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A DNA helicase induced by herpes simplex virus type 1

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ABSTRACT

We have identified and partially purified a DNA-dependent ATPase that is present specifically in herpes simplex virus type 1-infected Vero cells. The enzyme which has a molecular weight of approximately 440,000 differs from the comparable host enzyme in its elution from phosphocellulose columns and in its nucleoside triphosphate specificity. The partially purified DNA-dependent ATPase is also a DNA helicase that couples ATP or GTP hydrolysis to the displacement of an oligonucleotide annealed to M13 single-stranded DNA. The enzyme requires a 3' single-stranded tail on the duplex substrate, suggesting that the polarity of unwinding is 5'→3' relative to the M13 DNA. The herpes specific DNA helicase may therefore translocate on the lagging strand in the semidiscontinuous replication of the herpes virus 1 genome.

INTRODUCTION

Herpes simplex virus type 1 (HSV-1) is a eukaryotic DNA virus with an approximately 150 kilobase genome. In addition to cis acting elements (ori_S and ori_L) that very likely serve as origins of DNA replication, the HSV-1 genome codes for most and possibly all of the enzymes required for its replication (1-3). Genetic analyses of HSV-1 have shown seven complementation groups to be essential for DNA replication (4,5). Recently, seven open reading frames have been identified that correspond to the seven complementation groups. These have been shown to be both necessary and sufficient to support in trans the replication of plasmids containing an HSV-1 origin (ori_S or ori_L) (6). One of these open reading frames encodes the HSV-1 DNA polymerase and a second encodes the major single-stranded DNA binding protein (7,8). A third has very recently been identified with a protein that binds specifically to the ori_S sequence (9,10). The function of the other four open reading frames is unknown. By analogy to prokaryotic DNA replication these open reading frames might be expected to encode a DNA primase (11), a helicase and DNA polymerase accessory protein(s) (12).

Several of the enzymes that play an important role in DNA replication in

prokaryotes have an associated DNA-dependent ATPase activity. These include the E. coli dnaB protein (13) and the bacteriophage T4 gene 41 and dda proteins (14). Each of these DNA-dependent ATPases is a DNA helicase capable of coupling ATP hydrolysis to the unwinding of a DNA duplex (12-14). In an effort to identify an HSV-1 specific helicase we sought and ultimately found a DNA-dependent ATPase that upon purification proved to be a DNA helicase. This helicase acts only on DNA duplexes that contain a 3' single-stranded tail, implying that the direction of translocation on the strand opposite to that containing the tail is in the 5' to 3' direction.

MATERIALS AND METHODS

Materials

All reagents were obtained from the Sigma Chemical Company unless otherwise specified. [γ^{32}P]ATP (6,000 Ci/mmol), and [^{3}H]thymidine (60 Ci/mmol) were from New England Nuclear. Polynucleotide kinase was from New England Biolabs and the U. S. Biochemical Corp. Acetylated bovine serum albumin was from Bethesda Research Labs. Centricon 30 microconcentration devices were from Amicon and used according to the manufacturer's instructions. Phosphocellulose (P11) was obtained from Whatman. The prepacked Superose 12 gel filtration column (1.0 x 30 cm), molecular weight standards and the G-50 fine were from Pharmacia. Sodium dodecyl sulfate (SDS) was purchased from BDH Fine Chemicals. Acrylamide, bis-acrylamide, sodium persulfate, TEMED, and high and low molecular weight standards were obtained from Bio-Rad.

Cells and viruses. RΔ305 (15), a thymidine kinase-deficient mutant of HSV-1 [F], was used to infect roller-bottle cultures of Vero cells using a multiplicity of infection of 5.

DNA Substrates. Activated calf thymus DNA (16) and single-stranded M13mp18DNA were prepared as described (17). ^{3}H-labeled E. coli DNA (40,000 cpm/nmol) was prepared by labeling E. coli strain HB101 with [^{3}H]thymidine followed by density banding of the extracted chromosomal DNA in $CsCl_2$ as described (18). Oligonucleotides were synthesized by the phosphoramidate method (19,20) using a 380 A DNA synthesizer (Applied Biosystems). Deblocking was accomplished by incubation of the oligonucleotides in concentrated NH_4OH at 55°C overnight. The oligonucleotides were vacuum dried, resuspended in double-distilled H_2O, extracted three times with diethylether, dried again, and purified further by electrophoresis through 20% urea-containing polyacrylamide gels containing 89 mM Tris base, 89 mM boric acid, and 2 mM EDTA. The major DNA band was

visualized by long wave UV absorbance, excised, extracted from the polyacrylamide, and desalted into 10 mM Tris-HCl (pH 7.5) containing 1.0 mM EDTA by centrifugation through G-50 fine (21). The oligonucleotides were labeled with ^{32}P at their 5' termini by incubation with [γ ^{32}P]ATP and polynucleotide kinase (22). The unreacted [γ ^{32}P]ATP was removed by centrifugation of the labeled oligonucleotide through G-50 fine.

The sequences of the oligonucleotides used to prepare helicase substrates were: 5'-ACTCTAGAGGATCCCCGGGTACGTTATTGCATGAAAGCCCGGCTG-3' (45-mer, 3' tail), 5'-GTTATTGCATGAAAGCCCGGCTGACTCTAGAGGATCCCCGGGTACGTTATTGCATGAAAGCCCGGCTG-3' (68-mer 3' and 5' tails), 5'-GTTATTGCATGAAAGCCCGGCTGACTCTAGAGGATCCCCGGGTAC-3' (45-mer, 5' tail), and 5'-ACTCTAGAGGATCCCCGGGTAC-3' (22-mer, untailed) and designated as 3' tailed, 3',5' tailed, 5' tailed, and untailed, respectively. The untailed substrate is complementary to nucleotides 6243 to 6264 of M13mp18 single-stranded DNA (23), the only sequence to which the four oligonucleotides can be hybridized. The ^{32}P-labeled oligonucleotides were annealed to M13mp18 single-stranded DNA, and the duplex helicase substrates purified by gel filtration as described (24).

Buffers. Buffer A is composed of 20 mM Tris-HCl (pH 7.5), 10% v/v glycerol, 3.5 mM $MgCl_2$, 100 µg/ml bovine serum albumin, and 5.0 mM dithiothreitol. Buffer B consists of 20 mM Na-Hepes (pH 7.6), 10% v/v glycerol, 1.0 mM dithiothreitol, 1.0 mM EDTA, 1.0 mM EGTA, 2.0 µg/ml leupeptin, 2.0 µg/ml Pepstatin A, and 0.5 mM phenylmethylsulfonyl fluoride. The buffers were filtered (0.22 µm pore size) to remove particulate contaminants.

Methods

DNA-dependent ATPase activity. DNA dependent ATPase served to monitor DNA helicase activity during purification. The reaction mixture (50 µl) in buffer A contained 1.0 mM ATP, 2.0 µg of activated calf thymus DNA and enzyme fraction. After incubation for 40 min at 33°C, formation of P_i was determined by the addition of 0.75 ml of an acidic ammonium molybdate solution containing Malachite Green (25). After five min, color development was stopped by the addition of 0.10 ml of 34% trisodium citrate and the A_{650} was determined. One unit of DNA-dependent ATPase hydrolyzes 1.0 nmol of ATP/hr under these conditions.

DNase activity. The reaction mixture (25 µl) in buffer A contained 2.0 nmol (nucleotide) [^3H]thymidine-labeled *E. coli* DNA and 5.0 µl of enzyme fraction. After 30 min at 33°C, 10 µl of calf thymus DNA (2.0 mg/ml) and 0.125 ml of cold 10% trichloroacetic acid were added. After 10 min at 0°C, the precipitate was removed by centrifugation. 0.125 ml of the supernatant

was added to 1.0 ml of aqueous scintillation fluid and the radioactivity determined.

DNA helicase activity. The reaction mixture (10 µl) in buffer A contained 4.0 µM (nucleotide) helicase substrate; enzyme (Fraction IV) and 3.0 mM ATP were added as indicated. After 5 min at 30°C, one-third volume of a solution containing 1.0% SDS, 200 mM EDTA, and 50% glycerol at 0°C was added. The mixture was electrophoresed through a non-denaturing 15% polyacrylamide gel (14.5 cm x 13.5 cm x 0.75 mm) containing 89 mM Tris base, 89 mM boric acid, and 2.0 mM EDTA. After electrophoresis, the gels were dried under vacuum and autoradiographed overnight at -80°C using a Dupont Cronex Quanta II intensification screen. When applicable, the products of the reaction were excised from the gel, dissolved in 30% H_2O_2, and their radioactivity determined by scintillation counting.

Discontinuous SDS-polyacrylamide gel electrophoresis was performed as described (26) utilizing a 10% separating gel. After electrophoresis, the gels were stained with ammonical silver (27).

Protein concentrations were determined by the method of Bradford using bovine serum albumin as a standard (28).

RESULTS

Because of their potent DNase activity we were unable to detect DNA helicase activity in nuclear extracts of HSV-1 infected cells. Since helicases are invariably associated with a DNA-dependent ATPase activity that is relatively insensitive to nuclease action, we sought such an activity in high salt extracts of nuclei of HSV-1 infected cells. An HSV-1 specific DNA-dependent ATPase was identified which was then purified to the point at which it was free of nuclease activity. Assay of the purified DNA-dependent ATPase showed it to be a HSV-1 specific DNA helicase.

Identification of HSV-1 DNA-dependent ATPase

Chromatography on phosphocellulose of 1.7-M NaCl extracts of HSV-1 infected Vero cell nuclei (see below) revealed two peaks of DNA dependent ATPase activity, eluting at 0.15 M and 0.27 M NaCl, respectively (Fig. 1a). In contrast, only a single peak of activity eluting at 0.27 M NaCl appeared upon phosphocellulose chromatography of a comparable nuclear extract of uninfected Vero cells (Fig. 1b). Thus, the peak of DNA dependent ATPase activity eluting at 0.15 M NaCl appears to be HSV-1 specific. In the absence of DNA, ATP hydrolysis could not be detected at any point in the chromatogram.

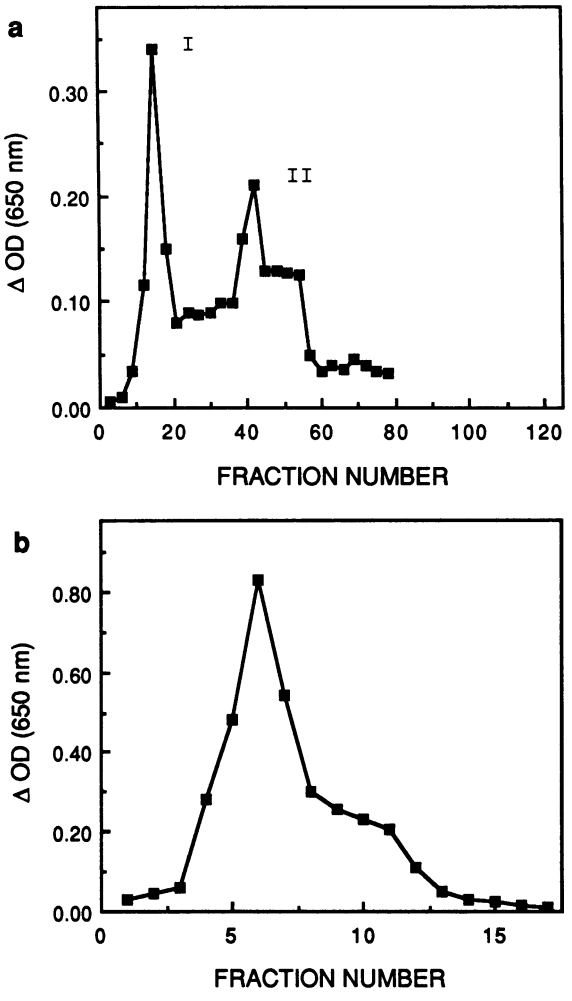

Figure 1. a. Phosphocellulose chromatography of 1.7-M NaCl extract of HSV-1 infected Vero cell nuclei. Nuclear extracts were prepared from 110 g of HSV-1 infected Vero cells and assays of DNA dependent ATPase activity were performed as described in Materials and Methods. b. Phosphocellulose chromatography of 1.7-M NaCl extract of uninfected Vero cell nuclei. Nuclear extracts were prepared from 15 g of Vero cells.

In addition to the difference in their chromatographic behavior the two DNA-dependent ATPases also differed in their nucleoside triphosphate specificity. As shown in Table I, the HSV-1 specific enzyme (Peak I) could hydrolyze both GTP and CTP. In contrast, the Vero cell enzyme (Peak II) showed only very low levels of activity with these two nucleotides.

Table I
Ribonucleoside Triphosphate Specificity of Host and HSV-1 DNA-dependent ATPase

Enzyme	DNA dependent Nucleoside Triphosphate Activity (% of ATPase)			
	ATP	GTP	CTP	UTP
Host	100	11	9	9
HSV-1 specific	100	48	28	16

Reaction mixtures were prepared as described under Materials and Methods with the nucleoside triphosphates (1 mM) added as indicated. 1.7 units of peak II of the phosphocellulose chromatogram were used for the host cell enzyme and 2.5 units of the peak I were used for the herpes-specific enzyme (Fig. 1).

Purification of HSV-1 specific DNA-dependent ATPase

All procedures were carried out at 4°C.

A 1.7-M NaCl extract of nuclei from 35 g of HSV-1 infected Vero cells was prepared as described (9). The extract was dialyzed against buffer B until the conductivity was below that of buffer B containing 0.1 M NaCl (Fraction I).

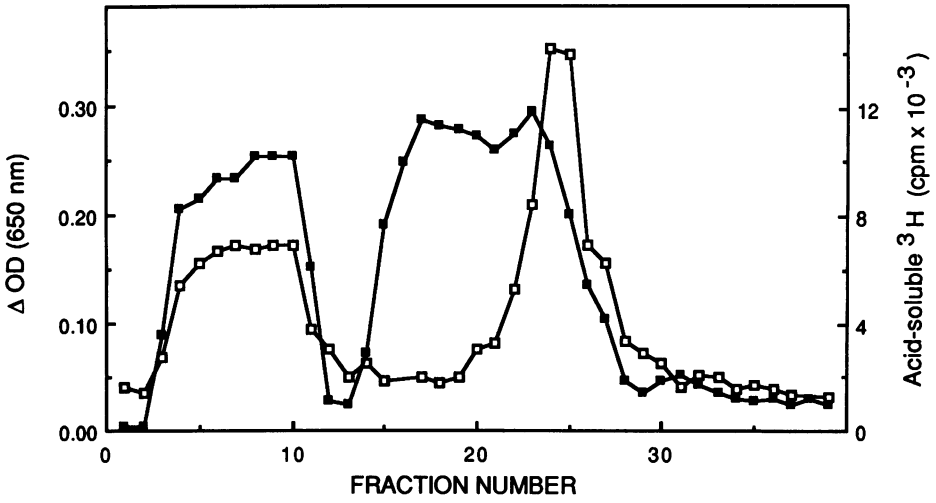

Figure 2. DEAE-Sephadex A25 chromatography of HSV-1 specific DNA-dependent ATPase. Peak I of DNA-dependent ATPase activity from phosphocellulose chromatogram was concentrated, then applied to a DEAE-Sephadex A25 column and chromatographed as described in Materials and Methods. DNA-dependent ATPase (□), DNase (■).

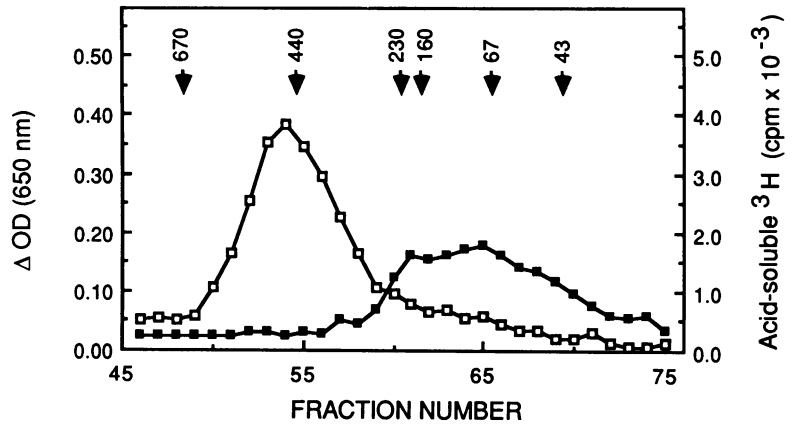

Figure 3. Superose 12 gel filtration of HSV-1 specific DNA-dependent ATPase. Fraction III was filtered through a Superose 12 column as described under Materials and Methods. DNA-dependent ATPase (□), DNase (■). The position of elution of proteins used to standardize the Superose 12 column are: thyroglobulin, 670 kDa; ferritin, 440 kDa; catalase, 230 kDa; aldolase, 160 kDa; bovine serum albumin, 67 kDa; and ovalbumin, 43 kDa.

Phosphocellulose chromatography. Fraction I was applied to a column of phosphocellulose (16 ml bed volume) that had been equilibrated with buffer B. The column was washed with 8 ml of buffer B containing 0.1 M NaCl and a linear gradient (192 ml) from 0.10 to 0.80 M NaCl in buffer B was applied (Fig. 1a). Peak I was pooled and dialyzed (4 hr) against 100 volumes of buffer B containing 60 mM NaCl (Fraction II).

DEAE-Sephadex chromatography. Fraction II was applied to a column (10-ml bed volume) of DEAE-Sephadex A25 equilibrated with buffer B. The column was washed with 10 ml of buffer B containing 50 mM NaCl, and a linear gradient (100 ml) between 0.05 and 0.4 M NaCl in buffer B was applied. Approximately one-half of the DNA dependent ATPase activity of Fraction II was retained by the DEAE-Sephadex column and was eluted by 0.3 M NaCl (Fig. 2). The peak fractions with low DNase activity were concentrated with a Centricon 30 microconcentrator to a volume of 0.175 ml (Fraction III). The remainder, which appeared in the pass through and wash fraction, was of lower molecular weight than the bound enzyme and may be a proteolytic product of the bound enzyme or alternatively, another HSV-1 specific DNA dependent ATPase.

Superose 12 gel filtration. Fraction III was applied to a prepacked column of Superose 12 that had been equilibrated with buffer B (lacking phenylmethylsulfonyl fluoride) containing 0.2 M NaCl. The column was eluted at a flow rate of 0.20 ml/min, and 1.0-min fractions were collected (Fig.

Table II
Purification of the HSV-1 DNA-dependent ATPase

Fraction		Total Protein (mg)	Total Activity (units)	Specific Activity (units/mg)
I.	Nuclear extract	150	-	-
II.	Phophocellulose	4.4	4.3×10^3	9.8×10^2
III.	DEAE-Sephadex	0.090	1.2×10^3	1.3×10^4
IV.	Superose 12	0.005	1.6×10^2	3.3×10^4

3). DNA-dependent ATPase activity eluted at a point corresponding approximately to the 440,000 molecular weight standard. The fractions that were devoid of nuclease activity (Fig. 3) were pooled and concentrated by Centricon 30 ultrafiltration (Fraction IV).

The HSV-1 specific DNA dependent ATPase was purified 34-fold from phosphocellulose peak I with a yield of 4% (Table II). We estimate that it

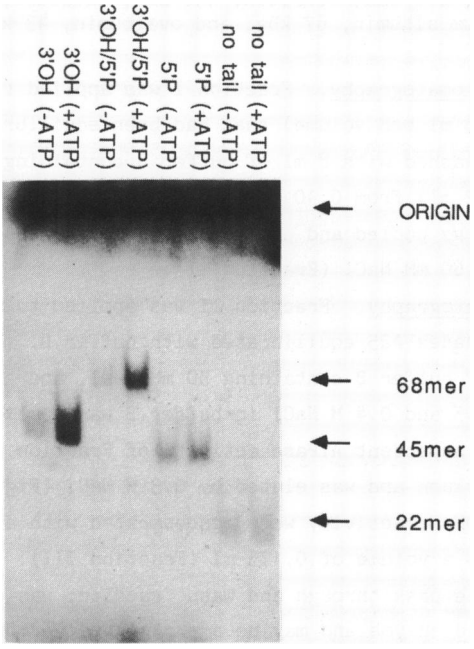

Figure 4. DNA helicase activity of DNA-dependent ATPase. DNA helicase assays, with 2.5 units of Fraction IV, were performed as described in Materials and Methods. Where indicated, ATP was added at 3.0 mM. 3'OH, 3'OH/5'P, 5'P and no tail represent assays in which the substrate was 3' tailed, 5' and 3' tailed, 5' tailed and untailed, respectively. The positions to which the displaced oligonucleotides migrated are indicated.

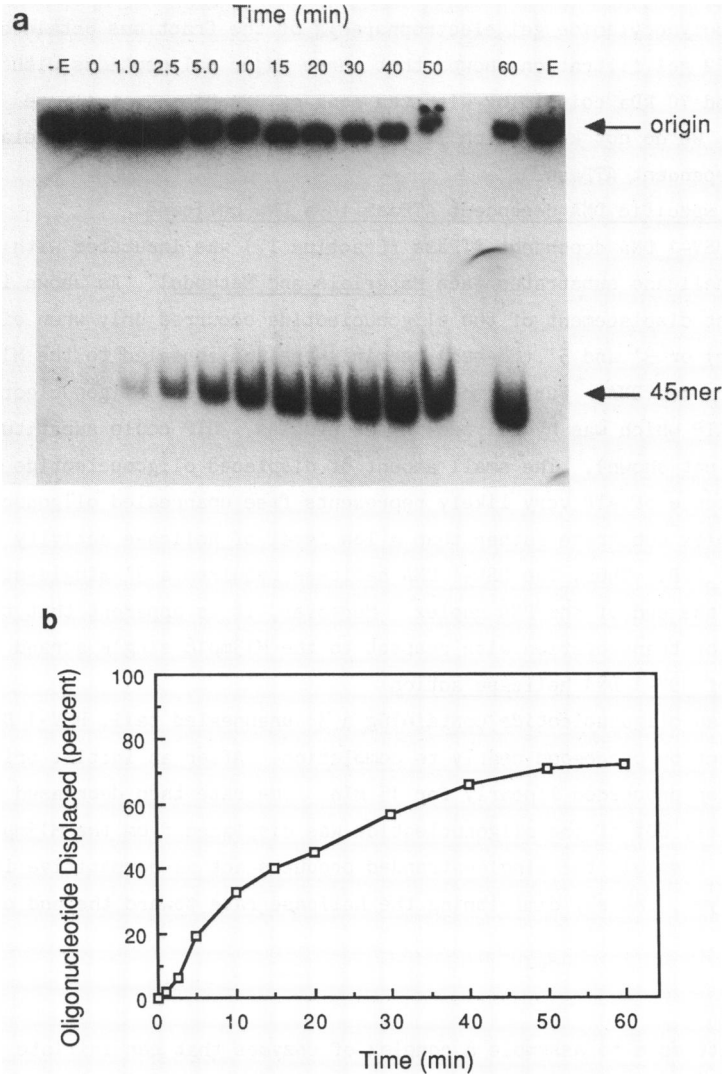

Figure 5. Time course of DNA helicase reaction. The reaction mixture was as described in Materials and Methods except that the volume was 70 µl and the concentration of the 3' tailed substrate was 2 µM (nucleotide). The ATP concentration was 3.0 mM, and 18 units (0.56 µg of protein) of Fraction IV were added. Samples (5 µl) were removed at the times indicated and electrophoresed as described in Materials and Methods. Autoradiography of the dried gel was for 36 h at -80°C. a. Autoradiogram of gel. b. Quantitation of helicase activity. The amount of oligonucleotide displaced was determined as described in Materials and Methods. (-E), no enzyme added.

was purified at least 1000-fold from the nuclear extract.

SDS polyacrylamide gel electrophoresis of the fractions obtained by Superose 12 gel filtration showed that three major polypeptides with masses of 130, 97 and 70 kDa coinciding with the peak of DNA dependent ATPase activity. We do not know which if any of these polypeptides are related to the DNA dependent ATPase.

The HSV-1 specific DNA-dependent ATPase is a DNA helicase

The HSV-1 DNA dependent ATPase (Fraction IV) was incubated with each of the four helicase substrates (see Materials and Methods). As shown in Fig. 4, significant displacement of the oligonucleotide occurred only when either the 3' (45-mer) or 3' and 5' (68-mer) termini were not annealed to the M13mp18 single-stranded DNA. Furthermore, displacement of these oligonucleotides required ATP which was hydrolyzed in the process. GTP could substitute for ATP (data not shown). The small amount of displaced oligonucleotide observed in the absence of ATP very likely represents free unannealed oligonucleotide in the duplex substrate rather than a low level of helicase activity in the absence of ATP. Thus, the HSV-1 DNA helicase requires a 3' single-stranded region at the end of the DNA duplex. Moreover, it is apparent that the direction of translocation with respect to the M13mp18 single strand is 5'→3'.

Kinetics of HSV-1 DNA helicase action

With an oligonucleotide containing a 3' unannealed tail, HSV-1 DNA helicase action proceeded nearly to completion. After an initial brief lag, the reaction proceeded linearly for 15 min. The rate then decreased until approximately 80% of the oligonucleotide was displaced from the M13mp18 DNA (Fig. 5). Possibly the single-stranded products act as competitive inhibitors of the enzyme, thereby diminishing the helicase rate toward the end of the reaction.

DISCUSSION

Our attempts to assemble a complex of enzymes that can initiate and sustain the semi-conservative replication of the HSV-1 genome have led to the identification and partial purification of a herpes-induced DNA helicase.

Attempts to observe DNA helicase activity in crude extracts of herpes infected Vero cells were unsuccessful, largely because of a potent DNase activity that rapidly hydrolyzed the helicase substrate. On the assumption that a DNA helicase would display a DNA-dependent ATPase activity that would be relatively unaffected by nuclease action, we sought and identified a DNA-dependent ATPase that was unique to HSV-1 infected cells. Unlike the host

enzyme(s) which was highly specific for ATP, the herpes enzyme was relatively non-specific with respect to its nucleotide cofactor and could hydrolyze GTP at approximately one-half the rate of ATP. Purification of the DNA dependent ATPase to the point that it was free of DNase activity then revealed that it was indeed a DNA helicase that could efficiently displace an oligonucleotide annealed to single-stranded M13mp18 DNA.

In its present state of purification the HSV-1 DNA helicase contains three major polypeptides with molecular weights of 130,000, 97,000 and 70,000 whose relative abundance coincides with the helicase activity as it elutes from a Superose 12 column. We do not know which, if any, of these polypeptides are associated with enzymatic activity. It is worth noting, however, that one of the open reading frames (UL5) in the HSV-1 genome that is required to support replication of ori_S containing plasmids codes for a protein with a predicted molecular weight of 99,000 containing an ATP binding site (7).

The HSV-1 helicase requires a 3' single-stranded tail on its duplex DNA substrate. Little, if any, activity was detected with a 5' terminated single strand. Thus, the enzyme very likely unwinds the duplex in the 5' to 3' direction on the strand opposite to that with the 3' tail. This polarity is the same as the two T4 phage-induced DNA helicases, the gene 41 and the dda proteins (14). Thus, the HSV-1 helicase may translocate on the lagging strand in advance of the HSV-1 replication machinery.

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