concentrated by means of a Centricon 30 microconcentrator (Amicon). Three preparations of OBP were pooled, mixed with 25 μl of sonicated calf thymus DNA (1 mg/ml), and diluted with buffer B so that the final concentration of NaCl was 0.1 M. The OBP was then rechromatographed on a 2-ml affinity column as described above. Fractions containing OBP were pooled and again concentrated using a Centricon 30 microconcentrator. The OBP preparations were stored at −80°C. They could be thawed and refrozen in liquid nitrogen several times without significant loss of activity.

**DNase I “Footprinting.”** OBP was incubated in the standard reaction mixture with 10–15 fmol of the 360-base-pair HindIII–EcoRI restriction fragment containing the ori₃ sequence, which had been end labeled with [α-32P]dCTP and DNA polymerase I large fragment. After 10 min at room temperature, 0.05 μg of DNase I was added. The digestion was stopped after 30 sec by the addition of 2.5 μl of 0.5 M EDTA. The reaction mixture was diluted with 70 μl of 10 mM Tris-HCl, pH 7.5/1 mM EDTA, and 5 μl of sonicated calf thymus DNA (0.1 mg/ml) was added. The mixture was extracted with phenol/chloroform, 50:50 (vol/vol) and precipitated with ethanol. The samples were run on 6% sequencing gels as described previously (12, 17).

**Polyacrylamide Gel Electrophoresis.** NaDODSO₄/polyacrylamide gel electrophoresis of OBP-containing fractions was performed as described (18). The polypeptides were visualized by silver staining. DNA–OBP complexes were analyzed by gel electrophoresis (mobility shift experiments) (19, 20) were formed in a 5-μl reaction mixture containing buffer A, 0.1% Nonidet P-40, 2.4 mM 32P-labeled oligonucleotide 17/18, OBP, and a 100-fold excess of competing oligonucleotide. The samples were incubated for 10 min on ice and loaded onto 7.5% polyacrylamide gels (acylamide/bis-acrylamide, 30:0.8). The gels (15 × 15 cm) were run at 4°C for 3 hr at 10 mV Tris-HCl, pH 8.5/1 mM EDTA at 150 V or 25 mA with circulating buffer. Subsequent to electrophoresis, the gels were soaked for 30 min in the buffer used for electrophoresis containing 2% glycerol and were dried on a gel dryer. Autoradiography was performed overnight at −80°C with an intensifying screen.

**RESULTS**

**Purification of OBP.** We previously described an activity in HSV-1-infected cells that specifically retains a restriction fragment containing the HSV-1 ori₃ sequence on nitrocellulose filters (13). This OBP was partially purified from nuclear extracts by phosphocellulose chromatography (13). DNase I footprint analysis revealed that the OBP specifically recognized a sequence of 18 nucleotides within ori₃ (13) (box I in Fig. 1). With this information, we could make use of sequence-specific DNA affinity chromatography to purify the OBP to near homogeneity (see *Materials and Methods*) (Fig. 2A). NaDODSO₄/polyacrylamide gel electrophoresis of the purified OBP showed one major component with a Mr of 83,000 (Fig. 2B). In addition, two minor bands (Mr values of 45,000 and 38,000, respectively) were observed (Fig. 2B). The latter may represent proteolytic breakdown products of the Mr 83,000 polypeptide. The purified OBP probably exists as a monomer; however, it tends to aggregate at low ionic strengths (<50 mM; data not shown).

**Interaction of OBP with ori₃.** DNase I footprint analysis of the interaction between homogenous OBP and ori₃ showed two protected regions (Fig. 3). These two sites, boxes I and II (Fig. 1), displayed different apparent affinities for OBP (see below). When the OBP concentration was increased, changes in the DNase I digestion pattern were evident, including less frequent cutting in the A + T-rich region of the dyad between boxes I and
II and an increased intensity of some of the bands immediately to the left of box I (Figs. 1 and 3). These alterations might indicate binding of OBP to additional sites within oriS and/or a change in the DNA conformation induced by OBP.

**Stability of OBP–oriS Complexes.** The stability of the complexes formed between OBP and duplex oligonucleotides representing sequences within oriS was determined by nitrocellulose filter binding. OBP could be titrated stoichiometrically at 0°C under conditions in which oligonucleotide 17/18 was present at a concentration of 1.2 nM; saturation was observed when 50% of the input oligonucleotide was trapped on the filter (Fig. 4). In an extension of this experiment in which the concentration of OBP was constant and the DNA concentration varied, an apparent K_d for box I of 0.1 nM at 0°C was determined (results not shown). The apparent affinity of OBP for box I within oriS was approximately 10-fold greater than for box II (Fig. 5). Thus, at 0°C the complex between OBP and box I (oligonucleotide 17/18) had a half-life of 8 min, whereas for the complex with box II (oligonucleotide 20/21) it was 54 sec. At 22°C the half-life of the complex between box I and OBP was 34 sec; for box II the rate of dissociation was too rapid to measure.

**Stoichiometry of Binding OBP to oriS.** The exact stoichiometry of the OBP–oriS complex was not determined; however, gel mobility shift electrophoresis (19, 20) resolved two distinct complexes. A major species remained in the loading wells at the top of the gel, whereas a second complex, present in smaller amounts, entered the gel readily (Fig. 6). The major species may correspond to a stable complex (or complexes) in which OBP is present in two or more copies.

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**Fig. 2.** (A) Sequence-specific DNA affinity chromatography of the HSV-1 OBP. A fraction (50 µl; 5 mg/ml) obtained after phosphocellulose chromatography of a nuclear extract of HSV-1-infected cells was mixed with sonicated calf thymus DNA and applied to a sequence-specific DNA affinity column containing the oriS high-affinity site (box I). The column was washed first with 0.1 M NaCl in buffer A and then with 0.4 M NaCl in buffer A. OBP was eluted with 1.0 M NaCl in buffer A. Fractions (0.5 ml) were collected and assayed. (B) NaDodSO4/polyacrylamide gel electrophoresis of purified OBP. OBP was purified through two cycles of sequence-specific DNA affinity chromatography. Five hundred units of OBP was applied to a 7.5% polyacrylamide gel, and the polypeptides were visualized by silver staining subsequent to electrophoresis.

**Fig. 3.** DNase I footprinting of oriS–OBP complex. A complex was formed between the indicated amounts of OBP and the 32P-labeled 360-base-pair EcoRI–HindIII fragment containing oriS and then was digested for 30 sec with 0.05 µg of DNase I in a volume of 25 µl. The products were extracted with phenol/chloroform, precipitated with ethanol, and analyzed on a 6% sequencing gel. The positions of the DNase I cleavages were identified as described previously (12).

**Fig. 4.** Stoichiometric binding of OBP to the box I sequence. The concentration of labeled oligonucleotide 17/18 was kept constant (1.22 nM), and the concentration of OBP was varied. All procedures were carried out at 0°C.
FIG. 5. Stability of OBP–ori5 complexes. Twenty femtomoles of 32P-labeled oligonucleotides 17/18 (box I; ○) or 20/21 (box II; △) was incubated with 10 units of OBP for 10 min at either 0°C (Left) or 22°C (Right). At the times indicated, 2.3 pmol of unlabeled oligonucleotide 7/8 was added in a total volume of 0.5 ml, and the samples were passed through nitrocellulose filters.

Whether the major species is stabilized by protein–protein interactions or by the presence of more than one recognition sequence within box I (each of which binds a single OBP molecule) remains to be determined. This complex was resistant to competition by dyad sequences contained in oligonucleotide 3/4 but was dissociated by an excess of oligonucleotide 7/8, which includes the entire high-affinity OBP binding site. The minor species, which entered the gel, may represent a less stable complex in which only a single OBP is bound, possibly at the high-affinity site.

Our earlier DNase I footprinting studies had indicated that the binding site for OBP is only partially located within the ori5 dyad but that the dyad sequence is essential for stable interaction between OBP and ori5 (13). In an experiment in which the amount of complex formed between OBP and box I (oligonucleotide 17/18) was measured in the presence of increasing amounts of the competing oligonucleotides 7/8 and 3/4, the affinity of OBP for the dyad sequence (oligonucleotide 3/4) was found to be three orders of magnitude lower than that for box I (oligonucleotide 7/8) (Fig. 7). Thus, the dyad is necessary but not sufficient for stable complex formation. The apparent Kd for the dyad sequence (100 nM as compared with an apparent Kd for box I of 0.1 nM) could reflect the interaction of OBP with either the isolated sequence or nonspecific sequences. A firm conclusion will have to await determination of the minimal binding site as well as accurate measurements of the stoichiometry of the complexes formed between ori5 and OBP.

DISCUSSION

The HSV-1 ori5 binding protein was purified to near homogeneity by a combination of phosphocellulose and sequence-

FIG. 6. Polyacrylamide gel electrophoresis of complexes formed between OBP and the box I sequence. OBP (22 units in 1 μl) was mixed with labeled oligonucleotide 17/18 (2.4 nM) and a 100-fold excess of competing unlabeled oligonucleotides as indicated. Electrophoresis was carried out for 3 hr at 4°C by using 10 mM Tris-HCl, pH 8.5/1 mM EDTA as the running buffer.
specific DNA affinity chromatography. The purified OBP interacts with ori\(_5\) at two sites, box I and box II (Fig. 1). The difference in affinity of OBP for the two sites probably reflects differences in nucleotide sequence within these sites. The 11-base-pair sequence CGTTCGACTT found within box I has also been observed at sites analogous to HSV-1 ori\(_5\) in the genome of varicella zoster virus and constitutes a crucial part of a sequence that can function as an origin of replication in transfection experiments when the appropriate cells are superinfected with HSV-1 (21). It is evident that the minimal recognition sequence for OBP is within this region. The role of particular sequences within as well as immediately outside of the ori\(_5\) dyad in high-affinity binding of OBP (Fig. 7; see also figure 4 in ref. 13) will require closer examination by, for example, site-directed mutagenesis.

Analyses of the interaction between OBP and duplex oligonucleotides by gel mobility shift electrophoresis suggest that at least two types of sequence-specific complexes may be formed. Indeed, these data are consistent with multiple OBP binding sites within each box. A plausible candidate for multiple binding of OBP to box I or II might be the sequence TTCCGCA, which is present as an almost perfect direct repeat within both boxes. This arrangement resembles the origin of replication of simian virus 40 (SV40), where the large tumor (T)-antigen recognition sequence occurs in a palindrome in which each arm consists of two closely spaced direct repeats (22, 23). The role of T antigen in directing the initiation of DNA replication on the SV40 genome has recently been clarified by the analysis of SV40 DNA replication in vitro (24). An understanding of the function of OBP in the initiation of herpes DNA replication and the need for multiple binding sites, protein–protein interactions, and/or cooperative binding will likely result from detailed in vitro DNA binding and replication studies.

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