**Herpes Simplex-1 Helicase-Primase**

IDENTIFICATION OF TWO NUCLEOSIDE TRIPHOSPHATASE SITES THAT PROMOTE DNA HELICASE ACTION*

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Herpes simplex virus type 1 (HSV-1) encodes a heterotrimeric helicase-primase comprised of the products of the UL5, UL8, and UL52 genes (Crute, J. J., and Lehman, I. R. (1991) J. Biol. Chem. 266, 4484-4488). A steady state kinetic analysis of the enzyme isolated from HSV-1-infected CV-1 cells or insect cells expressing the enzyme after infection with recombinant baculoviruses has shown it to possess two sites capable of hydrolyzing nucleoside triphosphates in a DNA-dependent manner. One site (Site I) hydrolyzes both ATP and GTP; the second (Site II) hydrolyzes only ATP. These two sites are contained within a subassembly of the helicase-primase by coexpression of the UL5 and UL52 genes in insect cells. Sites I and II are activated by separate DNA effector sites, both of which support DNA helicase action. These findings are likely to be of importance in understanding how helicases in general catalyze the unwinding of duplex DNA and, in particular, how the helicase-primase functions at the HSV-1 replication fork.

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The genome of herpes simplex virus type 1 (HSV-1) is composed of 152,260 nucleotides and contains 72 open reading frames (1, 2). Both cis- and trans-acting elements are essential for HSV-1 DNA replication. The cis-acting elements are the three DNA replication origins, OriL, and the diploid OriS (3-9). The trans-acting elements are the products of seven genes that encode enzymes that are required to replicate the viral chromosome (10-14). Notable among these is a helicase-primase, the product of the UL5, UL8, and UL52 genes (15-18).

In this report, we present evidence that the heterotrimeric HSV-1 helicase-primase contains two separate nucleoside triphosphatase sites; one of these (Site I) catalyzes the hydrolysis of both ATP and GTP, the other (Site II) specifically catalyzes the hydrolysis of ATP. These two sites are activated by independent DNA effector sites that support the helicase activity of the HSV-1 helicase-primase.

**EXPERIMENTAL PROCEDURES**

**Materials**

All reagents were obtained from sources previously described unless otherwise noted (15, 16). ADP and GDP were from Sigma. [3H]ATP (37 Ci/mmol) and [3H]GTP (7.0 Ci/mmol) were from Amersham Corp. Polyethyleneimine-cellulose F TLC plates were from EM Science (Gibbstown, NJ). Reagents for native and SDS-polyacrylamide gel electrophoresis were from Bio-Rad.

**Buffers**

Buffer A contained 40 mM Hepes, pH 7.5, 10% (v/v) glycerol, 100 μg/ml bovine serum albumin, and 2.0 mM dithiothreitol.

**Nucleic Acids**

The helicase substrate was prepared exactly as described previously (15). (dA) 200 was obtained from Midland Certified Reagent Co. (Midland, TX). Activated calf thymus DNA was prepared as described (19).

**Cells and Viruses**

R305 (20), a thymidine kinase deletion mutant of HSV-1[F], was used to infect roller bottle cultures of CV-1 cells at a multiplicity of infection of 5–10 plaque-forming units/cell. The recombinant baculoviruses AcmNPV/UL5 and AcmNPV/UL52 were used to doubly or, in combination with AcmNPV/UL8, triply infect Spodoptera frugiperda (SF9) cells as described (17).

**Purification of HSV-1 Helicase-Primase and Subassembly**

The HSV-1 helicase-primase from HSV-1-infected CV-1 cells (Fraction IV) was purified as previously described (15, 16). Recombinant baculovirus-expressed helicase-primase was isolated as described and purified through the gel filtration step (17). The UL5/UL52 subassembly of the HSV-1 helicase-primase that contains both helicase and primase activities was isolated from doubly infected SF9 cells. The purification procedure was the same as that described for the recombinant three-subunit enzyme; only the gel filtration step was omitted (21). It was approximately 80% pure, as assessed by SDS-polyacrylamide gel electrophoresis followed by staining with Coomassie Brilliant Blue (22).

**Enzyme Assays**

DNA-dependent NTPase—Reaction mixtures were assembled in buffer A (25 μl) with additions made as indicated. Either [3H]ATP (5.0 Ci/mmol) or [3H]GTP (3.0 Ci/mmol) served as the NTP substrate. MgCl2 was added to maintain the level of free Mg2+ at 3.5 mM, assuming 1 mol of Mg2+ complexed/mol of NTP, and activated calf thymus DNA (220 μM) was used as DNA cofactor unless otherwise indicated. Reactions were initiated by addition of enzyme and were stopped after 20 min at 34 °C by the addition of 5.0 μl of 0.5 M EDTA. To separate products from reactants, three 1.0-μl aliquots of the
reaction mixture were spotted onto polyethyleneimine-cellulose F TLC plates and allowed to dry. After development of the plates with 0.5 M formic acid, 0.5 M LiCl for assay of DNA-dependent ATPase, and 0.5 M formic acid, 1.0 M LiCl for assay of DNA-dependent GTPase, the products, ADP or GDP, were visualized with ultraviolet light using standards run in a parallel lane. Regions of the plates corresponding to the NDP were excised and dissolved in a liquid scintillation fluid, and their radioactivity was determined.

Helicase—DNA helicase activity was measured essentially as described (15) using the 3'-tailed substrate. Assay mixtures (10 µl) were assembled in buffer A on ice and contained the helicase substrate (15 µM in nucleotide) and the indicated concentration of either ATP or GTP. MgCl₂ was added to maintain the concentration of free Mg²⁺ at 3.5 mM. Reactions were initiated by the addition of 60 ng of the recombinant baculovirus-expressed HSV-1 helicase-primase. To ensure that the helicase assay was within the linear range, reactions were terminated after 10 min at 34 °C by the addition of one-third volume of a freshly prepared solution consisting of 200 mM EDTA, 1% SDS, 50% glycerol. Under these conditions, less than 10% of the labeled oligonucleotide was displaced from the single-stranded DNA.

Electrophoresis was performed as described (15); the gel was backed with DE-52 paper prior to drying, and the relative amounts of product in each reaction were determined by scanning with an AMBIS radioanalytic imaging system (AMBIS Systems, Inc., San Diego, CA).

RESULTS

The HSV-1 Helicase-Primase Has One DNA-dependent GTPase and Two DNA-dependent ATPase Sites—Examination of the DNA-dependent NTPase activity of the HSV-1 helicase-primase by Lineweaver-Burk analysis showed that ATP and GTP were utilized differently (Fig. 1a). When GTP was the substrate, a linear double-reciprocal plot was observed that yielded a $K_m$(GTP) of 1.0 mM. In contrast, when ATP hydrolysis was measured, a curvilinear graph resulted that produced two different $K_m$ values for ATP (1.3 mM and 170 µM). A similar result was obtained with an Eadie-Scatchard plot (Fig. 1b).

Competition experiments were performed to determine if the DNA-dependent GTPase ($K_m = 1.0$ mM) and the high $K_m$ DNA-dependent ATPase ($K_m = 1.3$ mM) represent a single site, distinct from the low $K_m$ DNA-dependent ATPase site ($K_m = 170$ µM). Low levels (250 µM) of either [³H]ATP or [³H]GTP were added to the reaction and then challenged with the reciprocal unlabeled NTP. If the high $K_m$ DNA-dependent ATPase site can also catalyze the hydrolysis of GTP, then addition of excess unlabeled ATP to reactions containing [³H]GTP should result in complete inhibition of DNA-dependent GTPase activity. Similarly, if the low $K_m$ DNA-dependent ATPase site hydrolyzes only ATP, then addition of excess unlabeled GTP to reactions containing sub-$K_m$ levels of [³H]ATP should produce only partial inhibition. Under these conditions, the contribution of the high $K_m$ site to the total DNA-dependent ATPase activity should be inhibited by the added GTP, leaving the activity of the low $K_m$ DNA-dependent ATPase site unchanged.

As shown in Fig. 2, in reactions performed in the presence of sub-$K_m$ levels of [³H]GTP (250 µM), addition of excess unlabeled ATP (up to 6.0 mM) resulted in complete inhibition of DNA-dependent GTPase activity. With [³H]ATP the addition of unlabeled ATP resulted in partial inhibition of the DNA-dependent ATPase activity until a plateau was reached. We conclude from these data that the HSV-1 helicase-primase has two DNA-dependent NTPase active sites. The higher $K_m$ site (i.e. $K_m$(ATP) = 1.3 mM and $K_m$(GTP) = 1.0 mM) can hydrolyze both ATP and GTP. This site will be referred to as Site I. The lower $K_m$ site, designated as Site II (i.e. $K_m = 170$ µM), is specific for ATP (Table I).

From measurements of DNA-dependent GTPase activity, we estimate the turnover number at Site I to be 35 s⁻¹. From measurements of DNA-dependent ATPase activity, we esti-
assembly Containing the Products of the HSV-1 UL5 and UL52 Genes—A baculovirus expressed subassembly of the helicase-primase, consisting of the products of the UL5 and UL52 genes, contains the DNA-dependent NTPase activity associated with the three-subunit holoenzyme (21). We therefore wished to determine whether Sites I and II are both retained in the subassembly. Competition experiments of the type described above were performed utilizing both the baculovirus expressed helicase-primase holoenzyme and the two-subunit subassembly. As summarized in Table II, the recombinant three-subunit helicase-primase and the two-subunit subassembly were indistinguishable. They were also indistinguishable from the enzyme isolated from HSV-1-infected cells (compare Table II and Fig. 2). With either recombinant enzyme, DNA-dependent GTPase activity was almost completely inhibited by the addition of excess unlabeled ATP. In contrast, approximately 30% of the DNA-dependent ATPase activity was retained in the presence of excess unlabeled GTP. These findings confirm the presence of both Sites I and II in the baculovirus-expressed HSV-1 helicase-primase and further localize these two sites to the two subunits encoded by the UL5 and UL52 genes.

Sites I and II Are Actuated by Distinct DNA Effector Sites—Since Sites I and II represent distinct DNA-dependent NTPase active sites in the HSV-1 helicase-primase, we wished to determine whether these two sites were responsive to the same or different DNA effector sites. By measuring DNA-dependent NTPase as described in the previous section, the $K_m$ (DNA) at Sites I and II can be measured independently. These findings confirm the presence of both Sites I and II in the baculovirus-expressed HSV-1 helicase-primase and further localize these two sites to the two subunits encoded by the UL5 and UL52 genes.

The HSV-1-encoded helicase-primase shows complex kinetics with respect to its NTP substrates. In the presence of a DNA cofactor, the enzyme displayed one $K_m$ for GTP (1.0 mM) and two for ATP (1.3 mM and 170 μM). Competition experiments showed the high $K_m$ (1.3 mM) DNA-dependent ATPase and GTPase sites to be the same (Site I), and distinct from the low $K_m$ DNA-dependent ATPase site with a $K_m$ of 170 μM (Site II). Both Sites I and II are present in the enzyme from HSV-1 infected CV-1 cells, as well as in the recombinant helicase-primase isolated from insect cells. The two-subunit subassembly composed of the products of the UL5 and UL52 genes also contained both sites, thereby localizing them to two of the polypeptides of the three-subunit holoenzyme.

In our initial studies of the helicase-primase from HSV-1,
infected Vero cells, we observed substantial proteolytic degradation in the course of purification (16). However, the major degradation product was separated from the intact enzyme at an early stage in the purification (15). The degradation product lacked DNA-dependent ATPase activity but retained DNA-dependent ATPase activity.2 Preliminary experiments showed this fragment to have a $K_m$(ATP) of 300 $\mu$M, close to the 170 $\mu$M that we have observed for Site II. These findings suggest that Site I can be differentially removed from the helicase-primase by proteolysis to yield an enzyme that retains a protease-resistant Site II.

Inspection of the nucleotide sequence of the UL5 gene has shown it to belong to a superfamily of NTP-binding proteins that may function as DNA helicases (25). A conserved ATP-binding motif, encompassing residues 92–119, within the UL5 gene can account for one of the two DNA-dependent NTPase sites, but not both. Analysis of the nucleotide sequence of UL52 has not revealed a consensus NTP-binding domain.2 The introduction of specific changes in the UL5 and UL52 genes by site-directed mutagenesis, followed by analysis of the mutant proteins, should permit the localization of Sites I and II within the helicase-primase.

Sites I and II are activated by different DNA effector sites. In the presence of GTP, the $K_m$(DNA) for Site I is 3.4 $\mu$M (nucleotide). At low concentrations of ATP, where only the DNA-dependent ATPase activity of Site II is measured, the DNA effector shows $K_m$ values of 12 and 0.62 $\mu$M. Thus, the helicase-primase contains two distinct DNA-dependent NTPase sites (Sites I and II), and these sites are activated by separate DNA effector sites. A single DNA effector site activates Site I, whereas two DNA effector sites appear to activate Site II. Site-directed mutagenesis studies comparable with those described for the NTP-binding sites should help to clarify the significance of the multiple DNA effector sites.

Both Sites I and II can support helicase action. However, when both sites are utilized at high levels of ATP, helicase activity is optimal. At present, we do not know whether the

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2 J. J. Crute and I. R. Lehman, unpublished observations.

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