Interaction between the DNA Polymerase and Single-stranded DNA-binding Protein (Infected Cell Protein 8) of Herpes Simplex Virus 1*

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The herpes virus-encoded DNA replication protein, infected cell protein 8 (ICP8), binds specifically to single-stranded DNA with a stoichiometry of one ICP8 molecule/12 nucleotides. In the absence of single-stranded DNA, it assembles into long filamentous structures. Binding of ICP8 inhibits DNA synthesis by the herpes-induced DNA polymerase on singly primed single-stranded DNA circles. In contrast, ICP8 greatly stimulates replication of circular duplex DNA by the polymerase. Stimulation occurs only in the presence of a nuclear extract from herpes-infected cells. Appearance of the stimulatory activity in nuclear extracts coincides closely with the time of appearance of herpes-induced DNA replication proteins including ICP8 and DNA polymerase. A viral factor(s) may therefore be required to mediate ICP8 function in DNA replication.

The herpes simplex virus 1 (HSV-1)[1]-encoded ICP8 is a 128-kilodalton DNA-binding protein (1, 2) that is essential for viral DNA replication (3–5). Conditional lethal mutants in the ICP8 gene have a quick-stop phenotype, indicating a direct role of ICP8 in replication fork movement (3). ICP8 is abundant in nuclei (6), the site of HSV-1 replication (7); and purified ICP8 binds tightly and cooperatively to ssDNA (8). Although the exact role of ICP8 in viral replication is still unknown, its cellular abundance and binding to ssDNA suggest that ICP8 is functionally analogous to the *Escherichia coli* SSB (9). *E. coli* SSB binds tightly and cooperatively to ssDNA, creating an activated surface for replication by DNA polymerase III holoenzyme (9). Similarly, SSB strongly stimulates the HSV-1-encoded DNA polymerase on singly primed ssDNA templates (10). In experiments described in this paper, we have examined the effect of ICP8 on DNA replication by the herpes-induced DNA polymerase. In contrast to SSB,

[1] The abbreviations used are: HSV, herpes simplex virus; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; ϕX, bacteriophage ϕX174; ssDNA, single-stranded DNA; RF I, closed circular duplex DNA; RF II, circular duplex DNA with a nick in one strand; SDS, sodium dodecyl sulfate; DTT, dithiothreitol; SSB, *E. coli* single-stranded DNA-binding protein; ICP8, infected cell protein 8.

ICP8 completely inhibits replication of singly primed ssDNA templates by the herpes polymerase. On the other hand, ICP8 strongly stimulates replication of duplex DNA. It does so, however, only in the presence of a nuclear extract from herpes-infected cells.

EXPERIMENTAL PROCEDURES

Materials—DNase I and venom phosphodiesterase were obtained from Worthington. The synthetic oligodeoxynucleotide (44-mer) was synthesized by the solid-phase coupling of protected phosphoramidate nucleoside derivatives (11). ^{3}H-Labeled ϕX ssDNA was a gift from Dr. R. Bryant (this department). pMOB45 (10.5 kilobases) linear duplex plasmid DNA was a gift from Dr. D. Julin (this department). M13mp18 RF I DNA and M13mp18ori$_S$ RF I DNA were prepared as described (12); HSV-1 DNA polymerase was prepared as described (10). Heparin-Sepharose was purchased from Pharmacia P-L Biochemicals. Glutaraldehyde (50%, v/v) and Alcian blue 86S were obtained from Electron Microscope Sciences. Bio-Gel A-5m was obtained from Bio-Rad. All other chemicals, nucleic acids, and enzymes not described here were from sources or prepared as in the preceding paper (10).

Buffers—Buffer A was 20 mM Tris-Cl (pH 7.5), 6 mM MgCl$_2$, 4% (w/v) glycerol, 0.1 mM EDTA, 40 μg/ml bovine serum albumin, 5 mM DTT. Buffer B was 20 mM Hepes/Na$^+$ (pH 7.6), 0.5 mM DTT, 0.5 mM EDTA, 0.5 mM phenylmethylsulfonyl fluoride, 10% (w/v) glycerol. Buffer C was 50 mM Tris-Cl (pH 7.5), 0.5 mM DTT, 0.1 mM EDTA, 150 mM (NH$_4$)$_2$SO$_4$. Buffer D was 25 mM Tris-Cl (pH 7.2), 10 mM MgCl$_2$, 5% glycerol. Buffer E was 25 mM Tris-Cl (pH 7.5), 1 mM DTT, 0.5 mM EDTA, 10% (w/v) glycerol. Buffer F was 20 mM Tris-Cl (pH 7.5), 20 mM NaCl, 8 mM MgCl$_2$, 1 mM DTT.

Purification of ICP8—ICP8 was purified by a modification of the method of Powell *et al.* (13). HSV-1-infected Vero cells (50 g, wet weight) from 100 roller bottles were harvested 18 h after infection with HSV-1 as described (12). Nuclei were prepared and extracted with 1.7 M NaCl, and the extract was fractionated by phosphocellulose chromatography as described (12). At all stages of purification, column fractions were analyzed by 7.5% SDS-polyacrylamide gel electrophoresis followed by staining with Coomassie Blue; ICP8-containing fractions were identified from the known mobility of ICP8 (14). ICP8 was eluted from the phosphocellulose column at 0.15 M NaCl as previously reported (12, 13) and was approximately 50% pure at this stage. The pooled phosphocellulose fractions were dialyzed against buffer B and applied to a ssDNA-cellulose column (20-ml bed volume) equilibrated with buffer B. The ssDNA-cellulose column was eluted with a linear gradient from 0.1 to 1.0 M NaCl in a total volume of 240 ml. Fractions (2 ml) were collected. The ICP8 eluted in a broad peak. Fractions containing ICP8 were pooled, dialyzed against buffer C containing 5% (w/v) glycerol, and concentrated to 4 mg/ml in 0.5 ml by ultrafiltration in a Centricon 30 (Amicon). At this stage, ICP8 was over 98% pure but was contaminated with a potent DNA exonuclease (assayed by digestion of 5.4-kilobase linear duplex DNA). The exonuclease contaminant was completely removed by sedimentation of ICP8 in a glycerol gradient. The ICP8 preparation was layered onto two 10.6-ml 10–30% glycerol gradients in buffer C and centrifuged at 40,000 rpm for 40 h at 4 °C in a Beckman SW41.Ti rotor. After centifugation, 0.2-ml fractions were collected from the bottom of the tubes. Total protein was measured by a modification of the method of Bradford (see Ref. 15). A total of 800 μg of ICP8 was obtained by this procedure.

Because of the relatively poor recovery of ICP8 following ssDNA-

cellulose chromatography and glycerol gradient sedimentation, we have devised an alternative procedure using heparin-Sepharose chromatography. The peak phosphocellulose fractions were pooled and precipitated by addition of $(NH_4)_2SO_4$ to 30% saturation (at 0°C). This procedure resulted in removal of several polypeptide contaminants to yield an ICP8 preparation that was over 80% pure.The pellet (7 mg of protein) was dissolved in 0.75 ml of buffer D and dialyzed against 1 liter of buffer E. Four mg of the dialyzed ICP8 was diluted to 8 ml in buffer E and applied to a heparin-Sepharose column (3-ml bed volume) equilibrated with buffer E. The column was eluted with a linear gradient from 0.1 to 0.6 M NaCl in a total volume of 40 ml of buffer E. Fractions (0.35 ml) were collected and analyzed for ICP8 and exonuclease activity. ICP8 was eluted from the column at 0.3 M NaCl as a sharp peak in 60% yield. The leading half of the ICP8 peak was homogeneous (>99% pure) and was completely free of exonuclease activity; however, the trailing half of the ICP8 peak was slightly contaminated with exonuclease. Exonuclease-free ICP8 prepared by both procedures was used interchangeably in the experiments to be described.

Electron Microscopy—A solution containing 1.6 μg of ICP8 and 25 ng of linear duplex DNA in 25 μl of buffer D was fixed in 0.2% glutaraldehyde for 15 min at room temperature. The cross-linked sample was gel-filtered through Bio-Gel A-5m in buffer D, and the void fraction was diluted 2-fold with buffer D prior to mounting. Two other samples of ICP8 were prepared as described above except that in one, 45 ng of ssDNA was added in place of the duplex DNA and DNA was omitted in the other.

Samples were adsorbed to carbon-coated copper grids treated with Alcian blue as described and blotted dry (16). Grids were either negatively stained with a solution of 1% uranyl acetate in H_2O (before drying) or rotary-shadowed with tungsten. Samples were examined in a Philips EM300 electron microscope. Instrument magnification was determined with commercially calibrated diffraction grating replicas.

Measurement of Stoichiometry of Binding of ICP8 to ssDNA—The reaction mixture (125 μl) contained 0.50 μg of ^{3}H-labeled φX ssDNA (120,000 cpm/μg) in buffer D. A 20-μl sample was removed to determine the total amount of acid-precipitable [^{3}H]ssDNA, and then the indicated amount of ICP8 was added. After 3 min at 37 °C, 1.25 μl of a solution containing 0.69 mg/ml DNase I and 0.69 mg/ml venom phosphodiesterase was added, and 20-μl samples were removed after a further 2, 5, 10, and 20 min at 37 °C. Samples were quenched by adding them to 100 μl of ice-cold 0.1 M pyrophosphate containing 5 μg of sonicated calf thymus DNA. The amount of DNA protected by ICP8 from nuclease digestion was inferred from the amount of DNA precipitated by 10% trichloroacetic acid as described (17). In the absence of ICP8, less than 1% of the [^{3}H]ssDNA remained acid-precipitable after 2 min of incubation. When ICP8 was present, the amount of acid-precipitable radioactivity reached a plateau after 2 min and then slowly decreased with further incubation (~5% of total in 20 min). The amount of [^{3}H]ssDNA protected from nuclease digestion by ICP8 was determined by extrapolation to the ordinate of a line drawn through the 2-, 5-, 10-, and 20-min points. When present, synthetic oligodeoxynucleotides were added to the solution prior to addition of the ICP8.

Kinetics of Transfer of ICP8 between ssDNA Molecules—The reaction mixture (69 μl) contained sufficient ICP8 (9 μg) to saturate partially (65%) the ^{3}H-labeled φX ssDNA (0.35 μg) in buffer D containing 40 mM NaCl. To determine the amount of DNA initially protected against nuclease by ICP8, an 8-μl sample was removed, diluted to 11 μl with buffer D, treated with 0.5 μl of a solution containing 0.75 mg/ml DNase I and 0.8 mg/ml venom phosphodiesterase, and incubated for 2 min at 37 °C before being quenched with ice-cold pyrophosphate (and carrier DNA) and analyzed for acid-precipitable radioactivity as described above. Measurement of the kinetics of transfer of ICP8 was initiated upon addition of 61 μl of buffer D containing 1.55 μg of unlabeled φX ssDNA at 37 °C. Samples (12 μl) of the reaction mixture were removed at timed intervals, treated with 2 μl of a solution containing 0.75 mg/ml DNase I and 0.8 mg/ml venom phosphodiesterase for 2 min at 37 °C, and then quenched and analyzed for acid-precipitable radioactivity as described above.

DNA Synthesis on Singly Primed ssDNA.—Compositions of reaction mixtures are given in the figure legends. All preincubations and reactions were at 30 °C. φX ssDNA was primed with a single synthetic oligodeoxynucleotide (15-mer). Synthesis, sequence, and annealing of the synthetic 15-mer to the φX ssDNA have been described (17). The concentration of herpes DNA polymerase was determined from the

amount of singly primed φX ssDNA that was fully replicated in 30 min at 30 °C by a given amount of enzyme as described in the preceding paper (10).

3′→5′ Exonuclease Assay—The 3′→5′ exonuclease activity of the herpes DNA polymerase was assayed using 3.1 pmol (as template molecules) of 3′-labeled hairpin templates having paired or unpaired 3′ termini in 12.5 μl of buffer A containing 83 fmol of polymerase and 60 mM NaCl as described in the preceding paper (10).

Preparation of Nuclear Extract—Nuclei from 10^9 18-hr infected or mock-infected cells were prepared, resuspended in 10 ml of buffer as described (12), and stored as 1-ml aliquots at −80 °C. A 1-ml sample of nuclei was thawed slowly on ice and then lysed by addition of 85 μl of 5 M NaCl followed by a 30-min incubation on ice. Insoluble chromatin was pelleted by centrifugation at 15,000 rpm in a Beckman JA-20 rotor for 30 min at 4 °C. The supernatant was dialyzed 3 h against 1 liter of buffer E containing 25 mM NaCl at 4 °C. The dialyzed extract (600 μl) was concentrated to 3 mg/ml (200 μl total) by ultrafiltration in a centrifuge (Centricon 10).

DNA Synthesis with Duplex DNA Templates—Reaction mixtures (40 μl) contained 0.6 μg of M13mp18 RF I DNA (or M13mp18ori$_S$ RF I DNA), 75 fmol of herpes DNA polymerase, 60 μM each dCTP, dGTP, and dATP, 20 μM [α-^{32}P]dTTP (2050 cpm/pmol), 200 μM each CTP, GTP, and UTP, 4 mM ATP, 20 mM phosphocreatine, 5 μg of creatine kinase, 9 μg of nuclear extract (protein determined according to a modification of the Bradford method (see Ref. 15), and the indicated amount of ICP8. Reactions were incubated at 30 °C for 1 h before quenching with 20 μl of 1.5% SDS, 60 mM EDTA. DNA synthesis was measured, and replication products were analyzed by agarose gel electrophoresis as described (17). An additional 75 fmol of herpes DNA polymerase (equivalent to the endogenous DNA polymerase activity in 9 μg of nuclear extract of infected cells) was present in reactions utilizing nuclear extracts of uninfected cells.

Measurement of Time Course of Appearance of Stimulatory Activity in Nuclear Extracts—Tissue culture cells were harvested at various times after infection with HSV-1 as described (12). Nuclei were prepared and stored at −80 °C as described above. One ml of resuspended nuclei (~10^8 cells) was lysed upon rapid mixing with 1 ml of buffer E containing 3.4 M NaCl followed by incubation for 30 min on ice. The solubilized extract was clarified by centrifugation at 55,000 rpm in a Beckman TSL55 rotor (Beckman TL100 ultracentrifuge) for 35 min at 4 °C. The extract was dialyzed overnight against 1 liter of buffer E and concentrated to 1 mg/ml (1 ml total) by ultrafiltration in a centrifuge (Centricon 10).

Reaction mixtures (40 μl) contained 3 μg of M13mp18 RF I DNA, 220 fmol of herpes DNA polymerase, 9 μg of ICP8, 60 μM each dATP, dCTP, and dGTP, 20 μM [α-^{32}P]dTTP (1050 cpm/pmol), 200 μM each CTP, GTP, and UTP, 4 mM ATP, 20 mM phosphocreatine, 5 μg of creatine kinase, and 5 μg of nuclear extract. Reactions were incubated at 30 °C for 30 min before quenching with 20 μl of 1.5% SDS, 60 mM EDTA. Incorporation of radioactivity into acid-insoluble material was measured as described (17).

RESULTS

ICP8 Assembles into Large Helical Filaments—ICP8 sedimented in a glycerol gradient containing 150 μM $(NH_4)_2SO_4$ as a monomer of 130 kDa (Fig. 1A). Analysis of ICP8 in buffer D by electron microscopy showed numerous long thick filaments which appeared as rope-like left-handed helices (Fig. 2, *upper*). The polymeric structure of ICP8 observed in the electron microscope is very likely induced by the Mg^{2+} and low ionic strength of buffer D. The filaments were usually long (>400 nM) but were not uniform in length. That the helical filament is composed of ICP8 is supported by the abundance of filaments, lack of other proteins in the ICP8 preparation (Fig. 1B), and complete disappearance of the filaments upon addition of ssDNA to which ICP8 binds (8). The appearance in the electron microscope of ICP8 bound to ssDNA was very different from Fig. 2 but similar to previously published photographs, although a beaded structure was not apparent under the conditions used in these experiments (18) (data not shown).

The tungsten-shadowed ICP8 helical filament (Fig. 2, *upper*) was left-handed with a pitch of about 32 nm and thickness of about 24 nm. The ICP8 filament did not appear to interact

A)

B) FRACTION NUMBER

FIG. 1. **Glycerol gradient sedimentation of ICP8.** *A*, sedimentation of ICP8 in a 10–30% glycerol gradient was as described under "Experimental Procedures." The position of migration of external standards in a parallel gradient is shown in *brackets*. *B*, SDS-polyacrylamide gel electrophoresis of peak fractions from the glycerol gradient of *A*. The *tick marks* correspond to the mobility of molecular mass standards in the same gel.
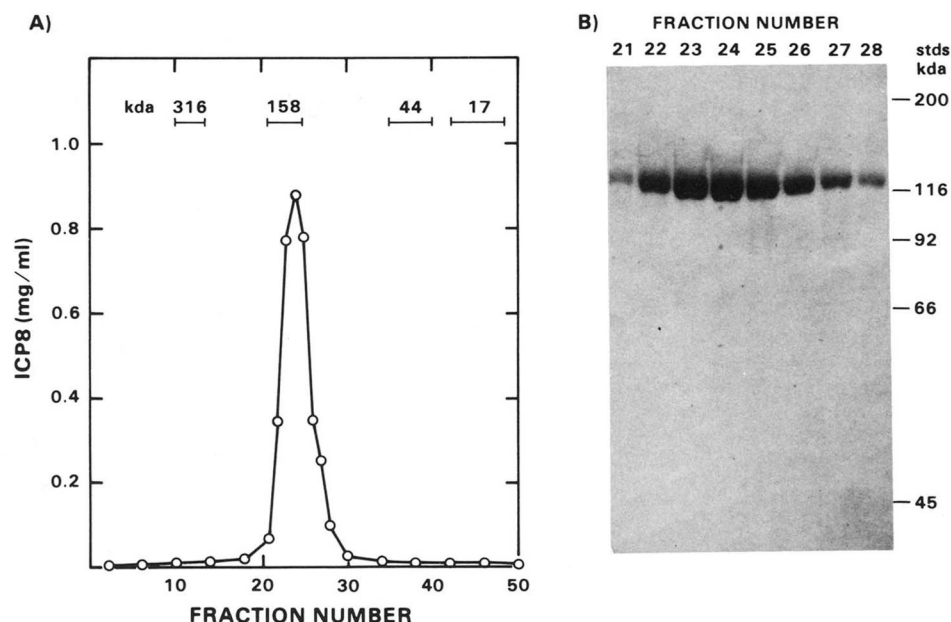
with linear duplex DNA (Fig. 2, *upper*). The abundance and appearance of the filaments were the same with ICP8 purified by both procedures (see "Experimental Procedures").

Negatively stained ICP8 produced a substantially different image (Fig. 2, *lower*); it appeared to consist of filaments about 20 nm in diameter that had collapsed slightly and wound around each other, possibly due to the acidic stain. However, the helical structure was not apparent. The image was similar if glutaraldehyde was omitted, indicating that the fixation step did not obliterate the helicity, as is the case for the helical fibers formed by the *E. coli* recA protein (19).

One ICP8 Molecule Protects 12 Nucleotides of ssDNA from Nuclease Digestion—The stoichiometry of binding of ICP8 to ^{3}H-labeled ϕX ssDNA was measured by nuclease protection of the ssDNA (Fig. 3). Maximum protection occurred at a weight ratio of 37:1 ICP8 to ssDNA, indicating a binding stoichiometry of one ICP8 molecule to 12 nucleotides of ssDNA. Since 87% of the ssDNA could be protected by ICP8, this method should not underestimate significantly the size of the ICP8-binding site. This binding site is substantially smaller than the previously reported estimate of 40 nucleotides of ssDNA/molecule of ICP8 (8). In the previous report, binding of ICP8 was measured by retention of ssDNA to nitrocellulose filters. This procedure may lead to an overestimate since less than fully saturated ssDNA may be bound to the filter.

At a weight ratio of ICP8 to ssDNA of 20:1, the level of protection against nuclease digestion (44%) was unaltered by increasing the reaction volume 30-fold (data not shown), indicating a very tight binding of ICP8 to ssDNA ($K_d < 0.2$ nM). With a 10-fold weight excess of an oligodeoxynucleotide 15-mer or a 5-fold weight excess of a 44-mer over the ^{3}H-labeled ϕX ssDNA, the level of protection was half that observed in the absence of these oligonucleotides (data not shown). Hence, the affinity of ICP8 for short ssDNA segments is substantially lower than for long ssDNA molecules, consistent with the previously observed cooperative binding of ICP8 to ssDNA (8). ATP had no effect on the stoichiometry of binding (data not shown). Less than 10% of duplex DNA was protected from digestion even at a weight ratio of 600:1 ICP8 to duplex DNA (data not shown), in agreement with previous reports of little or no binding of ICP8 to duplex DNA (18, 20).

To determine the time required for ICP8 to dissociate from ssDNA, ICP8 was bound to ^{3}H-labeled ϕX ssDNA and then incubated with a 5-fold excess of unlabeled ϕX ssDNA. The time for ICP8 to transfer to the unlabeled ssDNA was determined by removing samples of the reaction mixture at various time intervals and determining the amount of [^{3}H]ssDNA that remained protected against nuclease digestion. The results, shown in Fig. 4, indicates a $t_{1/2}$ of 4-min for dissociation of ICP8 from ssDNA under these conditions.

ICP8 Inhibits Herpes DNA Polymerase on Singly Primed ssDNA—Herpes DNA polymerase activity was inhibited upon binding of ICP8 to ϕX ssDNA primed with a synthetic 15-mer; complete inhibition correlated with saturation of the ssDNA with ICP8 (Fig. 5A). Addition of a saturating amount of ICP8 to a reaction proceeding in the absence of ICP8 resulted in the immediate cessation of DNA synthesis (not shown). ICP8 did not remove the 15-mer primer from the ssDNA. Thus, incubation of ssDNA annealed to the 5' ^{32}P-labeled 15-mer with a saturating level of ICP8 produced no measurable displacement of the primer from the ssDNA (data not shown).

Complete inhibition of DNA synthesis on primed ssDNA by a saturating amount of ICP8 was observed over a wide range of DNA polymerase concentrations (Fig. 5B). Herpes DNA polymerase was also inhibited by ICP8 with activated DNA as template (80% inhibition by addition of 6 μg of ICP8 to 1 μg of activated DNA (data not shown)). Inhibition of herpes DNA polymerase acting on activated DNA and singly primed ϕX ssDNA by ICP8 was not affected by ionic strength (0-100 mM ammonium sulfate or 150 mM KCl). ICP8 also inhibited T4 DNA polymerase and *E. coli* DNA polymerase III holoenzyme with activated DNA as template to the same extent as the herpes DNA polymerase (data not shown).

During DNA synthesis, deoxynucleoside monophosphates are produced by the low level of hydrolysis of the 3' terminus by the 3'→5' exonuclease activity of the herpes DNA polymerase (10, 21). At a concentration of ICP8 that completely inhibited the herpes DNA polymerase on the $(dA)_{1000} \cdot (dT)_{17}$ template, the production of dTMP was decreased by only 40% (Fig. 5C). The primed template is required for dTMP formation, ruling out a contaminating exonuclease in the polymerase or ICP8 preparations. A plausible explanation for these findings is that ICP8 prevents extension of primers but does
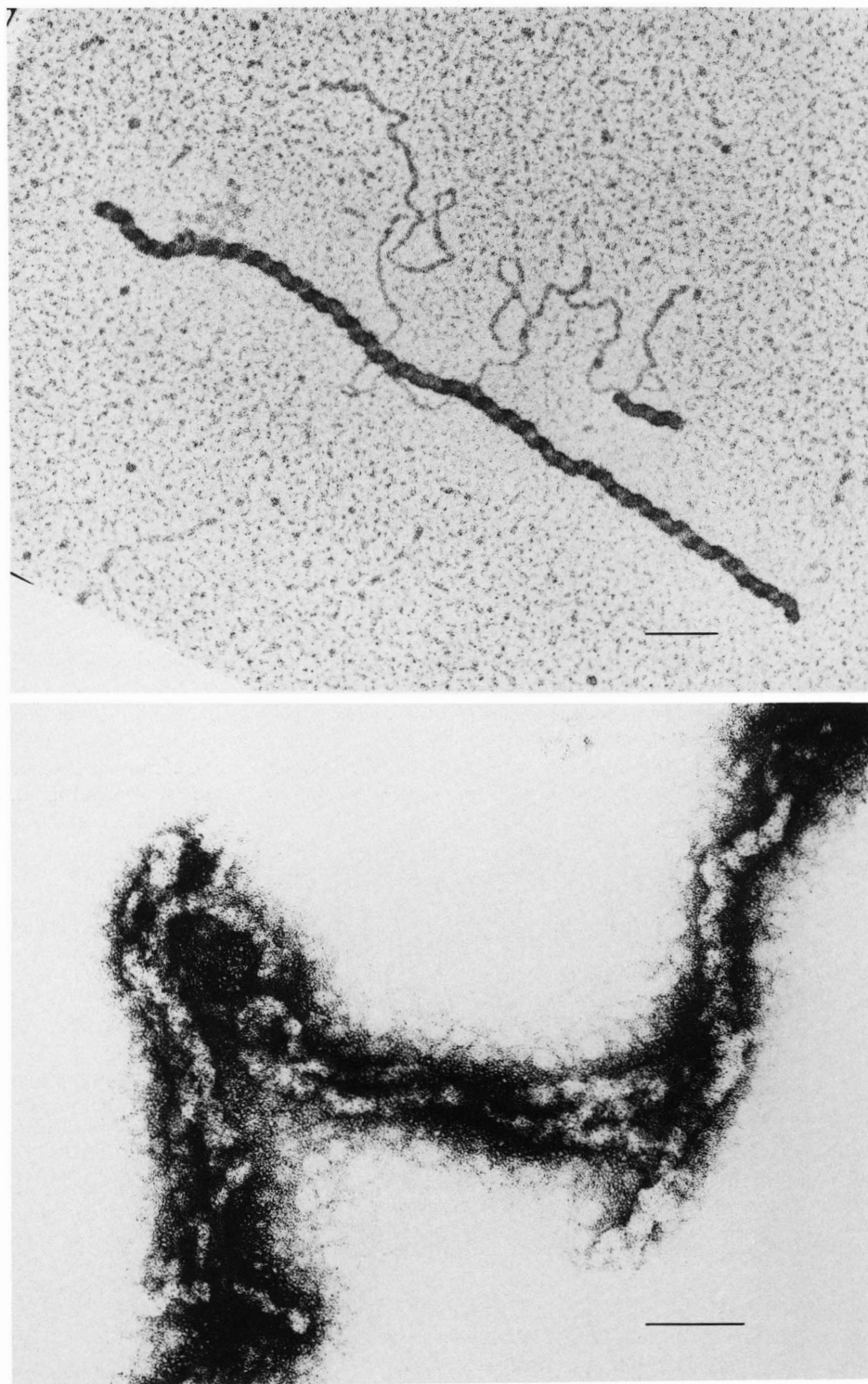
FIG. 2. **Electron micrographs of ICP8.** *Upper,* tungsten-shadowed helical ICP8 filaments in the presence of double-stranded DNA; *lower,* uranyl acetate-stained ICP8. The *bars* represent 100 nm.

not prevent polymerase from binding to primer termini. Under these conditions, the polymerase is constrained to idle by successive polymerization/excision reactions leading to the conversion of dTTP to dTMP.

In the absence of deoxynucleoside triphosphates, ICP8 stimulated the 3'→5' exonuclease of the herpes DNA polymerase 2.5-fold in removing the paired 3' terminus of a synthetic hairpin template (Fig. 6). *E. coli* SSB had no such effect.

*Replication of Duplex DNA Templates by Herpes DNA Polymerase in Presence of ICP8 and Virus-specific Factor—*Extraction of nuclei from herpes-infected cells with 0.4 M

NaCl releases most of the soluble proteins, leaving ICP8 bound to the insoluble chromatin. Such soluble nuclear extracts gave substantial DNA synthesis with M13mp18 RF I DNA as template, but only in the presence of added ICP8 (Fig. 7). Although herpes DNA polymerase was present in the nuclear extract, synthesis was stimulated approximately 2-fold by added polymerase. *E. coli* SSB could substitute for ICP8 in this reaction.

The amount of DNA synthesized corresponded to 3% of the input plasmid DNA; essentially all the products of replication migrated as RF II molecules during native agarose gel electrophoresis. Since DNA synthesis was linear after a slight
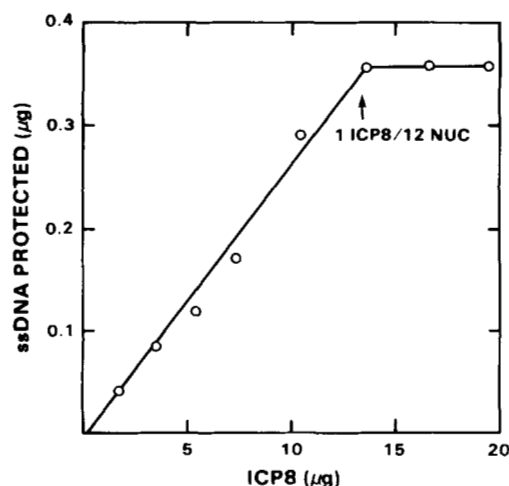
FIG. 3. **Protection of ssDNA from nuclease digestion by ICP8.** Reactions were performed as described under "Experimental Procedures." *Circles* represent the amount of ^{3}H-labeled ϕX ssDNA that remained trichloroacetic acid-precipitable after nuclease digestion. *NUC*, nucleotides.
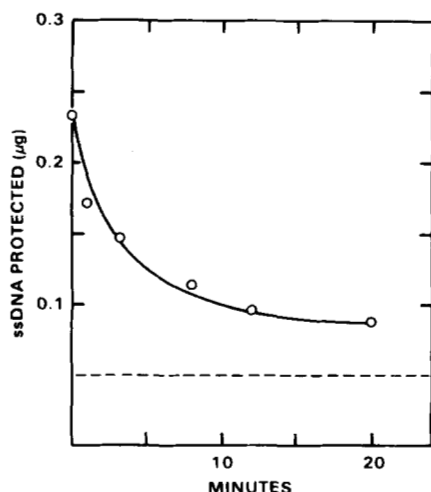

FIG. 4. **Time course of transfer of ICP8 between ssDNA molecules.** The transfer reaction was carried out as described under "Experimental Procedures." ICP8 was preincubated with ^{3}H-labeled ϕX ssDNA (65% saturation), and then a 5-fold excess of unlabeled ϕX ssDNA was added. Samples were withdrawn at the times indicated and analyzed for ICP8 remaining associated with the [^{3}H]ssDNA as measured by nuclease protection. The *broken line* represents the level of nuclease protection of the [^{3}H]ssDNA when the ICP8 is completely equilibrated between labeled and unlabeled ssDNA molecules.

FIG. 5. **Effect of ICP8 on DNA synthesis by herpes DNA polymerase.** *A*, effect of ICP8 concentration. The 25-μl reaction mixture contained the indicated amounts of ICP8, 0.06 μg of singly primed ϕX ssDNA (32 fmol as *circles*), 12 fmol of herpes DNA polymerase, 0.5 mM ATP, 60 μM each dATP, dCTP, and dGTP, 20 μM [α-^{32}P]dTTP (2,000 cpm/pmol), and 60 mM NaCl in buffer A. ICP8 was preincubated with the ssDNA for 3 min (30 °C), and then replication was initiated by addition of DNA polymerase. Incubation was for 30 min at 30 °C before the reaction was quenched, and synthesis was measured as described under "Experimental Procedures." The *arrow* marks the point at which one molecule of ICP8 was added per 12 nucleotides (*NUC*) of ssDNA. *B*, effect of ICP8 on DNA synthesis with increasing amounts of herpes DNA polymerase. The 25-μl reaction mixture contained 0.035 μg of singly primed ϕX ssDNA (20 fmol as *circles*), the indicated amount of herpes DNA polymerase, 60 μM each dCTP, dGTP, dATP, and 20 μM [α-^{32}P] dTTP (5,600 cpm/pmol) in buffer A. Incubation was for 20 min at 30 °C. O, reactions in the presence of 2.4 μg of ICP8; $\triangle$, no ICP8 added. *C*, effect of ICP8 on the 3'→5' exonuclease activity of herpes DNA polymerase. The reaction mixture (12.5 μl) contained 0.36 μg of (dA)$_{1000}$·(dT)$_{17}$ (100:1 weight ratio), 50 fmol of herpes DNA polymerase, 20 μM [α-^{32}P]dTTP (63,000 cpm/pmol), and 60 mM NaCl in buffer A. Samples (1 μl) were withdrawn at the times indicated and quenched immediately upon adsorption to a phenylethyleneimine thin layer chromatography strip. Chromatography and quantitation of dTMP and products of DNA synthesis (origin) were as described for the 3'→5' exonuclease assay under "Experimental Procedures." O, no other addition; $\triangle$, 15 μg of ICP8 was added to the DNA before addition of the DNA polymerase.

lag (Fig. 7), greater extents of synthesis could be achieved by longer incubation periods and higher levels of ICP8. The products of the reaction are most likely extensive stretches of newly synthesized DNA since most of the plasmid DNA visualized by ethidium bromide staining migrated as RF I (not shown). Inasmuch as no synthesis was observed with a nuclear extract prepared from uninfected cells even when the reaction was supplemented with purified herpes DNA polymerase, one or more factors produced during infection with HSV-1 are required in addition to the DNA polymerase and ICP8 for the replication of duplex DNA. Plasmid DNA containing an origin of HSV-1 replication (M13mp18ori$_S$) was replicated to the same extent as plasmid DNA lacking the origin sequence; the products of replication were also the same (data not shown). Pretreatment of plasmid DNA with DNase I such that 90% of DNA circles were nicked at least once had
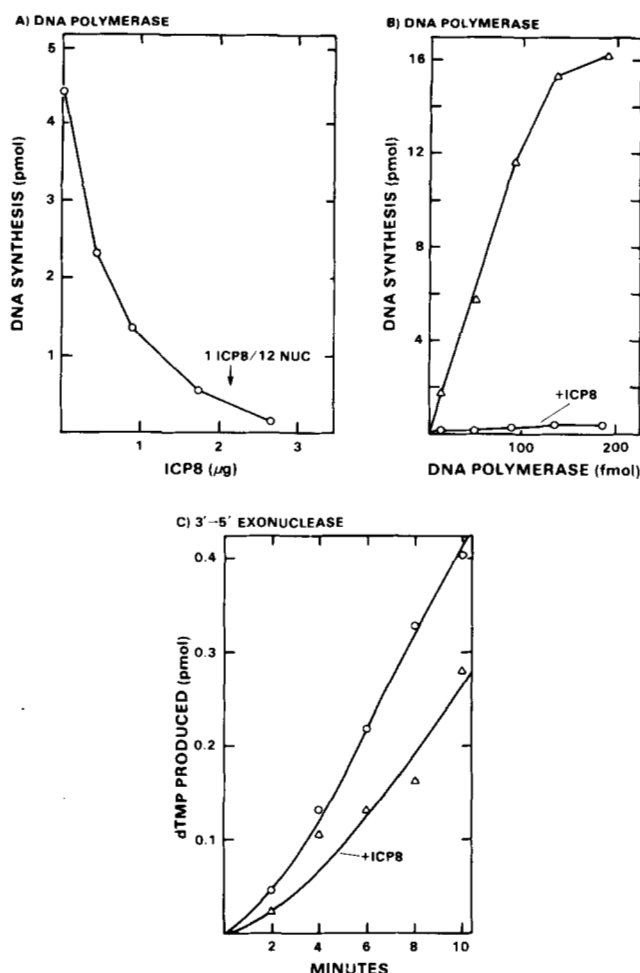
no significant effect on the ability to serve as template (not shown).

The ability of the nuclear extract from infected cells to stimulate DNA synthesis by the herpes DNA polymerase and ICP8 depends upon the time of infection (Fig. 8). The time
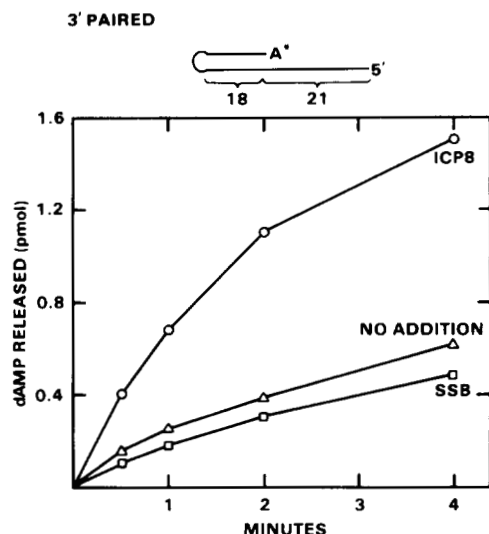
3' PAIRED

FIG. 6. **Stimulation of 3'→5' exonuclease of herpes DNA polymerase by ICP8 in absence of deoxynucleoside triphosphates.** Reactions were performed as described under "Experimental Procedures." The DNA substrate is diagramed above the plot. △, no addition; ○, 3 μg of ICP8 (23 pmol) was added to the substrate before adding herpes DNA polymerase; □, 1.3 μg of SSB was added to the DNA before adding DNA polymerase.
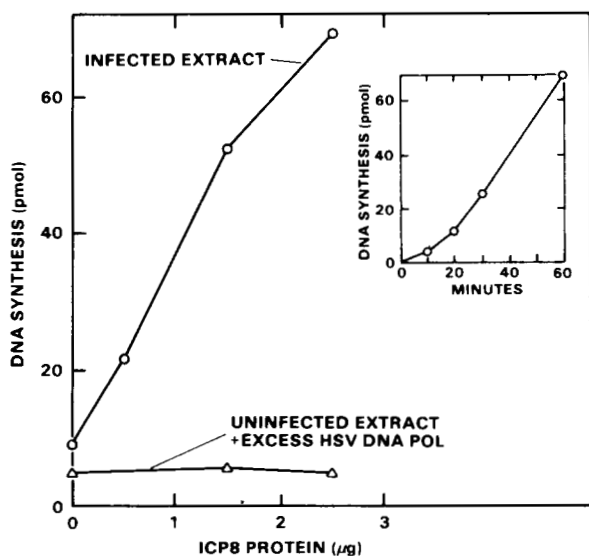

FIG. 7. **Stimulation of duplex DNA replication by ICP8 requires an extract of infected cell nuclei.** Preparation of nuclear extracts and conditions of replication are described under "Experimental Procedures." Substrate was M13mp18 RF I DNA. ○, the nuclear extract from 18-h infected cells; △, the nuclear extract from mock-infected cells supplemented with an additional 75 fmol of herpes DNA polymerase (*POL*) (the amount of endogenous herpes DNA polymerase in the infected extract). *Inset,* time course of DNA synthesis in the reaction containing nuclear extract from infected cells and 2.5 μg of ICP8.

course of appearance of stimulatory activity was similar to the time of appearance of the herpes replication proteins, ICP8, DNA polymerase, and the origin binding protein (ori$_S$ protein (12)). The nuclear extract used in Fig. 8 was prepared by solubilizing nuclei with 1.7 M NaCl which releases ICP8 from chromatin (1, 13). The endogenous ICP8 and herpes DNA polymerase in the solubilized extract were not, however, present in sufficient concentrations to support significant DNA synthesis in the presence of the large excess of plasmid DNA used in the experiment. Thus, even in the presence of
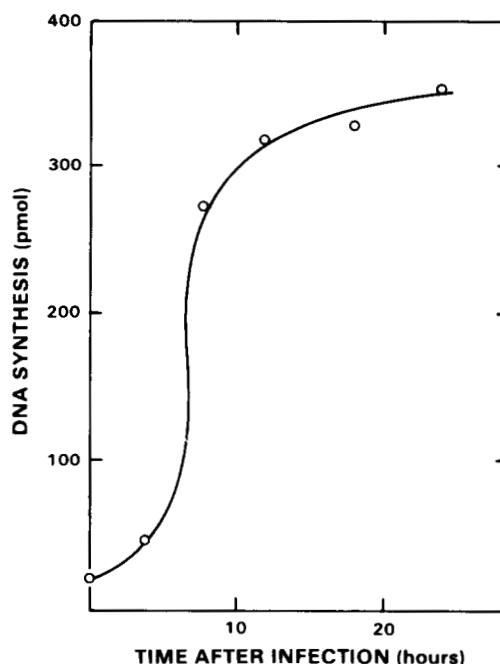

FIG. 8. **Time course of appearance of stimulatory activity after HSV-1 infection.** Nuclear extracts were prepared at the indicated times after infection with HSV-1, and reactions were performed as described under "Experimental Procedures."

an extract from 12-h infected cells, replication was highly dependent upon the addition of both herpes DNA polymerase and ICP8. The large amount of DNA synthesis in this experiment suggests that additional stimulatory factors may be released from nuclei by 1.7 M salt in addition to those released by 0.4 M NaCl (compare Figs. 7 and 8).

DISCUSSION

ICP8 is essential for HSV-1 DNA replication (3–5). The tight and cooperative binding of ICP8 to ssDNA (8) and its abundance during viral replication (1) suggest a role analogous to *E. coli* SSB (9). It was therefore surprising that binding of ICP8 to primed ssDNA resulted in the complete inhibition of DNA synthesis by the herpes DNA polymerase, particularly in view of the 20-fold stimulation by *E. coli* SSB with the same template (10).

Stimulation of duplex DNA replication by ICP8 in the presence of a nuclear extract of infected cells suggests that one or more HSV-1-induced protein(s) may mediate ICP8 function in viral DNA replication *in vivo.* Nuclease activation of the duplex DNA substrate is ruled out by several findings. (i) The unreacted plasmid DNA remained covalently closed, and the products of replication migrated during neutral agarose gel electrophoresis as intact RF II molecules. (ii) Pretreatment of the plasmid DNA with DNase I had no effect on the rate of replication. (iii) ICP8 inhibited the herpes DNA polymerase on nuclease-activated DNA. Previous reports of some stimulation (1.7-fold) of herpes DNA polymerase activity on activated DNA by ICP8 (18) might possibly be explained by contamination of the ICP8 or DNA polymerase preparation with the stimulatory factor(s).

The significance of the helical filaments of ICP8 formed in the presence of Mg^{2+} at low ionic strength is unknown. Depolymerization of ICP8 is presumably rapid since addition of ssDNA results in prompt loss of the heterogeneous filaments and appearance of a different and uniform structure composed of ICP8 bound to the ssDNA (8). The ICP8 filaments are reminiscent of the filamentous forms of *E. coli* recA

protein (19). However, the ICP8 filament differs from the recA protein filament in being left-handed and about twice as thick; more than one ICP8 molecule must therefore contribute to its width. In addition, under the conditions used here, there is no evidence that the ICP8 filament contains or interacts with duplex DNA.

The high cellular level of ICP8 is also consistent with a role as a recombinase or helicase, *e.g. E. coli* recA protein and DNA helicase II (22). However, we could not detect recombinase activity using a strand exchange assay (23) (data not shown). Furthermore, unlike recA protein and helicase II, ICP8 did not hydrolyze nucleoside triphosphates (ATP, GTP, CTP, dATP, dTTP) alone or in the presence of ssDNA or linear duplex DNA or a combination of the two (not shown). Despite these findings, a role for ICP8 in recombination and strand displacement must still be considered.

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