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The Herpes Simplex Virus Type I Origin Binding Protein

DNA-DEPENDENT NUCLEOSIDE TRIPHOSPHATASE ACTIVITY*

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A recombinant baculovirus overexpressing the herpes simplex virus type 1 (HSV-1) origin binding protein, encoded by the UL9 gene, was constructed. The purified recombinant protein has DNA-dependent nucleoside triphosphatase activity similar to the enzyme isolated from mammalian cells. Optimal nucleoside triphosphatase activity requires low salt (<50 mM), 2–3 mM Mg^{2+}, alkaline pH (8.3–9.5), high temperature (45 °C), and a single-stranded DNA coeffector containing minimal secondary structure. Enzymatic activity is subject to product inhibition, and there appears to be a single nucleotide binding site. The minimal length of single-stranded DNA that elicits enzymatic activity is 14 nucleotides, and activity increases as the length is increased. Saturation for various single-stranded DNA coeffectors is about 10 μM in nucleotide, but the maximum velocity is reduced 2–3-fold for coeffectors containing secondary structure. The HSV-1-encoded single-stranded DNA-binding protein ICP8 specifically stimulates the DNA-dependent nucleoside triphosphatase activity. The kinetics of nucleoside triphosphate hydrolysis exhibit a substantial lag period which can be shortened, but not eliminated, by reduced secondary structure in the DNA coeffector or by increased temperature.

DNA replication begins at specific genomic loci termed origins (1). In bacteria and several viruses, DNA replication starts with the binding of an initiator protein to specifically iterated sequences within an origin. The origin-bound initiator proteins associate with one another via protein-protein interactions and assemble into a higher order specialized nucleoprotein structure. This structure induces localized conformational changes in flanking A+T-rich sequences and initiates DNA replication by serving as a recognition site and foundation for the assembly of the replisome (2–4). Usually there is a specific ATP requirement for the correct assembly and function of the initiator nucleoprotein complex (3, 4).

Herpes simplex virus type 1 (HSV-1)[1] has three origins of replication, making it an attractive model for studying the coordinated use and regulation of multiple origins in cellular genomic replication. One of the HSV-1 origins, termed Ori_L, is located in the unique long segment of the HSV-1 genome (5–8). The other origin, termed Ori_S, is homologous in sequence to Ori_L and is diploid. Its two copies are located within the repeated termini of the unique short segment of the viral DNA (9, 10). Minimally, Ori_S is comprised of a 75-bp segment containing a 44-bp dyad of symmetry (11–13). The termini of the dyad each contains a 10-bp sequence composed of overlapping inverted partial repeats. These 10-bp sequences are homologous and termed Box I and Box II (14). A 31-bp A+T-rich spacer separates Boxes I and II. A third homologous partial inverted repeat, Box III, is located adjacent to the 44-bp dyad of symmetry (15).

HSV-1 contains seven genes that are necessary and sufficient for the initiation of HSV-1 DNA replication *in vivo* (16–18). One of these genes, UL9, encodes a 94-kDa protein that binds to both Boxes I and II of Ori_S, but with a 5–10-fold higher affinity for Box I (14, 19, 20). This origin binding protein, which we term the UL9 protein, binds specifically and cooperatively to the boxes and induces a conformational change in the intervening A+T-rich spacer sequence (10, 14, 15, 21–23). The DNA binding domain of the UL9 protein has been localized to a 269-amino acid long segment in the carboxyl terminus of the protein (24, 25). Mutations in origin-DNA sequences that inhibit the *in vitro* interaction of UL9 protein with the origin also inhibit origin-specific DNA replication *in vivo* (26, 27).

The UL9 protein is homodimeric and possesses both DNA-dependent nucleoside triphosphatase and DNA helicase activities (28). These enzymatic activities, along with its capacity to bind specifically origin DNA sequences, are reminiscent of the eukaryotic SV40 virus T antigen initiator protein (29–31)). In the presence of ATP, SV40 T antigen assembles into two double hexamers that untwist the SV40 origin as a prelude to initiating SV40 DNA replication (3). In contrast, binding of the UL9 protein to Ori_S does not appear to be affected by ATP (22).

A full understanding of the role of the UL9 protein in the initiation of HSV-1 origin-specific DNA replication will require a detailed study of its DNA-dependent ATPase and DNA helicase activities. In this paper we characterize in detail several parameters affecting the DNA-dependent nucleoside triphosphatase activity. In the following paper (66) we report the results of a detailed analysis of the DNA helicase activity associated with the UL9 protein.

EXPERIMENTAL PROCEDURES

Materials—Activated calf thymus DNA was prepared as described (32) and denatured by boiling for 3 min prior to use. M13mp18 ssDNA and oligo$(dT)_2$ to oligo$(dT)_{36}$ were from U. S. Biochemical

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[1] The abbreviations used are: HSV-1, herpes simplex virus type 1; bp, base pair; ss, single-stranded; TEMED, *N,N,N′,N′*-tetramethylethylenediamine; SSB, single-stranded DNA-binding protein; HSSB, human single-stranded DNA-binding protein; NTP, nucleoside 5′-triphosphate (either ATP, CTP, GTP, or UTP); CAPS, 3-(cyclohexylamino)-1-propanesulfonic acid; CHES, 2-(*N*-cyclohexylamino)-ethanesulfonic acid; EPPS, *N*-(2-hydroxyethyl)piperazine-*N*′-3-propanesulfonic acid; MES, 2-(*N*-2-morpholino)ethanesulfonic acid; MOPS, 3-(*N*-morpholino)propanesulfonic acid; TAPS, 3-[tris(hydroxymethyl)methyl]aminopropanesulfonic acid.

Corp. $(dT)_{3000}$ was from Midland Certified Reagent Co. (Midland, TX). Restriction and DNA modifying enzymes were from New England Biolabs. Acrylamide was from U. S. Biochemical Corp. TEMED, bisacrylamide, and ammonium persulfate were from Boehringer Mannheim. Sodium dodecyl sulfate and HEPES buffer were from U.S. Biochemical Corp. All other buffers were from Sigma. Heparin (Sigma) was coupled to Sepharose CL-4B (Pharmacia LKB Biotechnology Inc.) as described (33). Phosphocellulose P-11 was from Whatman. Hydroxylapatite was from Bio-Rad. Powdered Grace's medium was from Hazelton (Lexana, KS), ExCell 400 from J. R. H. Biosciences, fetal bovine serum from Irvine Scientific (Santa Ana, CA), and Fungizone and gentamycin sulfate were from GIBCO. Bovine serum albumin fraction V was from Sigma. Ribonucleoside 5′-triphosphates were from Pharmacia, and ribonucleoside 5′-diphosphates were from Sigma. α-^{32}P-Labeled ribonucleoside 5′-triphosphates (400 Ci/mmol) were from Amersham. Malachite green hydrochloride and ammonium molybdate(VI) tetrahydrate, used in the colorimetric DNA-dependent ATPase assay, were from Sigma and Aldrich, respectively. Polyethyleneimine-cellulose F plates were from EM Separations (Gibbstown, NJ). ICP8 and mammalian expressed HSV-1 UL9 protein were purified as described (28, 34). HSSB was a gift from Drs. Jerard Hurwitz and Frank Dean (Sloan-Kettering), and *Escherichia coli* SSB was a gift from Dr. Arthur Kornberg (Stanford).

Buffers—Buffer A, used during purification of the UL9 protein, contained 20 mM HEPES, pH 7.6, 10% (v/v) glycerol, 1.0 mM dithiothreitol, 0.5 mM EDTA, 0.5 mM EGTA, 4 μg/ml leupeptin, 4 μg/ml pepstatin A, and 0.5 mM phenylmethylsulfonyl fluoride. Buffer B was the same as buffer A except leupeptin and pepstatin A were 2 μg/ml. Buffer C was the same as buffer A except EDTA was 1.0 mM, EGTA was omitted, and leupeptin and pepstatin A were 2 μg/ml. The NaCl concentration in each buffer is indicated in brackets; for example buffer A [0.1] indicates that the buffer A contains 0.1 M NaCl. Buffer D was used for DNA-dependent NTPase assays, and, unless otherwise noted, consisted of 40 mM EPPS, pH 8.3, 10% (v/v) glycerol, 2 mM dithiothreitol, 3.0 mM $MgCl_2$, and 100 μg/ml bovine serum albumin. Stock buffers used for enzymatic assays were made at room temperature (22 °C) at a concentration of 150 mM, and the pH was adjusted with 5 M NaOH using an Orion Ross combination 81-15 pH electrode.

Cells, Plasmid DNA, and Viruses—Spodoptera frugiperda Sf9 cells were propagated at 27 °C in either Grace's medium supplemented with 0.33% TC yeastolate, 0.33% lactalbumin hydrolysate (TMNFH medium) and 10% fetal calf serum or in ExCell 400. The plasmid pET3a/OBP (15), which contains the UL9 gene of HSV-1 and encodes the origin binding protein, was obtained from Dr. Per Elias (Gothenberg University). The plasmid pVL941 and wild type *Autographa californica* nuclear polyhedrosis virus (35) were obtained from Dr. Max Summers (Texas A & M University). Recombinant baculoviruses were propagated in TMNFH medium containing 10% fetal calf serum, 50 μg/ml gentamycin, and 2 μg/ml Fungizone. Viral DNA was prepared as described (36).

Construction of Recombinant Baculovirus Expressing UL9—A 2.8-kilobase *Nde*I/*Hin*dIII DNA fragment containing the HSV-1 UL9 gene was cleaved from the plasmid pET3a/OBP and methylated with *Bam*HI methylase. The ends of the fragment were filled in with T4 DNA polymerase, and *Bam*HI linkers were added. The fragment was then ligated into the high expression vector pVL941 and used to transform *E. coli* HB101 cells. The recombinant plasmid pVL941/UL9 was then isolated and used to generate the recombinant *A. californica* nuclear polyhedrosis virus/UL9 baculovirus as described (36). Stocks of recombinant baculovirus were propagated in Sf9 cells grown in TMNFH plus 10% fetal calf, 50 μg/ml gentamycin sulfate, and 2 μg/ml Fungizone.

Infection of Sf9 Cells and Preparation of Infected Cell Extracts—Sf9 cells were grown to a cell density of 2.5×10^6 cells/ml in two 850-cm^2 roller bottles containing 500 ml of ExCell 400. The bottles were placed upright with caps loosened and shaken at 140 rpm in an orbital shaker at 27 °C. Thirty-five ml of prewarmed *A. californica* nuclear polyhedrosis virus/UL9 recombinant baculovirus at a titer of approximately 1.5×10^8 plaque-forming units/ml were added, and shaking at 140 rpm at 27 °C was resumed. Sixty hours later, the cells were pelleted by centrifugation at $350 \times g$ for 15 min. The cell pellet was resuspended in 100 ml of ice-cold lysis buffer consisting of 20 mM HEPES, pH 7.6, 1.0 mM dithiothreitol, 10 mM sodium bisulfite, pH 7.7, 4 μg/ml leupeptin, 4 μg/ml pepstatin, and 0.5 mM phenylmethylsulfonyl fluoride. The cells were transferred to a Dounce homogenizer and lysed using 10 strokes of a tight fitting pestle. The nuclei were pelleted by centrifugation at $1,500 \times g$ for 10 min at 4 °C, resuspended in lysis buffer supplemented with 10% sucrose, frozen in liquid nitrogen, and stored at −80 °C until use.

Purification of UL9 Protein—Unless otherwise noted, all steps were performed at 4 °C. Nuclei (37 ml) were quickly thawed at 37 °C, and an equal volume of ice-cold buffer A [3.4] was added all at once with vigorous stirring for 10 min. The extract was centrifuged at $95,000 \times g$ for 90 min, and the supernatant was dialyzed against two 4-h changes of buffer A [0.10]. The dialysate was cleared by centrifugation at $40,000 \times g$ for 20 min and applied to a 42-ml phosphocellulose column equilibrated with buffer A [0.10]. The column was washed with 42 ml of the same buffer and eluted with a 500-ml gradient from 0.15 to 0.75 M NaCl in buffer A. Fractions (7 ml) containing UL9 protein were identified using the colorimetric ATPase assay described below. The UL9 protein eluted between 0.40 and 0.46 M NaCl. Peak fractions were pooled (41 ml) and then diluted with an equal volume of buffer B[0.15]. The pool was then applied to a hydroxylapatite column (20 ml) that had been prewashed with buffer B containing 500 mM ammonium sulfate and equilibrated with buffer B[0.15]. The column was washed with 20 ml of equilibration buffer and eluted with a 260-ml gradient from 0.1 to 0.5 M ammonium sulfate in buffer B. Fractions containing the UL9 protein were identified by SDS-polyacrylamide gel electrophoresis. Approximately two-thirds of the UL9 protein was eluted between 0.16 and 0.26 M ammonium sulfate, with the remainder trailing throughout the rest of gradient. Peak fractions were pooled (35 ml) and concentrated to approximately 1.5 ml using a Centriprep 30 concentrator (Amicon). The concentrated UL9 protein was diluted with 1.2 ml of buffer C [0.10] and applied to a 4-ml heparin-Sepharose column equilibrated with the same buffer. The column was washed with 4 ml of equilibration buffer and eluted with a 40-ml gradient from 0.1 to 0.7 M NaCl in buffer C. Fractions (0.9 ml) were assayed for DNA-dependent ATPase and HSV-1 origin binding activity as described (28). Both DNA-dependent ATPase and origin binding activities were coincident and eluted between 0.45 and 0.60 M NaCl. Peak fractions containing the UL9 protein were aliquoted, frozen in liquid nitrogen, and stored at −80 °C. The yield of UL9 protein (3 mg) was determined using a calculated molar extinction coefficient of 89,220 based on the method of Gill and von Hippel (37). The purity was 94% as determined by densitometry of a Coomassie-stained SDS-polyacrylamide gel loaded with approximately 6 μg of protein.

DNA-dependent NTPase Assays—Depending on the required level of sensitivity, two different assays were used. In the first assay the formation of inorganic phosphate was determined by the addition of an acidic molybdate solution containing malachite green (38). Unless otherwise indicated, the standard reaction mixtures (25 or 50 μl) contained buffer D, 4.4 μg/ml UL9 protein, 44 μg/ml heat-denatured activated calf thymus DNA and 2 mM ATP. Mixtures were incubated for the indicated time at 37 °C, and 0.75 ml of acidic molybdenum/malachite green was added. After 5 min of color development, A_{650} was determined. A more sensitive radioisotopic assay using α-^{32}P-labeled nucleoside triphosphates at 0.4 Ci/mmol was used for kinetic measurements. Reaction mixtures (25 μl) were stopped by the addition of 3 μl of 0.5 M EDTA, pH 8.0, and chilled on ice. Aliquots (1-3 μl) were spotted onto polyethyleneimine-cellulose F thin layer chromatography plates and dried. Products of DNA-dependent ATPase, DNA-dependent CTPase, and DNA-dependent UTPase were separated using 1.0 M formic acid and 0.4 M LiCl as the solvent. Products of DNA-dependent GTPase were separated using 0.5 M formic acid and 1.0 M LiCl as the solvent. Nucleoside 5′-diphosphate products were located either by autoradiography or with ultraviolet using standards run in parallel lanes. Regions of the plate containing enzymatic products were excised and their radioactivity determined using Beckman Ready Safe as the scintillant.

HSV-1 Origin Binding Assay—The specific binding of HSV-1 UL9 protein to *Ori*$_S$-containing DNA was assayed by nitrocellulose filter binding as described using radiolabeled Box I DNA of *Ori*$_S$ as substrate (28).

RESULTS

Comparison of Mammalian and Insect Expressed HSV-1 UL9 Protein—The HSV-1 UL9 protein isolated from insect cells was indistinguishable from UL9 protein expressed in mammalian cells as judged by the following three criteria. First, the specific activities of the two proteins were the same. Mammalian and insect expressed HSV-1 UL9 protein bound 0.097 ± 0.01 and 0.11 ± 0.008 mol of *Ori*$_S$ Box I DNA/mol of

protein (monomer), respectively, and hydrolyzed 7.7 ± 0.3 × 10^3 and 8.0 ± 0.3 × 10^3 mol of ATP/mol of protein/h, respectively. Second, both proteins migrated identically as an 87-kDa polypeptide during SDS-polyacrylamide gel electrophoresis (data not shown). Finally, using denatured calf thymus DNA as coeffector, the two proteins displayed identical temperature profiles with an optimum temperature of 45 °C (Fig. 1). No activity was observed at 27 °C, which is the optimal growth temperature of the Sf9 insect cells. Similar results were observed using poly(dT) as the DNA coeffector (data not shown).

DNA-dependent ATPase Activity of UL9 Protein Has a Low Salt Optimum—Concentrations of NaCl or KCl of less than 50 mM had little or no effect on the DNA-dependent ATPase activity of the UL9 protein (Fig. 2*a*). However, at 150 mM the activity was decreased by 50% and was abolished at 300 mM. Presumably the chloride anion is inhibitory since the corresponding acetate salts required concentrations in excess of 225 mM before 50% inhibition was seen. Sulfate anion was extremely inhibitory, as evidenced by greater than 75% inhibition of activity at an ammonium sulfate concentration of 25 mM, whereas there was no inhibition at a similar concentration of ammonium acetate. Phosphate anion was also inhibitory, with greater than 75% inhibition of activity observed at a concentration of 25 mM potassium phosphate.

DNA-dependent ATPase Activity of UL9 Protein Requires a Divalent Cation—In the absence of any divalent cation, little DNA-dependent ATPase activity was observed. Optimal activity required the addition of Mg^{2+}. Assuming that ATP chelates Mg^{2+} at a 1:1 stoichiometry, a free Mg^{2+} concentration of 2–5 mM was required for optimal activity (Fig. 2*b*). However, substantial activity was still observed at greater than 10 mM Mg^{2+}. Similar concentrations of Mn^{2+} were equally as effective in supporting activity, and Ca^{2+} supported activity at 30% of the level seen with Mg^{2+} (data not shown).

Effect of Buffer Constituents on DNA-dependent ATPase Activity of UL9 Protein—The DNA-dependent ATPase activity of the UL9 protein was tested with omissions of each of the various constituents in the standard buffer (buffer D, "Experimental Procedures"). Omission of either bovine serum albumin or glycerol had little effect, decreasing the activity by less than 10%, whereas omission of 2 mM dithiothreitol increased the activity by 50%. The addition of spermidine at concentrations exceeding 7 mM inhibited by greater than 50%. Substitution of glycerol with polyethylene glycol 6000 resulted in a 70% increase in activity. Addition of dimethyl sulfoxide to 8% (v/v) in the standard reaction mixture doubled activity.

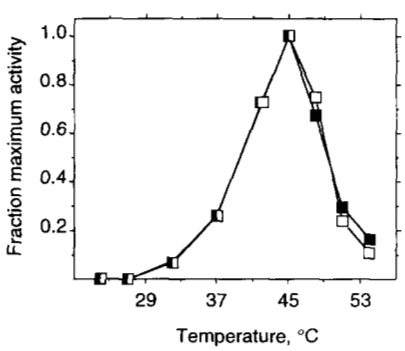

FIG. 1. **Temperature profile of DNA-dependent ATPase activity of mammalian and insect expressed UL9 protein.** DNA-dependent ATPase activity was determined after 30 min of incubation in the standard colorimetric assay described under "Experimental Procedures" except that the temperature was varied from 24 to 54 °C. □, mammalian expressed UL9 protein; ■, insect expressed UL9 protein.

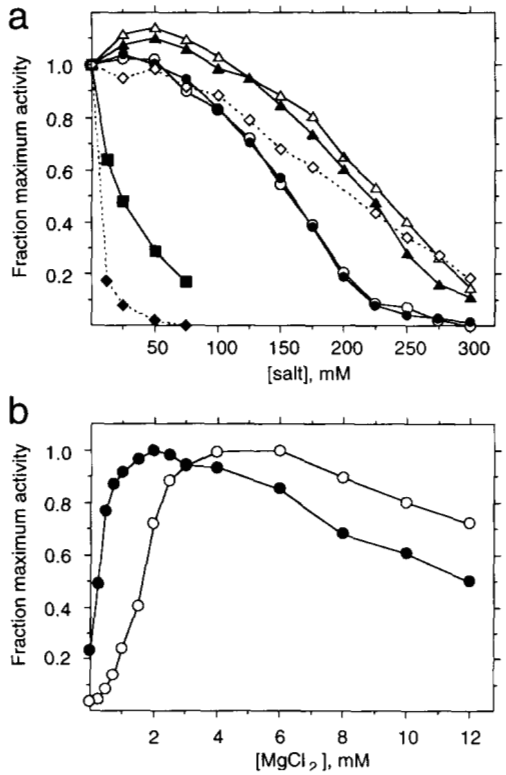

FIG. 2. **Effect of salt and Mg^{2+} on DNA-dependent ATPase activity of UL9 protein.** DNA-dependent ATPase activity was determined using the standard reaction mixture and colorimetric assay as described under "Experimental Procedures" except that the salt or Mg^{2+} concentration was varied as indicated. Incubation was for 45 min. *Panel a*, salt profile. ○, NaCl; ●, KCl; △, potassium acetate; ▲, sodium acetate; ◇, ammonium acetate; ◆, ammonium sulfate; ■, potassium phosphate, pH 8.3. *Panel b*, Mg^{2+} profile. ○, 2 mM ATP; ●, 0.5 mM ATP.

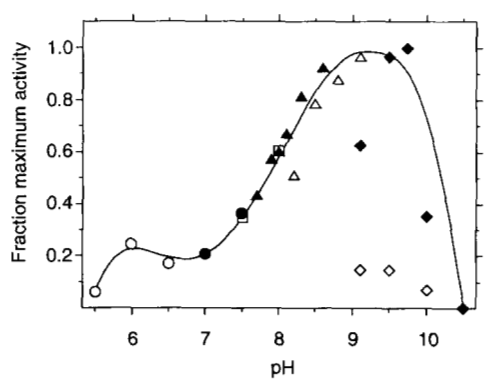

FIG. 3. **pH profile of DNA-dependent ATPase activity of UL9 protein.** DNA-dependent ATPase activity was determined using the standard reaction and colorimetric assay as described under "Experimental Procedures" except that the indicated buffer was substituted for EPPS, pH 8.3, in buffer D of the reaction mixture. Incubation was for 45 min. ○, MES; ●, MOPS; □, HEPES; ▲, EPPS; △, TAPS; ◇, CHES; ◆, CAPS.

DNA-dependent ATPase Activity of UL9 Protein Is Optimally Active between pH 8.3 and 9.5—The DNA-dependent ATPase activity of the UL9 protein was determined at pH values ranging from 5.5 to 10.5 (Fig. 3). There was a small peak of activity at pH 6.0. However, optimal activity was observed between pH 8.3 and 9.5, followed by a rapid decrease, diminishing to background at pH 10.5. CHES buffer was inhibitory relative to TAPS and CAPS buffers at a similar pH.

The Products of DNA-dependent ATPase Are Inhibitory—ADP and P_i, which are the products of the DNA-dependent ATPase activity of the UL9 protein, were tested for their ability to inhibit the ATPase activity. As shown in Fig. 4*a*, ADP was inhibitory. Approximately 50% inhibition was observed at equimolar ratios of ADP to ATP, and there was a greater than 90% inhibition at a molar ratio of 10:1. P_i was not as inhibitory. Fifty percent inhibition occurred when the molar ratio of P_i to ATP was 6:1, and only 65% inhibition was observed at a molar ratio of 18:1. The addition of both ADP and P_i to reaction mixtures resulted in only slightly more inhibition of activity than ADP alone. Thus, ADP is the predominant inhibitory product with no synergistic inhibition by ADP and P_i.

Ribonucleoside 5′-Triphosphates Compete as Substrates for the DNA-dependent NTPase Activity—Radiolabeled ATP or CTP substrates were challenged with increasing concentrations of unlabeled heterologous NTP-$MgCl_2$, and the DNA-dependent NTPase activity was measured (Fig. 4*b*). With labeled ATP as substrate, activity was reduced to background levels by the addition of increasing concentrations of unlabeled GTP and CTP. dGTP, which is not hydrolyzed by the UL9 protein (28), was also inhibitory. When labeled CTP was challenged with unlabeled ATP, activity was also reduced to background levels. These results suggest that in contrast to the HSV-1 helicase-primase, a single nucleotide binding site participates in UL9-mediated DNA-dependent NTPase activity.

DNA Structure and Concentration Affect DNA-dependent ATPase Activity—Double-stranded DNA has been reported to be ineffective as a coeffector for the DNA-dependent ATPase activity of the UL9 protein whether or not it contained an HSV-1 origin, whereas various ssDNAs differed in their capacity to act as a coeffector (28). We examined further the effects of ssDNA coeffectors on the DNA-dependent ATPase activity. As shown in Fig. 5*a*, poly(dT) was 3-fold more active than heat-denatured activated calf thymus DNA, whereas M13mp18 ssDNA was only 1.5-fold more active. Thus, either secondary structure or specific sequences in ssDNA significantly affect the interaction of the UL9 protein with DNA. However, all three of the DNA coeffectors saturated at a concentration of approximately 10 μM (in nucleotide). In the absence of DNA coeffector the activity was close to background.

Minimal Length of DNA Effector Required for Activity—Deoxythymidine oligonucleotides ranging in length from 2 to greater than 3,000 residues, at a concentration of 60 μM (in nucleotide) were tested as coeffectors for the DNA-dependent ATPase activity (Fig. 5*b*). The minimum length capable of eliciting activity was 14 nucleotides. Activity rose sharply with increasing lengths of oligonucleotide, appearing to saturate near 60 nucleotides, suggesting a processive mechanism of action.

ICP8 Specifically Stimulates DNA-dependent ATPase Activity—ICP8 is an SSB encoded by HSV-1 and required for

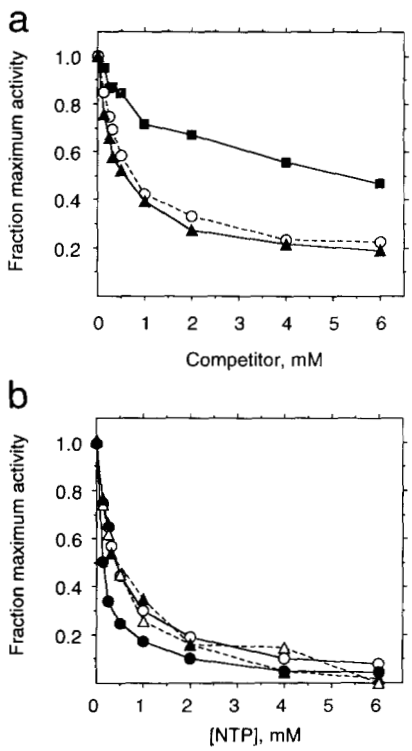

FIG. 4. *Panel a*, product inhibition of DNA-dependent ATPase activity of UL9 protein. DNA-dependent ATPase activity was determined using the standard radioisotopic assay described under "Experimental Procedures." The concentration of labeled ATP was 320 μM, and incubation was for 10 min. The concentrations of P_i (■), ADP (○), or P_i + ADP (▲) are indicated. *Panel b*, nucleoside 5′-triphosphate cross-competition for DNA-dependent NTPase activity. DNA-dependent NTPase activity was determined using the standard radioisotopic assay described under "Experimental Procedures." The concentration of labeled NTP was 320 μM, and incubation was for 10 min. The concentrations of unlabeled competitor NTP are indicated. ○, CTP *versus* labeled ATP; △, GTP *versus* labeled ATP; ▲, dGTP *versus* labeled ATP; ●, ATP *versus* labeled CTP.

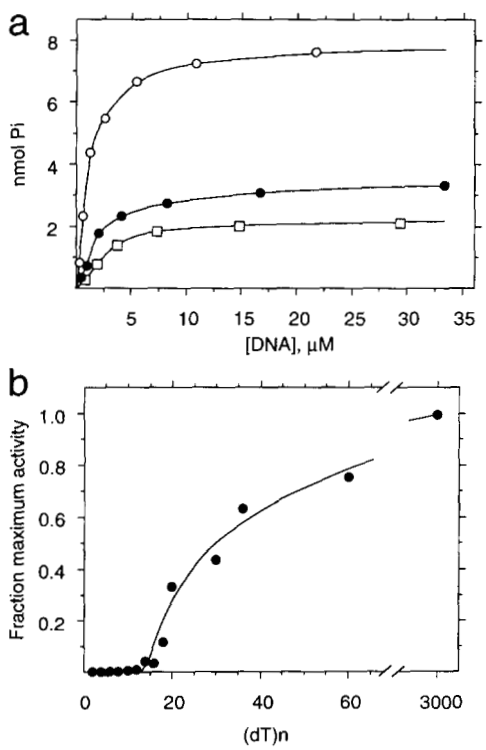

FIG. 5. **Effect of DNA structure, concentration, and length on DNA-dependent ATPase activity of UL9 protein.** DNA-dependent ATPase activity was determined using the standard colorimetric assay described under "Experimental Procedures" except the type and concentration of DNA coeffector were varied as indicated. Incubation was for 30 min. *Panel a*, DNA-dependent ATPase activity with varying concentrations of heat-denatured activated calf thymus DNA (□), M13 mp18 ssDNA (●), or $(dT)_{3000}$ (○). *Panel b*, activity in the presence of increasing lengths of poly(dT) at a constant concentration of 60 μM in nucleotide.

its replication (18). The addition of purified ICP8 to reaction mixtures containing DNA coeffectors with secondary structure stimulated DNA-dependent ATPase activity (Table I). With both native and heat-denatured activated calf thymus, DNA activity was doubled by the addition of ICP8. *E. coli* SSB had little effect, whereas HSSB inhibited activity more than 8-fold. The addition of purified ICP8 to reaction mixtures containing poly(dT) as the coeffector resulted in a modest increase in activity. *E. coli* SSB had no effect, whereas the HSSB inhibited more than 3-fold. These results suggest that in addition to removing inhibitory secondary structure from the DNA coeffector, ICP8 specifically interacts with the UL9 protein to stimulate its DNA-dependent ATPase activity.

DNA-dependent ATPase of UL9 Protein Exhibits Complex Kinetics—The effects of ribonucleoside 5′-triphosphate specificity and concentration on DNA-dependent NTPase activity were compared (Fig. 6*a*). The substrate specificity was similar to that reported for the UL9 protein derived from a mammalian overexpression system (28). ATP and CTP were effective substrates with heat-denatured activated calf thymus DNA as coeffector, and UTP and GTP were moderately effective. However, the kinetics were complex. A pronounced and extended lag was observed with increasing concentrations of ATP (Fig. 6*b*). Similar results were observed with CTP and UTP (data not shown).

To determine whether the lag is influenced by secondary structure in the DNA coeffector, a time course was performed comparing poly(dT) and M13mp18 ssDNA with heat-denatured activated DNA. For all three DNA coeffectors, products continued to accumulate throughout the reaction (Fig. 7). However, although differences in DNA secondary structure affected the rate of the DNA-dependent ATPase, they did not appear to affect the lag substantially. Preincubation of UL9 plus ATP, UL9 plus DNA, or UL9 plus Mg^{2+} for 30 min followed by the addition of the omitted component did not eliminate the lag. Incubation at higher temperature (45 °C) also failed to eliminate the lag but did shorten it by several minutes (data not shown).

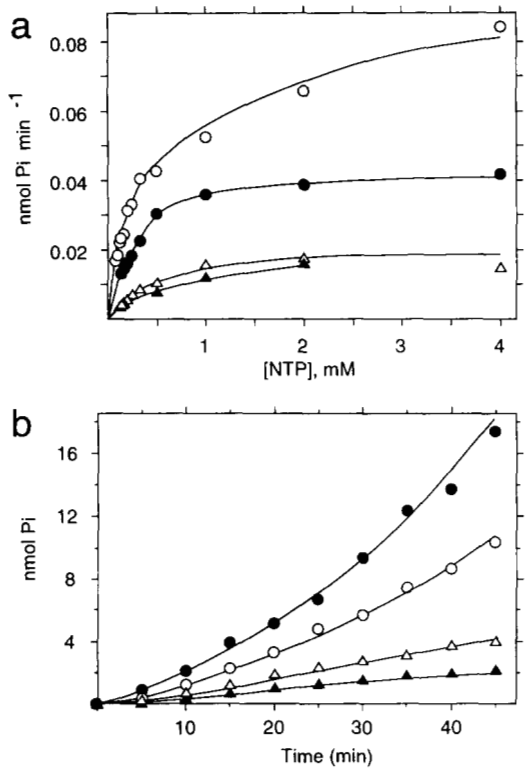

FIG. 6. *Panel a*, effect of ribonucleoside 5′-triphosphate specificity and concentration on DNA-dependent ATPase activity of UL9 protein. DNA-dependent ATPase activity was determined using the radioisotopic assay described under "Experimental Procedures." Reactions were initiated by mixing prewarmed enzyme and heat-denatured activated calf thymus DNA in reaction mixtures containing the indicated ribonucleoside triphosphate. ○, ATP; ●, CTP; △, UTP; ▲, GTP. *Panel b*, time course of DNA-dependent ATPase activity of UL9 protein. Assays in the presence of fixed concentrations of ATP were performed as above except that the period of incubation was varied as indicated. ●, 4 mM ATP; ○, 1 mM ATP; △, 0.25 mM ATP; ▲, 0.125 mM ATP.

DISCUSSION

HSV-1 encodes the minimal repertoire of proteins necessary to initiate origin specific replication (18). These include a DNA polymerase with intrinsic 3′-5′ exonuclease and RNase H activities (41–45), an accessory factor that enhances the processivity of the DNA polymerase (34, 46–49), an SSB (ICP8) (50–53), a heterotrimeric helicase-primase (39, 40, 54, 55), and an origin binding protein (UL9 protein) (19, 20).

TABLE I

Specificity of single-stranded DNA-binding protein stimulation of DNA-dependent ATPase activity of UL9 protein

Reaction mixtures (25 μl) contained 8 μg/ml of the indicated single-stranded DNA coeffector. The concentration of each of the indicated single-stranded DNA-binding proteins was 27 nM. After a 45-min incubation ATP hydrolysis was determined using the colorimetric assay.

Single-stranded DNA binding protein	Phosphate formed			
	None	ICP8	Human	*E. coli*
	nmol			
Single-stranded DNA coeffector				
Poly(dT)	6.4	8.3	1.7	5.7
Heat-denatured activated calf thymus DNA	1.6	3.2	0.19	1.5
Activated calf thymus DNA	0.32	0.77	0	0

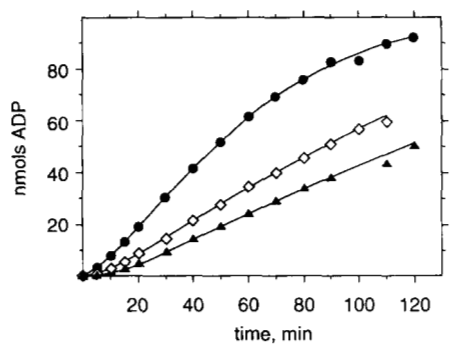

FIG. 7. **Effect of DNA structure on the kinetics of DNA-dependent ATPase activity of UL9 protein.** Reaction mixtures (70 μl) in buffer D contained 1.5 mM ATP, 4.5 mM Mg^{2+}, 2.3 μg/ml UL9 protein, and 12 μg/ml of the indicated DNA coeffector. Reactions were initiated by the addition of prewarmed enzyme and DNA to mixtures containing ATP. At the indicated times, 5-μl aliquots were withdrawn and assayed for ATP hydrolysis using the radioisotopic assay as described under "Experimental Procedures." ●, $(dT)_{3000}$; ◇, M13mp18 ssDNA; ▲, heat-denatured activated calf thymus DNA.

Presumably elongation and termination factors such as topoisomerase and ligase are supplied by the host. All of the herpes-encoded proteins have been purified in sufficient quantities for detailed study. The DNA polymerase, its accessory factor, the SSB, and the helicase-primase have been characterized and behave similarly to analogous replication proteins

from other sources. The interaction of the origin binding protein with Ori_S sequences has also been extensively characterized (14, 15, 20–23, 26, 27), but it is not clear how it functions in initiating replication, and little is known about its DNA-dependent NTPase and DNA helicase activities. This gap in our knowledge probably relates to the inability, thus far, to reconstitute HSV-1 origin-specific replication *in vitro* with the purified herpes-encoded proteins. Of course, there may be a requirement for an as-yet undiscovered host factor(s). Also puzzling is the finding that ATP does not alter the nature of the interaction of the UL9 protein with Ori_S sequences (22). Other origin binding proteins possessing DNA-dependent ATPase activity, *e.g.* the *E. coli dnaA* protein and the SV40 T antigen utilize ATP in assembling an initiator-origin complex that untwists origin DNA (1, 3, 4). In light of these observations we have initiated a detailed study of of the enzymatic properties of the UL9 protein in the hope of gaining further insight into how it functions in initiating replication.

In this paper we have shown that UL9 protein isolated from an insect overexpression system is indistinguishable from mammalian expressed UL9. We have investigated the parameters affecting the DNA-dependent ATPase activity of the insect expressed UL9 protein. Optimal activity requires low salt, a divalent cation, high pH, and high temperature. The low salt optimum of the DNA-dependent ATPase activity contrasts with the high salt optimum of the HSV-1 DNA polymerase. At 150 mM NaCl the DNA-dependent ATPase activity is reduced by half. However, the alkaline pH optimum is consistent with that of the other HSV-1 replication enzymes (39, 54). Also consistent with the DNA polymerase and helicase-primase is the requirement for a divalent cation. Maximal DNA-dependent ATPase activity requires 2–5 mM Mg^{2+}, but even at 12 mM there is considerable activity. This contrasts with the extremely sharp Mg^{2+} optimum of 2–3 mM for the helicase-primase (54), and 0.5–1 mM for the DNA polymerase.[2] High temperature is necessary for optimal activity. Below 30 °C, enzymatic activity is extremely weak. Previous studies on the binding of the UL9 protein to origin sequences were carried out at low temperature, low pH, and high salt, conditions under which the UL9 protein would have been enzymatically inactive (14, 15, 20–23). This may possibly account for the observed lack of any effect of ATP on the interaction of UL9 protein with Ori_S.

Several compounds influence enzymatic activity. Dithiothreitol and ammonium sulfate, which are often used in purifying overexpressed origin binding proteins, are inhibitory. Macromolecular crowding agents, such as polyethylene glycol, substantially stimulate, as does dimethyl sulfoxide. The enzyme is also subject to product inhibition. Both ADP and P_i are inhibitory, although P_i is much weaker in its effect than is ADP. Consideration of these observations should facilitate future attempts at reconstitution of HSV-1 origin-specific replication using purified proteins.

The ability of unlabeled heterologous nucleoside triphosphates to compete with labeled nucleoside triphosphate substrates suggests that there a single nucleotide effector site on the UL9 protein. This observation is consistent with the appearance of a single consensus nucleotide binding site in the UL9 sequence (17, 28). In contrast, the HSV-1 helicase-primase has two distinct sites for NTP hydrolysis, each of which affects the DNA helicase activity of the helicase-primase (55).

The composition of the DNA coeffector affects the DNA-dependent ATPase activity. Double-stranded plasmid DNA containing Ori_S has been reported to be relatively inert as a coeffector, whereas ssDNA is effective (28). However, variable rates of activity are observed with different ssDNAs (28, this work). Presumably the UL9 protein binds to ssDNA and utilizes the energy of ATP hydrolysis to traverse unidirectionally along the bound DNA coeffector. When a double-stranded region is encountered, the opposing strand must be displaced before the UL9 protein can continue. This helicase activity necessarily introduces an additional mechanistic step and accounts for the diminished rates of ATP hydrolysis with coeffectors containing regions of secondary structure. Alternatively, the UL9 protein may simply have a higher affinity for double-stranded DNA than it does for ssDNA, enabling regions of secondary structure in a single-stranded coeffector to decrease activity simply by sequestering it.

The minimal length of coeffector that will elicit activity is 14 nucleotides, and as length increases so does activity. Assuming that the UL9 protein binds DNA and translocates by hydrolyzing ATP, this length-dependent increase in DNA-dependent ATPase activity suggests a processive mechanism. A distributive mechanism should be unaffected by variation in DNA coeffector length.

A replisome is comprised of several protein constituents that are in intimate association with one another via protein-protein contacts (1–4). The UL9 protein may initiate replication by assembling into a multimeric structure at the origin and untwisting the A+T-rich region into an open complex receptive to entry of the remaining replicative enzymes. Open complex formation may be facilitated by the coupled DNA-dependent ATPase and DNA helicase activities. ICP8, the HSV-1-encoded SSB, may facilitate these functions through specific contacts with the UL9 protein and nonspecific contacts with the DNA. The observation that ICP8 specifically stimulates the DNA-dependent ATPase activity using DNA coeffectors that both possess and lack secondary structure lends credence to this conjecture.

The complex kinetics of the DNA-dependent ATPase activity exhibit a pronounced lag period that persists even when the secondary structure of the DNA coeffector and temperature are varied. These complex kinetics are reminiscent of other origin binding proteins, notably SV40 T antigen. In the presence of ATP, T antigen binds to the SV40 origin and assembles into a double hexameric structure that encases the origin (3, 56, 57). The formation of the double hexamer causes origin sequences to untwist into an open complex (58–60). Replication ensues beginning with the bidirectional unwinding of duplex origin sequences through the combined action of the T antigen-associated DNA helicase activity with HSSB and topoisomerase (61–63). However, SV40 origin sequences are not required for T antigen to manifest its DNA-dependent ATPase and DNA helicase activities. Complementary DNA fragments annealed to M13 ssDNA are also unwound by T antigen in an ATP-dependent fashion (64, 65). This origin-independent activity also exhibits a pronounced lag period. The observed lag in the DNA-dependent ATPase activity of the UL9 protein may therefore reflect a similar mechanism of action.

Note Added in Proof—Fierer and Challberg have also purified and characterized baculovirus expressed UL9 protein and have reported similar results (67).

[2] T. Hernandez and I. R. Lehman, unpublished observation.

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