A human cellular factor (OF-1) has been previously implicated in replication of herpes simplex virus, type 1. This protein binds to three conserved regions (Boxes I, II, and III) in the viral replication origin and appears to be required for viral DNA synthesis (Dabrowski, C. C., Carmillo, P. J., and Schaffer, P. A. (1994) Mol. Cell. Biol. 14, 2545-2555). In the present study, we have partially purified and characterized OF-1 from human cells. This protein appears to consist of a tetramer composed of two heterodimers with subunits of 73 and 90 kDa. The smaller subunit contains the DNA binding activity. We have investigated the binding specificity of OF-1 using a mobility shift analysis. These studies reveal that binding is specific for both duplex and single-stranded Box I sequences and that the strongest preference is for the bottom strand of Box I. We present evidence suggesting that the binding of OF-1 to Box I DNA is enhanced in the presence of the herpes simplex-encoded UL9 protein, which also binds to Box I in oriS and is required for viral replication. Implications of these findings for the initiation step in viral replication are discussed.

Initiation of DNA replication in eukaryotic cells occurs within localized chromosomal regions and involves binding by a series of origin-binding proteins. Although initiation occurs at specific DNA sequences in yeast (Saccharomyces cerevisiae) (1), the corresponding process in higher eukaryotes is less well understood (2), and only recently has evidence for the existence of specific origin sites in mammalian cells been presented (3). Hence, studies of human DNA viruses, such as herpes simplex virus, type 1 (HSV-1),  have been useful as models for understanding the role of origin recognition sequences in the initiation of DNA synthesis in human cells.

HSV-1 replication (reviewed in Refs. 4 and 5) initiates at one of three viral replication origins, called oriL and oriS (present in two copies). This process requires the viral initiator protein, UL9, which binds the origins in a sequence-specific manner. This protein appears to consist of a tetramer composed of two heterodimers with subunits of 73 and 90 kDa. The smaller subunit contains the DNA binding activity. We have investigated the binding specificity of OF-1 using a mobility shift analysis. These studies reveal that binding is specific for both duplex and single-stranded Box I sequences and that the strongest preference is for the bottom strand of Box I. We present evidence suggesting that the binding of OF-1 to Box I DNA is enhanced in the presence of the herpes simplex-encoded UL9 protein, which also binds to Box I in oriS and is required for viral replication. Implications of these findings for the initiation step in viral replication are discussed.

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Viral replication also appears to require host factors, since attempts to establish replication of oriS-containing plasmids in vitro have been unsuccessful (discussed in Ref. 4). A recently identified human host protein, OF-1 (8), appears to be one of these required factors. This possibility is based on observations that OF-1 specifically binds the viral replication origins at the Box I, II, and III sites and that plasmids carrying oriS mutations that inhibit OF-1 binding, but not UL9 binding, fail to replicate efficiently in virally infected cells. Hence, OF-1 likely plays a role in viral replication in vivo.

The binding properties of OF-1 implicate this factor during the initiation step of replication. OF-1 specifically binds within oriS (8) to sequences that overlap the sites bound by UL9. The finding that OF-1 interacts with multiple origin sites suggests that OF-1, like UL9, may also participate in a nucleoprotein complex at the origin. However, although both OF-1 and UL9 bind with the highest affinity to the Box I site, their relative affinity for the other boxes differs significantly (4, 8). Binding by OF-1 shows decreasing relative affinities of Box I > Box III > Box II, whereas binding by UL9 shows affinities of Box I > Box II > Box III. Thus, the complexes formed by UL9 and OF-1 are likely to differ and may play distinct roles during viral replication.

We describe the first partial purification and physical characterization of OF-1 from human cells. We have characterized the binding specificity of this protein, and we find that it binds to the Box I site in oriS in a manner that involves specific interactions with one of the two DNA strands. This binding appears to be enhanced in the presence of UL9. Our results support the possibility that OF-1 participates in the initiation step of viral replication.

EXPERIMENTAL PROCEDURES

Oligonucleotides—Oligonucleotides (Genosys Biotechnologies, Inc.) had the sequences shown in Fig. 1. Duplex oligonucleotides were prepared by mixing the complementary top and bottom strands for a given set of oligonucleotides in 10 mM Tris-HCl (pH 7.6) and 1 mM EDTA, heating the mixture to 95 °C, and allowing the mixture to cool slowly to room temperature. Where indicated, duplex or single-stranded oligonucleotides were labeled at their 5’ ends with [γ-32P]ATP by polynucleotide kinase (9). Unincorporated radioactivity was removed using either NEN-Sorb-20 cartridges (New England Nuclear) or Sephadex G-25 spin columns (9). Homopolymer DNA (Amersham Pharmacia Biotech) consisted of poly(dC)-oligo(dG)12-18.

Purification of UL9—UL9 protein was overexpressed in Autographa california-infected SF9 cells, purified as described (10), and stored at −80 °C in UL9 storage buffer, containing 20 mM HEPES (pH 8.3), 20% (v/v) glycerol, 1 mM EDTA, 0.5 mM dithiothreitol (DTT). Purity was 95% as determined by densiometry of a Coomassie-stained SDS-polyacrylamide gel loaded with approximately 6 μg of protein.

Purification of OF-1—Throughout the purification, OF-1 activity was assessed by gel mobility shift analysis by using a series of labeled molecular marker oligonucleotides.
monitored by a mobility shift assay showing specific binding to a duplex Box I oligomer (see “Mobility Shift Assays in Non-denaturing Gels”), and protein concentrations were determined by the method of Bradford (11). Where indicated, the species of protein present in a given fraction were visualized by electrophoresis on 6% polyacrylamide gels containing SDS, followed by silver staining (9). Gels were scanned by a Hewlett-Packard optical scanner to create a digitized image. Where required, the species of protein present in OF-1-specific bands was determined relative to the sum of the intensities of all bands within the lane, using the NIH Image program.

Ammonium Sulfate Precipitation—Suspension-grown HeLa S3 cells (National Cell Culture Center) were obtained as cell pellets that had been washed twice in 2.8 mM NaH2PO4, 13.6 mM Na2HPO4, 145 mM NaCl. Pellets totaling 2.3 × 10^8 cells were resuspended in 10 ml of Buffer A, containing 50 mM Tris-HCl (pH 7.8), 100 mM NaCl, 1 mM EDTA, 20% (v/v) glycerol, and supplemented with 20% (w/v) (NH4)2SO4. Each extraction was followed by centrifugation. The four supernatants were then combined, mixed with (NH4)2SO4 to a final concentration of 20% (w/v), held on ice for 15 min, and centrifuged as above. The supernatant was collected. To recover additional OF-1, the pellet was re-extracted three times with 1 ml of Buffer B, containing 20 mM HEPES (pH 7.6), 0.5 mM DTT, 50 mM NaCl, 1 mM EDTA, 20% (v/v) glycerol, and supplemented with 20% (w/v) (NH4)2SO4 (10 to 0% in Buffer B). OF-1 eluted between 150 and 500 mM NaCl. Fractions containing peak OF-1 activity were pooled and stored at −80 °C.

**Host Factor OF-1**

**Oligonucleotides:**

| TOP Strands | BOTTOM Strands |
|-------------|----------------|
| 5′-GGGTGCAACCTCGCTCCAACT-3′ | 3′-CCCATCCGCGCACTCTTCCGTGGTCTG-5′ |
| 5′-GGGTAAAGAAGTGAGAAGCC-3′ | 3′-CCGTTCGCACTCTGTCCAGCAGTACC-5′ |
| 5′-CGAAGGTCTCGGACATCGTCCG-3′ | 3′-CGACGCTGCTGAGCTGAGTGC-5′ |
| 5′-CCACGCGTCTGCGAGCTGAG-3′ | 3′-CGACGCTGCTGAGCTGAGTGC-5′ |

**Sequence of oriS:**

Box I → Box III → Box II

**Calculation of Binding and Kinetic Parameters—**Amounts of bound and unbound oligomer from electrophoretic analyses on non-denaturing (mobility shift assays) and denaturing (cross-linking assays) gels were determined using ImageQuant. Where more than one bound complex was observed (e.g. in cross-linking analyses), the intensities of the bound complexes were combined. As appropriate, lines of best fit were applied to the data points, using the curve-fit function of Kaleidograph (Synergy Software). Where indicated, Scatchard plots were produced, and Kd or Ka,app values were calculated from the negative reciprocals of the slopes of the lines. For analysis of the kinetic data from the ATPase assay, the data were fit to a sigmoidal curve derived from a rearrangement of the Hill equation (13). The equation used was $v = \frac{V_{max}[S]}{1 + \left(\frac{K_d}{[S]}\right)^n}$.
Host Factor OF-1

OF-1 was purified by ammonium sulfate precipitation and chromatography on the indicated matrices, as described under "Experimental Procedures." After each step, peak OF-1 fractions were pooled and assayed for protein concentration and specific activity. To measure specific activity (nmol of DNA bound/mg of protein), protein was diluted and assayed for a mobility shift using a 32P-labeled duplex Box I oligomer (12.5 nM), and the fraction of shifted DNA was determined from scans of the gels. For each measurement, several protein dilutions were used, and specific activity was determined with those dilutions that showed a linear increase in binding proportional to concentration. Since nucleases may have been present during purification and since the DNA concentrations used did not exceed the subsequently determined $K_d$ value for binding; the specific activity determined at each step is a minimal estimate of activity.

### Table I

| Purification step  | Total protein (mg) | Total activity (nmol DNA bound/mg protein) | Specific activity (fold) |
|--------------------|-------------------|------------------------------------------|-------------------------|
| Crude extract      | 787.5             | 31.5 (1.00)                              | 0.040                   |
| Ammonium sulfate   | 99.0              | 47.7 (1.51)                              | 0.482                   |
| Phenyl-Sepharose   | 51.3              | 76.7 (2.43)                              | 1.50                    |
| Phosphocellulose   | 1.70              | 63.1 (2.00)                              | 37.1                    |
| Heparin-Sepharose  | 0.0182            | 2.48 (0.08)                              | 136                     |

$K_v + [S]^n$, where $v$ = reaction velocity, $V_{max}$ = maximum reaction velocity, $K_{v0}$ = substrate concentration at one-half $V_{max}$, $[S]$ = substrate concentration, and $n$ = the Hill coefficient, a measure of the cooperativity of the system.

### RESULTS

**Purification of OF-1**—To gain insight into the role of host factor OF-1 in HSV-1 replication, we have purified this factor from human cells and characterized it with respect to polypeptide composition, DNA binding specificity, and its potential interaction with the HSV-1 origin-binding protein, UL9.

OF-1 activity was isolated from HeLa cell extracts as shown in Table I and was purified 3400-fold. It was detected by its ability to produce a mobility shift during electrophoresis of a duplex Box I oligomer. This activity remained in the supernatant after ammonium sulfate (20% w/v) precipitation of crude extracts. Further purification was achieved by sequential chromatography on phenyl-Sepharose, phosphocellulose, and heparin-Sepharose, as shown in Fig. 2, panel A. We noticed that early steps in the purification resulted in an increase in total activity (see Table I), most likely due to removal of contaminating nucleases. Heparin-Sepharose chromatography resulted in substantial loss of activity but removed several major contaminating polypeptides. Since only a single peak of activity was recovered from this purification step (see Fig. 2, panel A), and since we observed only a single band in our mobility shift assay throughout the purification (not shown), we believe that OF-1 represents a single DNA binding activity.

The protein species present after each purification step were analyzed by denaturing gel electrophoresis, as shown in Fig. 2, panel B. Four major polypeptide species predominated after heparin-Sepharose purification (Fig. 2, panel B, lane 5). As discussed below, OF-1 consists of two of these polypeptides (73 and 90 kDa), designated by arrows in Fig. 2, panel B. Based on densitometry scans of these protein bands, we estimate that the OF-1 polypeptides comprised 28% of the total protein in the heparin-purified sample.

**OF-1 Contains Two Subunits and May Exist as a Tetramer**—To determine which of the peptides in our OF-1 preparation are involved in binding to HSV-1 origin (Box I) sequences, we employed a modified two-dimensional electrophoresis protocol. OF-1 protein was first run on a non-denaturing gel in the presence of labeled duplex Box I oligomer under mobility shift conditions. Polypeptides associated with the shifted band were then separated by denaturing gel electrophoresis. As shown in Fig. 3, this analysis resulted in recovery of two polypeptides of 73 and 90 kDa. These peptides correspond to two of the four species seen in the purified OF-1 preparation (arrows in Fig. 2, panel B). Densitometry of the OF-1-associated bands showed that the two polypeptides are present in approximately equimolar amounts, suggesting that OF-1 exists as a heterodimer.

Further insight into the subunit structure of OF-1 was obtained using gel filtration chromatography. A 32P-labeled duplex Box I DNA oligomer was chromatographed on Sepharose CL-4B either alone or after being cross-linked to OF-1. As shown in Fig. 4, a comparison of these two chromatograms revealed a large peak from both columns representing the free DNA and two additional smaller peaks from the column loaded with the cross-linked material. These smaller peaks presumably represent OF-1-DNA complexes and exhibit apparent mo-
masses (in kDa) of protein standards run on the same column. DNA (OF-1 protein that had been cross-linked to radioactive duplex Box I (kDa) that were run on the same gel. Stained material (to described previously (30). The scan (using NIH Image to create a graphical plot) of the region of the stained gel containing the OF-1-associated peptides.

FIG. 3. Composition of OF-1. Heparin-purified OF-1 protein (3 μg) was subjected to a modified two-dimensional gel electrophoresis protocol, as described under “Experimental Procedures.” Arrows indicate the positions of the two major peptides recovered and correspond to molecular masses of 73 (right) and 90 (left) kDa, based on standard markers (kDa) that were run on the same gel. Stained material (to right of arrows) is an artifact that appeared across the gel, even in the absence of protein, and may be due to formation of an SDS-complex (60–70 kDa) described previously (30). The inset below the gel depicts an intensity scan (using NIH Image to create a graphical plot) of the region of the stained gel containing the OF-1-associated peptides.

FIG. 4. Gel filtration chromatography of OF-1. Heparin-purified OF-1 protein that had been cross-linked to radioactive duplex Box I DNA (●) or unbound DNA (○) was applied to a Sepharose CL-4B column, and the radioactivity of the column fractions was measured, as described under “Experimental Procedures.” Arrows indicate molecular masses (in kDa) of protein standards run on the same column.

FIG. 5. OF-1 binds specifically to Box I DNA. Aliquots (0.175 μg) of heparin-purified OF-1 protein (approximately 0.3 pmol) were incubated with 12.5 nM 32P-labeled Box I DNA; the reaction products were run on a non-denaturing polyacrylamide gel, and the gel was analyzed by autoradiography as described under “Experimental Procedures.” Labelled DNA was either duplex (lanes 1–4), containing both strands of Box I, or single-stranded (lanes 5–7), containing the bottom strand of Box I. Two additional reactions contained labeled duplex Box I (lane 8) or duplex Alternate (Alt) Box I (lane 9). Reactions were run either in the absence of competitor DNA (lanes 1, 5, 8, and 9) or in the presence of a 10-fold molar excess of unlabeled competitor DNA consisting of duplex Box I (lane 2), a randomized duplex oligomer (Random Box I) (lane 3), poly(dC)-oligo(dG) homopolymer (lane 4), single-stranded Box I bottom strand (lane 6), or duplex Random Box I that had been denatured by boiling, quick cooled to 4 °C, and maintained on ice prior to addition (lane 7). Molar excesses of competitor were calculated with respect to the number of molecules in the case of Box I or randomized oligomers or the number of nucleotides in the case of the homopolymer. Sequences of the Box I oligomers and strand designations are shown in Fig. 1.

go(dG). Reaction products were then analyzed by non-denaturing polyacrylamide gel electrophoresis and autoradiography.

Consistent with the previous results of Dubrowski et al. (8), we find that OF-1 binds specifically to duplex Box I DNA. As shown in Fig. 5, OF-1 bound to the labeled Box I probe yielding a single-shifted band, in the absence of competing DNA (lane 1). Specificity of binding was revealed in competition experiments that showed no shift when the competing DNA was the same duplex Box I sequence (lane 2) but a complete shift when the randomized duplex oligo (lane 3) or poly(dC)-oligo(dG) homopolymer (lane 4) was used.

Our results also indicate that OF-1 binds specifically to single-stranded Box I sequences. For this analysis, we conducted similar mobility shift experiments to those described above, except that the labeled probe consisted of the bottom strand of Box I, and the competing DNA was also single-stranded. As shown in Fig. 5, in the absence of competition, OF-1 bound to the single-stranded probe producing a mobility shift (lane 5). This binding is specific, since it was eliminated by an excess of unlabeled Box I bottom strand (lane 6) but not by an excess of the randomized duplex oligomer which had been denatured prior to addition to the reaction (lane 7).

A similar mobility shift analysis with a related Box I sequence revealed that residues at the 3’ end of Box I are critical for binding. This related sequence (called Alternate Box I in Fig. 1) is shifted to the left relative to the original Box I sequence and, hence, lacks three nucleotides from the right end (AAT).
As shown in Fig. 5, whereas OF-1 bound strongly to Box I DNA (lane 8), it bound very poorly to this alternate duplex (lane 9). This result is significant since Dabrowski et al. (8) showed that OF-1 from crude extracts fails to bind an oligomer carrying deletions or mutations in the residues CAAT at the 3’ end of the Box I sequence. They further showed that base substitutions at the 3’ CA residues eliminated replication of oriS-containing plasmids transfected into HSV-1-infected cells. Our finding indicates that our purified OF-1 retains a binding requirement for the 3’ CAAT residues in this sequence and further suggests that these residues may be critical for OF-1 function during viral replication.

**Fig. 6. Determination of dissociation constants (K_d values) of OF-1 binding to DNA.** Aliquots (0.29 ng) of heparin-purified OF-1 were incubated with 0.005–10.5 ng of 32P-labeled DNA oligomers (0.11–167 nt). Oligomer sequences and strand designations are shown in Fig. 1. The reaction mixtures were run on native polyacrylamide gels and analyzed as described under “Experimental Procedures.” The concentration of bound DNA for each reaction was determined, and the K_d values were obtained from Scatchard plots of the data.

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**OF-1 Binds Box I Sequences Preferentially Over Box III Sequences and Shows the Highest Overall Affinity for the Box I Bottom Strand**—To define and quantitate the binding preferences of OF-1, we conducted the experiments shown in Fig. 6. Mobility shift assays similar to those described above were performed, and dissociation constants (K_d values) were calculated from Scatchard plots of the data. The plots obtained were biphasic (except for panel F), suggesting that OF-1 contains two sites with different affinities for DNA. Since the K_d value for the higher affinity site in each case is substantially lower than that for the second site, we have reported only the higher affinity values.

First, we examined the preference of OF-1 for Box I and III duplex sequences, since Dabrowski et al. (8) had previously shown that OF-1 from crude extracts binds preferentially to Box I duplexes. Our results confirm this finding using partially purified OF-1 and indicate that OF-1 has a 3.2-fold higher affinity for duplex Box I (panel A) than for duplex Box III (panel D) (K_d = 24 nM and 78 nM, respectively).

Next, we compared the binding affinities of OF-1 for duplex and single-stranded Box I DNAs. This comparison reveals that OF-1 binds with the strongest affinity to the bottom strand of Box I. The K_d value for binding to the Box I bottom strand was 10 nM (panel C) and was 2.4-fold lower than that seen for duplex Box I (K_d = 24 nM, panel A) and 6.2-fold lower than seen for the top strand of Box I (K_d = 62 nM, panel B). Hence, we suspect that OF-1 may prefer a single-stranded Box I site for binding in vivo.

Finally, we observed that OF-1 also binds to single-stranded Box III sequences but greatly prefers the top strand. In this case, we obtained K_d values of 61 nM for the Box III top strand (panel E) and 780 nM for the bottom strand (panel F). Since Box I and Box III are inverted with respect to each other in oriS (see Fig. 1), OF-1 shows a preference for the equivalent strand in each case. The affinity of OF-1 for the preferred Box III strand is higher than that seen for the Box III duplex DNA, although the preference in this case is not as striking as that seen with Box I DNA.

**The 73-kDa Subunit of OF-1 Is the DNA Binding Subunit**—To identify the polypeptide(s) in OF-1 that directly contact DNA in OF-1-DNA complexes, we cross-linked OF-1 to 32P-labeled duplex Box I or Box III oligomers, boiled the complexes in SDS to denature the protein and DNA, and analyzed the products by SDS-polyacrylamide gel electrophoresis and autoradiography, as shown in Fig. 7, panel A. OF-1 formed three major bands following cross-linking to either Box I or Box III DNA (lanes 2 and 7, respectively). The most intense band (lowest arrow) had an apparent molecular mass of 75 kDa, suggesting that it represents a complex between the smaller OF-1 subunit (73 kDa) and a single DNA strand (6.6 kDa). Most likely, this DNA strand is the bottom strand in the case of Box I and the top strand in the case of Box III, since OF-1 preferentially interacts with these specific strands. A second band migrated with an apparent molecular mass of 82 kDa, consistent with a complex between the smaller OF-1 subunit and two DNA strands. The third band was broad with a maximum molecular mass of 168 kDa. We suggest that this band consists of a complex between DNA (1 or 2 strands) and both the smaller (73 kDa) and larger (90 kDa) OF-1 subunits. Taken together, these results suggest that the smaller subunit binds DNA, whereas the larger subunit makes only incidental contact with the DNA through interaction with this smaller subunit.

**Effects of UL9 Protein on DNA Binding by OF-1**—Since the OF-1-binding site in Box I overlaps that of the viral UL9 protein (8, 14), we suspected that these proteins might interact at this site. In contrast, since UL9 binds poorly to Box III DNA (14), we did not predict an interaction at the Box III site. To test these possibilities, we measured the binding of OF-1 to Box I and Box III oligomers in the presence of UL9, using the cross-linking procedure described above.

As shown in Fig. 7, panel A, the OF-1 preparation forms cross-linked complexes with Box I in both the absence and presence of UL9 (compare lane 2 with lanes 3–5). The mobilities of these bands are the same in the absence and presence of UL9, suggesting that the same complexes are being formed in both cases. However, the intensities of the bands increase as the concentration of UL9 increases. We have quantitated these increases (as shown in Fig. 7, panel B) by determining the relative intensities of the major (75 kDa) OF-1-associated band.
Panel B, bands and are 95 and 208 kDa. OF-1-specific bands and correspond to molecular masses of 75, 82, and 168 kDa.

Of the Box I-associated bands cannot be compared directly with the determinations of protein purity in each preparation and the assumption that OF-1 exists as a heterodimer. Reactions were cross-linked and analyzed by SDS-polyacrylamide gel electrophoresis and autoradiography to determine the amounts of bound and unbound DNA. The data are presented in a Scatchard plot, and the $K_{d(app)}$ values are indicated for reactions conducted in the presence ($\Delta$) and absence (○) of UL9. (True $K_d$ values cannot be determined here because equilibrium conditions do not exist due to the cross-linking, but relative affinities can be estimated.) Reactions contained 0.175 $\mu$g of OF-1 (approximately 0.3 pmol), 0 or 1.2 pmol UL9, and 0–1.5 pmol of DNA (0–150 nM).

**FIG. 7.** Binding of OF-1 to Box I and Box III DNA and the effects of UL9. Panel A, 12.5 nm $^{32}$P-labeled Box I (lanes 1–5) or Box III (lanes 6–10) duplexes were incubated with either UL9 protein alone (1.2 pmol) (lanes 1 and 6), with 0.175 $\mu$g of heparin-purified OF-1 alone (approximately 0.3 pmol) (lanes 2 and 7), or with a constant amount of OF-1 (0.175 $\mu$g) and increasing quantities of UL9 (0.3–2.4 pmol). These latter reactions contained apparent UL9:OF-1 molar ratios of 1:1 (lanes 3 and 8), 4:1 (lanes 4 and 9), or 8:1 (lanes 5 and 10), based on our determinations of protein purity in each preparation and the assumption that OF-1 exists as a heterodimer. Reactions were cross-linked and analyzed by SDS-polyacrylamide gel electrophoresis and autoradiography, as described under “Experimental Procedures.” Since the specific radioactivities of the Box I and Box III DNAs differed and since the Box I and Box III experiments were conducted independently, the intensities of the Box I-associated bands cannot be compared directly with the Box III-associated bands. Molecular weights of each band were determined using standard protein markers run on the same gel (not shown). Arrows to the right of each gel panel mark the positions of the major OF-1-specific bands and correspond to molecular masses of 75, 82, and 168 kDa. Arrows to the left indicate molecular masses of UL9-specific bands and are 95 and 208 kDa. Panel B, the intensity of the OF-1-associated 75-kDa band in panel A is plotted versus the molar ratio of UL9:OF-1 for Box I (○) or Box III (△) DNA.

FIG. 8. The effect of UL9 on the apparent affinity of OF-1 for duplex Box I DNA. Heparin-purified OF-1 was mixed with duplex Box I DNA and, where indicated, an apparent 4:1 molar ratio of UL9 over OF-1. As described under “Experimental Procedures,” OF-1-DNA complexes were cross-linked by ultraviolet irradiation and analyzed by SDS-polyacrylamide gel electrophoresis and autoradiography to determine the amounts of bound and unbound DNA. The data are presented in a Scatchard plot, and the $K_{d(app)}$ values are indicated for reactions conducted in the presence (△) and absence (○) of UL9. (True $K_d$ values cannot be determined here because equilibrium conditions do not exist due to the cross-linking, but relative affinities can be estimated.) Reactions contained 0.175 $\mu$g of OF-1 (approximately 0.3 pmol), 0 or 1.2 pmol UL9, and 0–1.5 pmol of DNA (0–150 nM).

**Effect of OF-1 on UL9 Binding to Box I DNA**—As shown in Fig. 7, panel A, our cross-linking experiments are consistent with previous findings that UL9 binds strongly to Box I and poorly to Box III sequences (14). With Box I DNA (lane 1), we detected two UL9-DNA complexes (95 and 208 kDa), consistent with complexes containing a UL9 monomer (94 kDa) and dimer, respectively. We also observed that the vast majority of the Box I DNA was apparently trapped in higher order UL9 complexes and did not enter the gel, as previously reported (15). In contrast, few or no UL9-cross-linked products were observed when the Box III probe was used (lane 6). In the presence of our OF-1 preparation, we observed a large reduction in UL9-DNA complexes (especially those Box I complexes trapped in the wells), and we failed to observe any supershifted OF-1-associated bands indicative of a UL9-OF-1-DNA complex. These findings suggest that OF-1 may compete with UL9 for Box I binding.

**Effect of OF-1 on the ATPase Activity of UL9**—Our results in Fig. 7 suggested that OF-1 might compete with UL9 for binding to Box I DNA. Hence, to test the possibility that OF-1 might also interfere with UL9 enzymatic activities that require DNA binding, we assayed UL9 ATPase activity in the presence and absence of OF-1. This activity is specifically and potently stimulated by a DNA duplex containing Box I (12). As shown in Fig. 9, the presence of a 5-fold molar excess of OF-1 substantially reduced UL9 ATPase activity at low DNA concentrations, resulting in a 3.9-fold increase in the $K_{d(app)}$ over the value in the absence of OF-1. In contrast, relative $V_{max}$ values in the presence and absence of OF-1 were similar (0.81 and 0.88, respectively). Hence, our OF-1 preparation appears to affect the affinity of UL9 for Box I DNA without altering the catalytic activity of UL9.

The effect of UL9 on the apparent affinity of OF-1 for duplex Box I DNA. Heparin-purified OF-1 was mixed with duplex Box I DNA and, where indicated, an apparent 4:1 molar ratio of UL9 over OF-1. As described under “Experimental Procedures,” OF-1-DNA complexes were cross-linked by ultraviolet irradiation and analyzed by SDS-polyacrylamide gel electrophoresis and autoradiography to determine the amounts of bound and unbound DNA. The data are presented in a Scatchard plot, and the $K_{d(app)}$ values are indicated for reactions conducted in the presence (△) and absence (○) of UL9. (True $K_d$ values cannot be determined here because equilibrium conditions do not exist due to the cross-linking, but relative affinities can be estimated.) Reactions contained 0.175 $\mu$g of OF-1 (approximately 0.3 pmol), 0 or 1.2 pmol UL9, and 0–1.5 pmol of DNA (0–150 nM).
Host Factor OF-1

FIG. 9. The effect of OF-1 on the Box I-stimulated ATPase activity of UL9. ATPase activity of UL9 was measured as described under "Experimental Procedures" in the presence (○) and absence (△) of an apparent 5:1 molar ratio of phosphocellulose-purified OF-1 over UL9. The molar ratio is based on our determination of protein purity of each preparation and the assumption that OF-1 exists as a heterodimer. Reactions contained UL9 (6 nM), DNA (0–80 nM), and OF-1 (200 ng) or an equivalent volume of OF-1 buffer. $K_{\text{d}}$ values from these data are shown on the graph. For the reaction with UL9 alone, $K_{\text{d}} = 3.3 \pm 0.2$ nM, $V_{\text{max}} = 0.88 \pm 0.02$, and the correlation coefficient for the curve fit = 0.9656. For the reaction with UL9 and OF-1, $K_{\text{d}} = 9.3 \pm 0.6$ nM, $V_{\text{max}} = 0.81 \pm 0.03$, and the correlation coefficient = 0.9850.

**DISCUSSION**

This study examines the properties of OF-1, a human protein that has been implicated in the initiation of replication by HSV-1. Our results support a role for OF-1 in viral replication and suggest that OF-1 might participate in a nucleoprotein complex at the viral origin.

**DNA Binding Specificity of OF-1 and Formation of Origin Complexes**—Dabrowski et al. (8) previously showed that human cell extracts contain a factor, OF-1, that binds to Box I, II, and III regions within oriS and shows the strongest preference for Box I. We have partially purified this factor and have characterized its properties. Our results indicate that OF-1 binds specifically to duplex Box I, thus confirming previous studies using crude extracts (8). We also show that OF-1 exhibits a 3-fold lower affinity for Box III than for Box I.

A novel finding from our studies is that OF-1 binds specifically to single-stranded Box I DNA and shows a high affinity for a Box III single strand. Binding is significantly stronger for one of the two strands at each site (the bottom strand of Box I and the top strand of Box III). Since these strands contain functionally equivalent sequences, due to inversion of the Box I and III sequences within oriS, the binding to OF-1 in both cases may involve similar protein-DNA interactions. Furthermore, since OF-1 strongly prefers the single-stranded form of Box I over the duplex form of Box I, it seems likely that some of the OF-1 interactions with oriS in vivo involve single-stranded DNA. Although several eukaryotic proteins are known that bind single-stranded DNA at specific origin sequences (16–20), the target sequences of these proteins show no apparent correlation with the OF-1-binding sites.

The sequence specificity of OF-1 binding was demonstrated by binding studies with various single-stranded and duplex competitor DNAs. These experiments also rule out the possibility that OF-1 binds nonspecifically to generic DNA structures such as duplex DNA ends or single strands. In addition, our binding affinity assays show a significant decrease in the affinity of OF-1 for Box III relative to Box I, demonstrating that binding affinity is sequence-dependent. It is possible, however, that the observed specificity is partially due to a preference of OF-1 for a secondary structure stabilized by the sequence of Box I.

**The Quaternary Structure of OF-1 and Identification of the DNA Binding Subunit**—OF-1 appears to be a tetramer containing two identical heterodimers. Evidence for a heterodimeric structure is provided by a two-dimensional electrophoresis procedure that identified two OF-1-associated polypeptides (73 and 90 kDa) present in approximately equal molar amounts. Additional evidence for a heterodimeric structure is provided by determination of the sizes of cross-linked OF-1-DNA complexes by denaturing gel electrophoresis. The heterodimers appear to dimerize to form a tetrameric structure, as shown by the profile of cross-linked complexes observed during non-denaturing gel filtration chromatography. The smaller OF-1 subunit (73 kDa) is probably responsible for the DNA binding activity of this protein. This conclusion is based on our observation that the sizes of cross-linked products are consistent with complexes between DNA and either the heterodimer or the smaller subunit but never between DNA and the larger subunit.

The fact that OF-1 binds to multiple sites within the HSV-1 origin suggests that OF-1 may form a large nucleoprotein complex involving interactions between proteins bound at multiple sites. This possibility is further supported by our finding that OF-1 may exist in a tetrameric form. Initiation complexes involving protein-protein interactions at multiple DNA sites have been observed in other replication systems. For example, initiation in *Escherichia coli* and bacteriophage λ requires binding of initiator proteins (dnaA and O, respectively) at multiple origin sites (22, 23). Electron microscopy studies of the *E. coli* and λ systems reveal that the initiator proteins bound at different sites interact by protein-protein contacts to form a larger structure (22, 23). Similar complexes have been proposed for the HSV-1 initiator protein, UL9, that binds as a dimer to Boxes I and II origin sequences (24) and may form a larger complex via tetrameric interactions (reviewed in Ref. 4).

At least two possible hypotheses for the interaction of OF-1 with the full-length origin in vivo are suggested by the DNA binding preferences of OF-1. First, since OF-1 prefers Box I single-stranded DNA, it is possible that OF-1 binds to single-stranded DNA resulting from unwinding of the origin by UL9. The UL9 helicase activity is known to unwind a partial duplex DNA containing Box I in the presence of ATP and ICP8 (7) and to form extruded single-stranded loops of DNA upon binding to oriS in the presence of ATP (27). In the second hypothesis, OF-1 may bind to an alternative conformation of the origin, known as oriS*. This conformation, recently proposed by Aslani et al. (28), consists of an extruded stem-loop structure formed by the top strand of oriS. In this structure, which is stabilized by UL9 binding (28), the top strand of the Box I motif is paired with the top strand of Box II. An analogous stem-loop structure is presumably formed by the bottom strand of oriS. This analogous structure would contain the Box I bottom strand, which is the preferred strand for OF-1 binding, and, thus, could serve as an optimal binding site for OF-1.

**Possible Functional Interactions between OF-1, UL9, and oriS**—When OF-1 was cross-linked to Box I DNA in the presence of UL9, we obtained substantial increases in the yield of cross-linked products compared to similar reactions without UL9. Hence, UL9 appears to enhance DNA binding by proteins in the OF-1 preparation. We believe that the increased products consist of OF-1-DNA complexes, although we cannot rule out the possibility that UL9 promotes binding of other proteins in our OF-1 preparation. However, arguing against this possibility is our finding that the increased products have the same mobilities as OF-1-DNA complexes formed without UL9.
We suggest two possible mechanisms that might explain the putative functional interactions between OF-1 and UL9. On the one hand, UL9 may physically interact with OF-1 to increase its affinity for DNA. Alternatively, UL9 may alter the conformation of the DNA to favor OF-1 binding. Consistent with this latter possibility, UL9 causes an increase in cross-linking of our OF-1 preparation to Box I but not to Box III. Since UL9 binds strongly to Box I but poorly to Box III sequences (14, 25, 26), the enhancement effect appears to correlate with UL9 binding to the DNA.

Our experiments further suggest that UL9 is displaced from Box I DNA in the presence of our OF-1 preparation. Evidence for displacement is provided by our observations that the formation of UL9-DNA complexes is reduced in the presence of OF-1 and that the Box I-stimulated UL9 ATPase activity is suppressed by OF-1. However, we presently cannot rule out the possibility that proteins other than OF-1 in our preparation are responsible for the apparent displacement. Competition between OF-1 and UL9 for binding to oriS sequences could result in formation of a nucleoprotein initiation complex at the viral origin.

**Summary**—We have partially purified and characterized OF-1, a human protein that binds specifically to the HSV-1 replication origins (8). We find that OF-1 binds specifically to both duplex and single-stranded sequences from oriS with the strongest preference for the lower strand of the Box I region. Based on its interaction with multiple origin sites, we speculate that OF-1 participates in a large origin complex. We present evidence that UL9, the viral initiation protein, facilitates binding of OF-1 to Box I DNA. This result suggests that UL9 either interacts with OF-1 directly or alters the DNA structure to favor OF-1 binding. The OF-1-origin complex may be involved in initiation of viral replication.

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