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Properties of the Novel Herpes Simplex Virus Type 1 Origin Binding Protein, OBPC

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We have recently identified a novel 53-kDa herpes simplex virus type 1 (HSV-1) protein encoded by, and in frame with, the 3′ half of the UL9 open reading frame, designated OBPC (K. Baradaran, C. Dabrowski and P. A. Schaffer, J. Virol. 68:4251–4261, 1994). Here we show that OBPC is a nuclear protein synthesized at both early and late times postinfection. In gel-shift assays in vitro-synthesized OBPC bound to oriS site I DNA to form a complex identical in mobility to complex A, generated with infected cell extracts and site I DNA. OBPC inhibited both plaque formation and viral DNA replication in transient assays, consistent with its ability to bind to site I DNA and its limited ability to interact with other essential DNA replication proteins. These properties suggest that OBPC may play a role in the initiation, elongation, or packaging of viral DNA.

The protein products of seven herpes simplex virus type 1 (HSV-1) genes are required for viral DNA replication (reviewed in references 11, 12, 27, and 52; 10, 40, 53, 55). These genes include UL9, which encodes the origin binding protein, OBP; UL29, which encodes a single-stranded DNA binding protein with (ICP8)helix-destabilizing properties; UL5, UL8 and UL52, which encode components of the helicase-primase complex; UL30, which encodes the viral DNA polymerase; and UL42, which encodes the polymerase accessory protein.

These seven gene products are sufficient for the replication of plasmids containing an HSV-1 origin in transfected or baculovirus-infected cells (48, 55). Attempts to reconstitute a cell-free, origin-dependent DNA replication system using these seven proteins have been unsuccessful, however, suggesting that additional viral or cellular factors are required. The cellular factor, OF-1, whose binding to HSV-1 oriS has been shown to affect origin function may be such a factor (13). As we demonstrate herein, an additional viral factor, OBPC, may also be involved.

OBP, the product of the UL9 gene (Fig. 1), binds to specific sequences within the HSV-1 origins, oriS and oriL, and is required for the initiation of DNA replication at these origins. OBP is an 82-kDa nuclear phosphoprotein, 851 amino acids in length (19, 20, 36, 39), that forms stable homodimers in solution (21). OBP binds to DNA and is capable of wrapping supercoiled DNA and looping and distorting DNA at the origin (17, 18, 21, 23, 25, 28, 43). OBP also has ATP-dependent 3′-5′ helicase activity (6, 8, 21, 48). By analogy with *Escherichia coli* DnaA protein, bacteriophage lambda O, and simian virus 40 large T antigen (30), OBP likely unwinds DNA following binding to viral origins, serving both to recognize these origins and to facilitate initiation of DNA synthesis.

OBP interacts with three viral replication proteins, the products of the UL8, UL29, and UL42 genes (5, 7, 37, 38), suggesting that it may act as a "docking" protein around which the DNA replication complex forms at viral origins. The N terminus of OBP is required for its interactions with the products of the UL8 and UL42 genes (37, 38). The C-terminal 317 amino acids of OBP contain all of the elements needed to bind to viral origins (2, 16, 33, 34, 47, 48, 50, 51). This polypeptide has been shown to inhibit virus replication and viral DNA synthesis in a dominant negative manner (41, 48, 49).

We have shown previously that a novel HSV-1 protein, designated OBPC, is encoded in frame with, and overlaps, the C terminus of the OBP open reading frame (ORF) (3) (Fig. 1). Because OBPC contains the DNA-binding domain of OBP, we hypothesize that like OBP, OBPC plays a role in origin-dependent viral DNA replication.

Although OBP localizes to the nucleus (9), its nuclear localization signal has not been identified definitively. A motif present in all members of the SF2 superfamily of helicases (22, 35), as well as OBP and OBPC, has been proposed to function as a nuclear localization signal (35) (motif VI, Fig. 1). To determine whether OBPC, like OBP, localizes to the nucleus, indirect immunofluorescence assays of cells transfected with an OBPC-expressing plasmid were conducted essentially as described previously (57). Vero cells were transfected with plasmids expressing either OBPC (pCMVOBPC$^+$; Fig. 1) or OBP (pCMVUL9; Fig. 1) under the control of the human cytomegalovirus (HCMV) immediate-early promoter. Transfected cells were tested for immunofluorescence with antibody specific for the C terminus of OBP at a dilution of 1:100 (Fig. 2). These tests demonstrate that OBPC accumulates in nuclei of Vero cells (Fig. 2A) in the absence of other viral proteins. The greater cytoplasmic component of OBPC staining (Fig. 2A) relative to OBP staining (Fig. 2B) was evident in repeat experiments and may reflect a higher level of OBPC expression in transfected cells or less efficient nuclear localization of OBPC than OBP. These observations demonstrate that at least one nuclear localization signal is common to both OBP and OBPC and suggest that OBPC functions in the nucleus.

The transcript specifying OBPC (UL8.5) is synthesized with delayed-early kinetics (3). To determine the kinetics of OBPC protein synthesis, Western blot analysis of nuclear extracts was conducted using an antibody that recognizes both OBP and OBPC (3). OBPC was detected at very low levels at 3 h and continued to accumulate through 24 h postinfection (Fig. 3A and B). In contrast, OBP, an early protein, was evident at 3 h,

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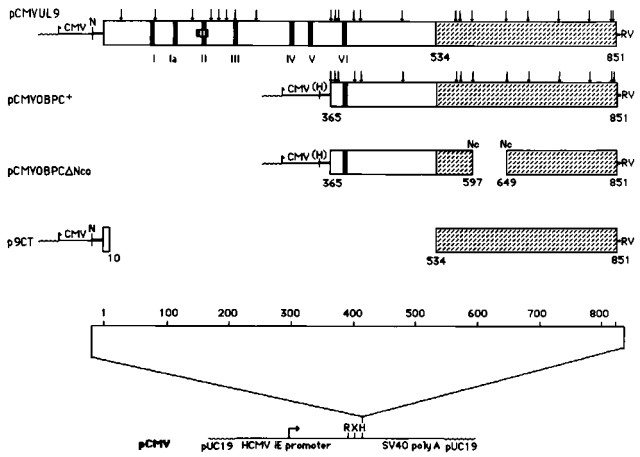

FIG. 1. Diagrams of the structures of full-length OBP (the product of the UL9 gene, pCMVUL9), OBPC (the product of the UL8.5 gene, pCMVOBPC$^+$), and mutant polypeptides OBPCΔNco (pCMVOBPCΔNco) and 9CT (p9CT) used in these studies. The numbers beneath each diagram and the scale at the bottom of the figure indicate amino acid positions in full-length OBP. The black bars indicate the locations of conserved helicase motifs, the small lined box indicates the location of the leucine zipper, and the hatched box denotes the DNA binding domain. The N terminus of the OBPC ORF begins at amino acid 365 with respect to full-length OBP. In-frame methionine codons are indicated by vertical arrows. Polypeptide ΔNco lacks 52 amino acids in the DNA binding domain of OBPC. Polypeptide 9CT consists of the N-terminal 10 amino acids fused to the C-terminal 317 amino acids of OBP (49). Plasmid pCMV, the vector used to construct the plasmids that express these polypeptides under control of the strong HCMV immediate-early promoter, is illustrated at the bottom of the figure. Restriction sites are as indicated: H, *Hin*dIII; N, *Nar*I; Nc, *Nco*I; R, *Eco*RI; RV, *Eco*RV; X, *Xba*I.

reached a peak at 6 h, and maintained this level through 12 h postinfection, dropping slightly at 24 h postinfection. These findings support our previous observation that UL8.5 RNA is expressed with delayed-early kinetics whereas UL9 RNA is expressed with early kinetics (3).

Western blotting analysis also facilitated a more careful assessment of the molecular mass of OBPC in HSV-1 infected cells. The predominant species migrated as a 50-kDa species rather than the 53-kDa product generated by in vitro translation (Fig. 3A). This difference may be due either to posttranslational processing or to initiation at a downstream AUG that occurs in infected cells, but not during in vitro translation. Both the 50- and 53-kDa estimates are consistent with a protein of 486 amino acids.

Three complexes (A, B, and M) form following incubation of infected cell extracts with binding sites I, II, and III of oriS and oriL in mobility shift assays (14, 15, 24, 29, 32, 50). A complex identical in mobility to complex M is also observed when the source of protein is uninfected cell extracts (13, 14). Hence complex M in infected cells likely contains only cellular proteins. Complexes A and B are observed only when infected cell extracts are used as the source of protein and oriS or oriL site I or site II DNAs are used as probes (14). Complex B, but not complex A, is observed when infected cell extracts are incubated with site III DNA or when extracts are prepared from cells incubated in the presence of the DNA replication inhibitor, phosphonoacetic acid (PAA) (14, 51). Although both complexes A and B are supershifted with antibody to the C terminus of OBP, the binding site required for stable complex A formation is 4 nucleotides longer than that required for stable complex B formation (14, 24). Taken together, these observations demonstrate that the proteins in complexes A and B share at least one epitope but are fundamentally different in electrophoretic mobility, nucleotide binding requirements, and ability to form in the presence of inhibitors of viral DNA replication.

Complex B was initially postulated to contain full-length OBP and complex A, a truncated "protease-resistant" segment of OBP (50). A major inconsistency with this theory is the observation that the formation of complex A, but not complex B, is sensitive to PAA (14), as early viral genes such as OBP are expressed in the presence of PAA. It is therefore unlikely that formation of a complex consisting of a stable, truncated por-

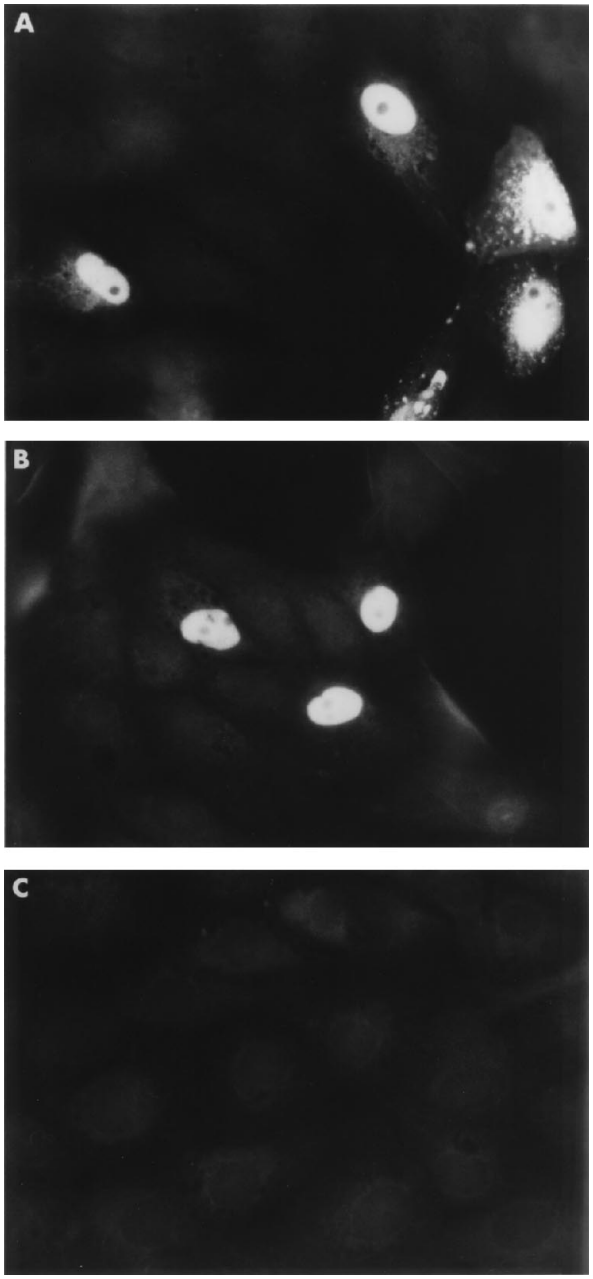

FIG. 2. Comparison of the intracellular localization of OBPC and OBP. Shown are Vero cells transfected 24 h previously with plasmid pCMVOBPC (A) or pCMVUL9 (B). Cells were fixed and treated with a primary rabbit antibody directed against the C-terminal 10 amino acids of OBP and with fluorescein-conjugated goat anti-rabbit antibody. (C) Mock-transfected cells. Magnification, ×60.

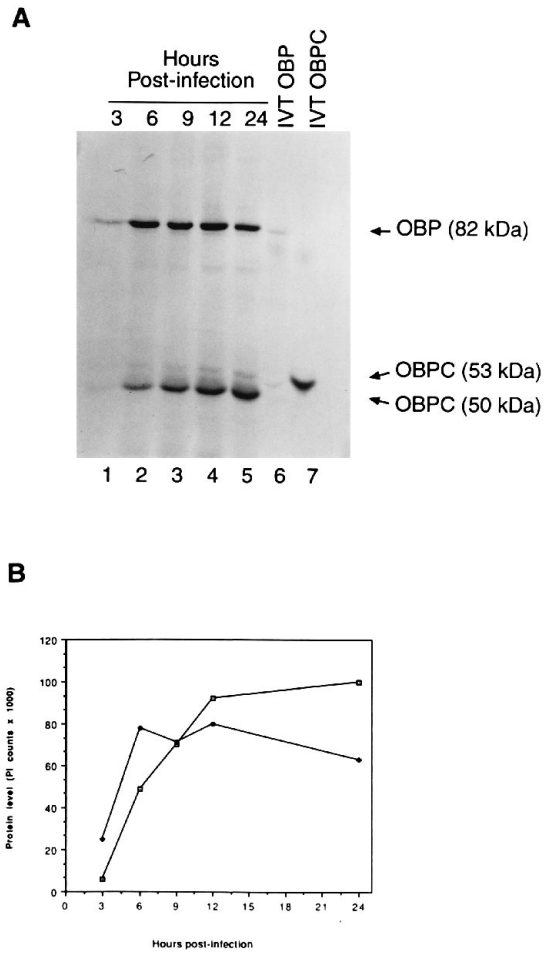

FIG. 3. Kinetics of OBP and OBPC expression. (A) Western blot of nuclear extracts from infected cells (10 PFU/cell) harvested at 3, 6, 9, 12, and 24 h postinfection (lanes 1 to 5, respectively), in vitro-translated OBP (lane 6), and in vitro-translated OBPC (lane 7). (B) Graph of protein levels as quantified by PhosphorImager analysis of the data shown in Fig. 3A. □, OBPC; ■, OBP.

tion of OBP would be sensitive to PAA. With the discovery that a delayed-early (and hence PAA-sensitive) HSV-1 protein (OBPC) is encoded by the C-terminal DNA-binding half of the OBP ORF, we asked whether OBPC is the viral protein in complex A that is recognized by antibody to OBP. For this purpose ^{35}S-labeled proteins were synthesized in vitro and analyzed by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (3) to assess their sizes and stabilities. Full-length OBP generated from plasmid pSP6UL9 migrated with a molecular mass of approximately 82 kDa (Fig. 4A, lane 2), consistent with published data (8, 19, 39, 40). Several faster-migrating minor bands were also observed. OBPC, generated from plasmid pSP6UL8.5, migrated with a mobility of approximately 53 kDa (Fig. 4A, lane 3). Mutant OBPC, generated from plasmid pSP6UL8.5ΔNco (Fig. 1), migrated with a molecular mass of approximately 48 kDa (Fig. 4A, lane 4), consistent with the size of the deleted sequence relative to OBPC (Fig. 1). No ^{35}S-labeled proteins were detected when no RNA was added to the in vitro translation mix (Fig. 4A, lane 1).

To determine whether OBPC synthesized in vitro can bind to recognized OBP binding sites, mobility shift assays were conducted in which in vitro-synthesized OBPC was incubated with radiolabeled oriS site I DNA essentially as described previously (14, 24) (Fig. 4B and C). In vitro-translated OBPC formed a complex identical in mobility to complex A seen when infected cell extracts were used as the source of protein and site I DNA was used as the probe (Fig. 4B, lanes 3 and 4). Complex B was not observed with in vitro-translated OBPC, indicating that complex B contains OBP but not OBPC. The specificity of the OBPC-site I complex was demonstrated by the ability of antibody to the C terminus of OBP to supershift this complex (Fig. 4B, lane 5). In contrast, no complex was observed when OBPCΔNco was used in gel shift assays (Fig. 4B, lane 6), consistent with the previous observation that the sequences missing in OBPCΔNco are required for OBP DNA binding (2, 16, 33). The results obtained with oriL site I DNA were indistinguishable from those observed using oriS site I DNA (data not shown).

The binding specificity of OBPC was further demonstrated by competition analysis (Fig. 4C). Radiolabeled site I DNA was incubated with in vitro-translated OBPC and increasing concentrations of unlabeled site I DNA or nonspecific competitor DNA (NS). Although a 10-fold excess of unlabeled site I DNA reduced complex formation by greater than 80%, a 10-fold excess of unlabeled nonspecific competitor produced only a minor reduction in OBPC complex formation (Fig. 4C, compare lanes 4 and 5). These results are consistent with previous analyses in which the formation of complex A using infected cell extracts and site I DNA was reduced in the presence of excess specific competitor (14). Furthermore, in vitro-synthesized OBPC failed to form a complex with radiolabeled site III DNA (data not shown), consistent with the observation that complex A is not seen when infected cell extracts are incubated with radiolabeled site III DNA (14). This is the first demonstration that a naturally occurring viral protein, OBPC, forms a specific complex of identical mobility to that of complex A observed with site I DNA and infected cell extracts.

An ~35-kDa polypeptide consisting of the N-terminal 10 amino acids of OBP fused to the C-terminal 317 amino acids (9CT, Fig. 1) has been shown to inhibit viral DNA replication in transient cotransfection experiments (49) and in recombinant baculovirus-infected cells (48). This polypeptide is also able to inhibit virus replication in virus-infected cells when expressed constitutively in cultured cells (41) and in cells cotransfected with plasmid p9CT and infectious viral DNA (49). To test whether the 50-kDa OBPC protein can inhibit virus replication, infectious viral DNA (56) was cotransfected with plasmids expressing OBP, OBPC, or subsets of OBPC sequences. A plasmid containing only vector sequences was included as a negative control (pCMV, Fig. 1). The effects of two concentrations of each plasmid on the plaque-forming ability of infectious HSV-1 DNA (equivalent to ~30 PFU) were determined. The results of one representative experiment and the average of three independent experiments are presented in Table 1.

Approximately 30 plaques were produced when plasmid pCMV or pCMVOBPC$^-$ (inverted OBPC ORF) was cotransfected with infectious viral DNA at either plasmid concentration tested. The addition of 0.2 μg of plasmid pCMVOBPCΔNco did not affect the number of plaques formed, whereas a slight increase in plaque numbers was observed with 1.0 μg of this plasmid in a single experiment. Thus, plaque formation by infectious viral DNA was not affected by vector alone (pCMV), plasmid DNA containing the OBPC ORF in the inverted orientation (pCMVOBPC$^-$), or plasmid DNA expressing OBPC lacking the DNA binding domain. In contrast, the addition of 0.2 or 1.0 μg of plasmid pCMVOBPC$^+$ resulted in 50 and 75% inhibition of plaque formation, respectively (Table 1). As shown previously, both pCMVUL9 (expressing OBP) and

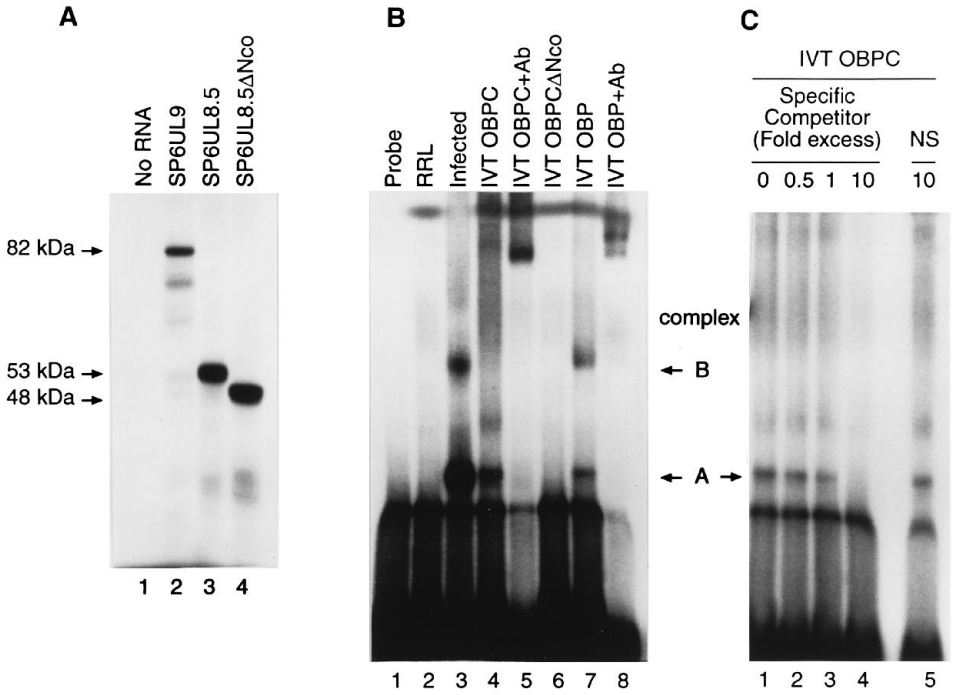

FIG. 4. Properties of in vitro-translated (IVT) proteins. (A) SDS-polyacrylamide gel electrophoresis analysis. No RNA (lane 1), SP6UL9 RNA (OBP, lane 2), SP6UL8.5 RNA (OBPC, lane 3) or RNA synthesized from plasmid pSP6ΔNco, which contains a 52-amino-acid deletion in the DNA binding domain of OBPC (lane 4). (B) Gel mobility shift analysis using site I DNA. Radiolabeled oriS site I DNA was incubated alone (lane 1) or with 1 μl of programmed rabbit reticulocyte lysate containing no exogenous RNA (lane 2), infected cell extracts harvested at 16 h postinfection (2 μg, lane 3), in vitro-synthesized OBPC (lanes 4 and 5), in vitro-synthesized ΔNco (lane 6), or in vitro-synthesized OBP (lanes 7 and 8). Antibody directed against the C terminus of OBP was added to the incubation mixture where indicated (lanes 5 and 8), and protein-DNA complexes were resolved by electrophoresis. (C) Competition analysis. In vitro-translated OBPC was incubated with radiolabeled oriS site I DNA in the presence of increasing concentrations (0 to 10-fold molar excess) of unlabeled oriS site I competitor DNA (lanes 1 to 4) or a 10-fold excess of a nonspecific competitor (NS, lane 5).

p9CT significantly reduced the efficiency of plaque formation (49). Thus, at the plasmid concentrations tested, OBPC was able to inhibit plaque formation by infectious HSV-1 DNA, albeit less efficiently than either OBP or 9CT.

Because OBPC binds specifically to site I DNA and inhibits plaque formation by infectious viral DNA, we attempted to determine whether OBPC affects the efficiency of origin-dependent viral DNA replication from oriS. The effect of OBPC was tested by protein-protein competition analysis using a transient DNA replication assay in which plasmids expressing all seven essential viral DNA replication proteins were cotransfected with an oriS-containing test plasmid, pOS822 (54, 55). The plasmid containing the OBPC ORF in the positive orientation (pCMVOBPC$^+$) inhibited pOS822 replication by 65%, whereas the vector plasmid alone (pCMV) and the plasmid containing OBPC sequences in the negative orientation (pCMVOBPC$^-$) had no effect on the level of pOS822 DNA replication (Fig. 5A and B). As anticipated, the mutant form of OBPC lacking the DNA binding domain (pCMVOBPCΔNco) had no inhibitory effect, but rather had a stimulatory effect, on pOS822 replication, consistent with its ability to stimulate plaque formation (Table 1). Thus, the ability of OBPC to bind to DNA is important for its ability to inhibit origin-dependent DNA replication. Finally, pCMVUL9 and p9CT reduced replication by 68 and 91%, respectively (Fig. 5A, lanes 5 and 6, and 5B), as initially demonstrated by Stow et al. (49) and consistent with the findings presented in Table 1. This experiment was repeated twice with essentially the same results. Collectively, these findings are consistent with the hypothesis that OBP, OBPC, and the protein expressed by plasmid p9CT can inhibit origin-dependent DNA replication, and hence plaque formation by infectious viral DNA, by a mechanism involving sequence-specific binding to origin DNA.

TABLE 1. Effect of OBPC on plaque formation by infectious HSV-1 DNA

Plasmid	No. of plaques			
	Representative expt[a]		Avg of 3 expt	
	0.2 μg	1.0 μg	0.2 μg	1.0 μg
pCMVOBPC$^-$	28	26	29 ± 1	28 ± 5
pCMVOBPC$^+$	15	6	18 ± 3	4 ± 2
pCMVUL9	3	1	3 ± 2	1 ± 1
p9CT	0	0	0	0
pOBPCΔNco	32	47	—[b]	—
pCMV	31	25	—	—

[a] Vero cells were cotransfected with 0.2 or 1.0 μg of the indicated plasmid and 500 ng (~30 PFU) of infectious KOS DNA by the calcium chloride method. Transfected cells were overlaid with medium containing methylcellulose and stained with neutral red, and on day 5 posttransfection, plaques were counted. The results of one representative experiment and the average of three independent experiments are indicated.

[b] —, not done.

Attempts to reconstitute an in vitro HSV-1 origin-dependent DNA replication assay using the seven essential replication proteins in purified form have been unsuccessful. Since six of the seven proteins (all but OBP) can replicate DNA by an origin-independent mechanism (26, 42), the block in origin-dependent DNA replication using all seven proteins likely occurs at the initiation step. These observations indicate that

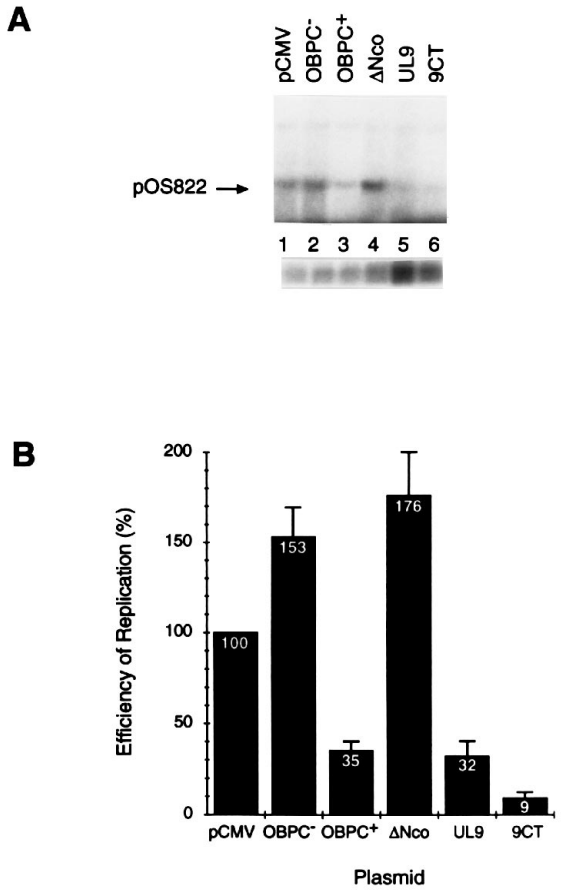

FIG. 5. Effect of OBPC on origin-dependent DNA replication. (A) Southern blot analysis of replicated plasmid DNA. Vero cells were cotransfected with 1 μg each of the indicated plasmids and 1 μg each of the following plasmids: the oriS-containing plasmid pOS822 (54), plasmids pCW8, pMC160-1, pMC160-2, pNN1, pNN3, pNN4, pNN5 (55), pSH (ICP0) (8a), pN11 (ICP4) (16a). Replication was analyzed by *Dpn*I digestion of cellular DNA and Southern blot analysis (top). Input DNA was analyzed by *Mbo*I digestion (bottom). The position of replicated pOS822 is indicated. Lanes: 1, pCMV; 2, OBPC$^-$; 3, OBPC$^+$; 4, ΔNco; 5, OBP; 6, 9CT. (B) Graph of replication efficiency. The replication efficiency of pOS822 (see panel A) was normalized to transfection efficiency (input DNA as determined by *Mbo*I digestion) and set relative to pCMV as 100%. Standard error bars are shown.

factors in addition to OBP may be required for initiation. One such factor may be OF-1, a cellular factor whose binding is critical for efficient origin-dependent DNA replication in cell culture (13, 14). A second such factor may be OBPC. In this study we have shown (i) that OBPC is a virus-coded nuclear protein able to bind to the same DNA sequence element as OBP in site I of oriS and oriL and (ii) that in vitro-translated OBPC forms a complex with site I (but not site III) DNA identical to infected-cell complex A.

To date, studies of origin-dependent HSV-1 DNA replication have been conducted without the knowledge that two HSV-1-encoded origin-binding proteins, OBP and OBPC, exist. Because the gene encoding OBP encodes the OBPC gene in its entirety, OBPC was likely present in all DNA replication assays conducted in vitro or using infected cell extracts. By extension, studies that employed mutant forms of OBP likely included mutant forms of OBPC. As a result, it is likely that the "OBP$^-$" phenotypes reported are a consequence of mutations in OBP or both OBP and OBPC. For this reason, isolation of OBPC$^-$ OBP$^+$ and OBPC$^+$ OBP$^-$ mutant viruses will be required to define the roles of these two proteins in DNA replication. Efforts to isolate viruses containing the desired mutations are ongoing.

Because OBPC$^-$ OBP$^+$ and OBPC$^+$ OBP$^-$ viruses have not been isolated, little information concerning the possible role of OBPC in viral DNA replication is available. One observation, however, suggests that OBPC may perform a critical role in origin-dependent viral DNA replication. In attempting to elucidate the roles of complexes A (OBPC), B (OBP), and M (OF-1) in transient DNA replication assays, 1- and 2-bp substitution mutations were introduced into the complex A, B, and M binding sites of site I DNA in an oriS-containing plasmid. These mutations decreased the formation of each of the three complexes significantly and differentially, as determined by gel-shift analysis (13). One mutation, R, disrupted the formation of complex A and decreased B formation by approximately threefold, but had minimal effects on the formation of complex M. The oriS test plasmid that contained the R mutation replicated only 3% as efficiently as the wild-type plasmid, suggesting that the binding of both OBP and OBPC to site I DNA is critical for origin-dependent DNA replication (13).

Critical for an understanding of the mechanism of HSV-1 DNA replication, and of the potential role of OBPC in this process, is a consideration of the structure of replicative intermediates. Recent studies using pulse-field gel electrophoresis and two-dimensional agarose gel electrophoresis have demonstrated that, like bacteriophage lambda (4, 44, 46), HSV-1 DNA is likely replicated by a theta mechanism early in infection and by a rolling circle mechanism at later times (31, 45). Implicit in this model is a mechanism by which the DNA replication apparatus is modified so that theta replication ceases and rolling circle replication commences. OBPC may function to mediate this switch. In this study we have shown that OBPC can inhibit origin-dependent viral DNA replication. A hypothesis consistent with available information is that by binding to its cognate DNA binding sites at viral origins, OBPC serves to inhibit de novo initiation by competing with OBP for binding. Because OBPC does not contain the N terminus of OBP, it lacks both the helicase function believed to be required for destabilization of duplex DNA at HSV origins and the sites of interaction with other proteins (the products of the UL8 and UL42 genes) in the DNA replication complex. Thus, although OBP is likely required for de novo initiation, OBPC may serve to block de novo initiation, thereby allowing passage of the replication complex through the origin and facilitating the switch from theta to rolling circle replication.

Additionally or alternatively, newly replicated genomes whose origin binding sites are occupied by OBPC and not OBP would not serve as templates but rather would be directed to the packaging machinery. Bacteriophage ϕx174 utilizes such a mechanism to direct newly synthesized DNA to the packaging machinery (1). The ϕx174-encoded gene C protein, which inhibits further rounds of replication following synthesis of the viral DNA strand, is specifically recognized by gene J protein and the prohead (1) and thus serves to direct newly synthesized viral DNA to the packaging machinery. Identification of the proteins that interact with OBPC would help to establish the veracity of either or both of these hypotheses. Notably, both hypotheses are consistent with the observation that OBPC is expressed somewhat later than OBP. The overexpression, purification, and characterization of OBPC in in vitro DNA replication assays and the isolation and characterization of OBPC$^-$ OBP$^+$ and OBPC$^+$ OBP$^-$ viruses will be useful in elucidating the roles of these two proteins in the HSV-1 life cycle.

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