The Herpes Simplex Virus Type 1 Origin-binding Protein

SEQUENCE-SPECIFIC ACTIVATION OF ADENOSINE TRIPHOSPHATASE ACTIVITY BY A DOUBLE-STRANDED DNA CONTAINING BOX I

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Lauren B. Murata and Mark S. Dodson‡

From the Department of Biochemistry, University of Arizona, Tucson, Arizona 85721-0088

Origin-dependent replication of the herpes simplex virus type 1 genome requires the virally encoded origin-binding protein, UL9. UL9 binds specifically to the herpes simplex virus type 1 replication origin at two high affinity binding sites on the DNA, Boxes I and II. UL9 also has ATP-dependent DNA helicase and DNA-stimulated ATPase activities that are used to unwind the origin DNA. Origin-specific binding is mediated by the C-terminal domain (C-domain) of the enzyme. ATPase and helicase activities are mediated by the N-terminal domain (N-domain). Previous studies have shown that single-stranded DNA is a good coeffector for ATPase activity. We have analyzed several DNAs for their ability to stimulate the ATPase activity of UL9 and of a truncated UL9 protein (UL9/N) consisting only of the N-domain. We report here that duplex Box I DNA specifically and potently stimulates the ATPase activity of UL9 but not of UL9/N. We also find that removal of the C-domain significantly increases the ATPase activity of UL9. We have incorporated these results into a model for initiation in which the C-domain of UL9 serves to regulate the enzymatic activity of the N-domain.

A recurrent theme in the initiation of DNA replication is the assembly of a highly organized nucleoprotein complex at the replication origin, initiated by an origin-specific DNA-binding protein (OBP)1 (reviewed in Refs. 1 and 2). The OBPs found in certain eukaryotic DNA viruses (simian virus 40 (SV40), bovine and papilloma viruses, and herpes simplex viruses) also actively unwind the origin DNA to nucleate assembly of a complete replication complex at the origin (3–6). We have been interested in the mechanism by which the OBP of herpes simplex virus type 1 (HSV-1), UL9, coordinates its DNA binding and DNA unwinding activities to initiate replication of the HSV-1 genome at the replication origin.

The HSV-1 genome contains three highly homologous origins of replication, OriL and two copies of OriS (reviewed in Refs. 6 and 7). The minimal sequence necessary for origin-specific replication, OriS, contains three homologous inverted repeat sequences known as Boxes I, II, and III, each containing a partially overlapping pentanucleotide dyad (8–11) (Fig. 1). Boxes I and II flank an 18-bp A + T-rich region (ATR). UL9 binds cooperatively and with high affinity to Boxes I and II (8, 12) and binds with very low affinity to Box III (9, 11, 13, 14). High affinity binding by UL9 is critically dependent upon residues within the pentanucleotide dyad (8, 9, 15–17). The ATR undergoes structural changes as a result of UL9 binding and is required for normal replication (15, 18, 19). In addition to UL9, six virally encoded proteins are required for origin-specific replication of the HSV-1 genome in vivo (6, 20, 21): a single-stranded DNA (ssDNA) binding protein (ICP8), a processive heterodimeric DNA polymerase, and a heterotrimeric primosome with 5' → 3' DNA helicase activity.

A generally accepted model for initiation of HSV-1 replication proposes that protein-protein interactions between Box I- and Box II-bound dimers of UL9 cause formation of a nucleoprotein core complex in which the ATR is distorted into a partially single-stranded conformation (13, 15, 18, 22–26). ICP8 binds to the distorted ATR, stabilizing the single-stranded conformation, and also interacts with UL9. This UL9-ICP8-ssDNA initiation complex opens the origin DNA, rendering it accessible to other replication proteins. The ensemble of replication proteins that subsequently assembles at the origin then carries out the subsequent unwinding, priming, and elongation events of replication. In this model, formation of a ssDNA segment at the ATR is essential for UL9 activity contributing to origin unwinding.

UL9 has ATPase and ATP-dependent 3' → 5' helicase activities that are specifically stimulated by ICP8 but not by heterologous ssDNA-binding proteins (23, 27–30). The ATPase activity has previously been reported to be greatly stimulated by the presence of ssDNA but not by the presence of duplex DNA (28, 29). Helicase activity has also been found to be dependent on ssDNA; UL9 unwinds partially single-stranded DNA substrates in the presence of ATP but has not been observed to unwind fully duplex DNAs or plasmids, even if they contain origin DNA or if ICP8 is present (23, 26, 30). These observations further support a model in which distortion of the ATR is a prerequisite for initiation of replication.

Sequence analysis and mutagenesis of the 851-amino acid UL9 polypeptide have led to the assignment of functional domains (Fig. 2). The domain comprised of the N-terminal two-thirds of the polypeptide chain (the N-domain) contains conserved ATP binding and helicase motifs that are critical for origin-specific replication (31–33). The N-domain also contains the regions critical for dimerization (12, 13), cooperative binding to OriS (13, 34), and interaction with the UL8 subunit of the HSV-1 primosome (35). The N-domain has been expressed independently and comprises a functional helicase (36). On the
FIG. 1. The minimal origin of replication for HSV-1, OriIS. Boxes I, II, and III and the intervening ATR are shown in capital letters. The relative orientations of the boxes are shown by solid arrows. The overlapping pentanucleotide repeats believed to bind the dimeric HSV-1 initiator protein, UL9, are underlined; open arrows show their relative orientations.

FIG. 2. Schematic diagram of the 851-amino acid HSV-1 UL9 protein. Sequence comparisons to other DNA helicases, as well as deletion and mutagenesis studies, have led to the identification of the functional domains shown.

The overlapping pentanucleotide repeats believed to bind the dimeric HSV-1 initiator protein, UL9, are underlined; open arrows show their relative orientations.

OTHER HAND, the domain comprised of the C-terminal one-third of the protein (the C-domain) is responsible for OriIS-specific binding. This domain, when expressed independently, is an OriS-specific DNA-binding protein (37, 38) that is monomeric in both solution and when bound to OriS (12, 13, 15, 39). The C-domain also contains the region of interaction with ICP8 (40). Thus, the critical DNA binding and helicase functions of UL9 are present on two separable but linked domains. This structural arrangement may provide for regulation of UL9 activity, as discussed later in this report.

Although the various functions of UL9 as an OBP, an ATPase, and a helicase have been identified and characterized, the exact mechanism by which UL9 functions to unwind DNA at the origin is not well understood. The enzymatic functions of UL9 have been consistently observed to require a single-stranded or partially single-stranded DNA substrate, implying that distortion of the origin to form a ssDNA region is a prerequisite for UL9 enzymatic activity in vivo. In this study, we have more rigorously examined the stimulation of UL9 ATPase activity by DNA effectors. We present evidence that duplex DNA containing Box I can act to specifically and dramatically stimulate UL9 ATPase activity. We also show that this DNA is a poor effector for the ATPase activity of the isolated N-domain and that the isolated N-domain is a more active enzyme than full-length UL9. The implications of these findings for the role and regulation of UL9 in unwinding origin DNA are discussed.

EXPERIMENTAL PROCEDURES

Materials—Restriction and DNA-modifying enzymes were from New England Biolabs and Life Technologies, Inc. Spodoptera frugiperda Sf9 cells were from Invitrogen. Reagents for insect cell culture were from Life Technologies, Inc. The baculovirus transfer vector (pBacPAK9), viral DNA (BacPAK6), and transfection reagent (Bacfectin) were generous gifts from N. grapha californica nuclear polyhedrosis virus (baculovirus) AcNPV/UL8/H expressing HSV-1 UL8 protein were generous gifts from Dr. I. R. Lehman (Stanford University). UL8/H expressing HSV-1 UL8 protein were generous gifts from N. grapha californica nuclear polyhedrosis virus (baculovirus) AcNPV/UL8/H expressing HSV-1 UL8 protein were generous gifts from Dr. I. R. Lehman (Stanford University).

Oligonucleotides—Synthetic DNAs were obtained from Genosys Bio-technologies, Inc. The sequences of the oligonucleotides used as effectors in ATPase assays are shown in Fig. 3. The complementary oligonucleotides BoxIU (Box I upper) (5’-CGAAGCGCTTGCGACTCTCCGTC-3’) and BoxIL (Box I lower) (5’-GGGAGACCGTGGCAAGCGGCTTTAGC-3’) comprised the 22-bp segment of OriS containing Box I. The complementary oligonucleotides MutU (Mutant upper) (5’-CGAAGCGGCGGCGACTCTCCGTC-3’) and MutL (Mutant lower) (5’-GGGAGACCGGCTGGGCGGCTTTAGC-3’) were identical to BoxIU and BoxIL, respectively, with the exception of the 4 underlined base changes. The complementary oligonucleotides RanU (Random upper) (5’-CCATCGCTCTCGTGTGCGACAC-3’) and RanL (Random lower) (5’-GCTGAGTGCGAGCCGAGCGGAGC-3’) were equal in base composition to BoxIU and BoxIL, respectively, but were of randomized sequence. The concentration of each oligonucleotide was confirmed by measurement of A260, using the extinction coefficients provided by the manufacturer. Duplex DNAs BoxDS, MutDS, and RanDS were made by heating equimolar mixtures of the component upper and lower strands to 95 °C and allowing them to cool slowly to 25 °C. For use in ATPase activity assays 8 μM single-stranded or duplex oligonucleotide stocks were serially diluted in 10 mM Tris-HCl, pH 8.0, 0.1 mM EDTA.

Construction of Recombinant Baculovirus Expressing UL9/N—Plasmid pGAD-UL9t contains the UL9 gene derived from pVL941/UL9 (28) in pGAD-424 (CLONTECH), flanked by EcoRI and XbaI sites. A partially self-complementary DNA (5’-GATCCCTAACCGGTTAGG-3’) containing a stop codon (TAA) and RanHI ends was self-anneled and then inserted into the distal RanHI site in the UL9 gene on pGAD-UL9t to truncate the gene at amino acid 536. The 3651-bp EcoRI-XbaI DNA fragment containing the truncated UL9 gene (UL9/N) was then inserted into the baculovirus transfer vector pBacPAK9. The sequence of the entire UL9/N gene in the resultant recombinant plasmid pBP-UL9/N was confirmed by the sequencing laboratory at the University of Arizona. pBP-UL9/N was then used to generate the recombinant baculovirus AcNPV/UL9/N as described by the CLONTECH manual. Stocks of recombinant baculoviruses were propagated in Sf9 cells grown in TMNFH containing 10% fetal calf serum, 50 μg/ml gentamycin sulfate, and 2 μg/ml fungizone (TMNFH medium) (42).

Buffers—Buffer A, used during purification of UL9/N and UL8 protein, contained 20 mM HEPS, pH 7.6, 10% (w/v) glycerol, 1.0 mM DTT, 150 mM NaCl, 3 mM MgCl2, and 0.5 mM phenylmethylsulfonyl fluoride, 100 μg/ml leupeptin, 1 μg/ml pepstatin A, 0.5 mM benzamidine HCl. Buffer B was the same as Buffer A except it contained 100 mM NaCl.

Infection of Sf9 Cells and Preparation of Infected Cell Cytosolic Extracts—A 400-ml suspension culture of Sf9 cells was grown to a cell density of 2.4 × 10⁶ cells/ml in TMNFH medium containing 10% fetal bovine serum (FBS; TMNFH/F medium). The cells were pelleted by centrifugation at 100,000 × g for 15 min and resuspended in fresh TMNFH/F medium. Fifty ml of AcNPV/UL9/N viral stock (at approximately 2 × 10⁹ plaque-forming units/ml) were added to the cells for a final concentration of 9.4 × 10⁷ cells/ml. After 1 h at 24 °C, the cells were divided into two 1-liter roller bottles, and fresh TMNFH/F medium was added to dilute the cells to 9.4 × 10⁶ cells/ml. The roller bottles were placed upright in an orbital shaker at 27 °C and shaken at 140 rpm with loosened caps. Seventy hours later, the cells were pelleted by centrifugation at 2000 × g for 10 min at 4 °C. The cells were gently resuspended in 20 mM HEPS-NaOH, pH 7.6, containing 1.0 mM DTT, 150 mM NaCl, and 5% (w/v) glycerol. Centrifugation as described yielded 2.8 g wet cells that were frozen in liquid nitrogen and stored at −80 °C. The cells were subsequently thawed at 37 °C, immediately resuspended in 30 ml of cold buffer A transformed E. coli sonication buffer, and pelleted by centrifugation at 300 × g for 15 min. The cytosolic extract was frozen in liquid nitrogen and stored at −80 °C.

Purification of Recombinant UL9/N Protein—All purification steps were performed at 4 °C. After thawing on ice, the cytosolic extract was brought to 100 mM NaCl (as determined by conductivity measurements) by dropwise addition of 4 mM NaCl while stirring. The extract was cleared.
BoxI/U 5‘-CGAACGGTTCCGACTTGGCCTCCC-3’
BoxI/L 3‘-GGTTCCGGAGCGTGAAGCCAGGG-5’

Mut/I 5‘-CGAACGGGccGacCGTCCGGCCTCCC-3’
Mut/L 3‘-GGTTCCGGCcGtcGAGGCGAGGG-5’

Run/A 5‘-CCATCGCTGTCGGACGGTACCC-3’
Run/L 3‘-GGTACCGAGCAGCGTACCAG-5’

Fig. 3. DNA sequences of oligonucleotide coeffectors examined in this report. The 22- bp portion of OriS containing Box I (see Fig. 1) comprises BoxI/DS, with the upper and lower strands corresponding to Box D/U and Box I/L, respectively. The Box I pentanucleotide dyad is underlined. The Mut sequences are identical to the Box I sequence except for 4 nucleotide changes, shown in lowercase, which disrupt UL9 binding. The BoxI sequences were scrambled to make the Ran sequences.

by centrifugation at 24,000 rpm in a Beckman Ti50 rotor for 30 min. The outlet of a 12-mL DEAE-Sepharose column was connected directly to a 2-mL heparin-Sepharose CL-4B column, and both columns were equilibrated with Buffer B. The cleared extract was loaded onto the columns at 10–20 ml/h. The columns were washed extensively with Buffer B (about 38 ml) until A280 returned to base line, at which point the DEAE-Sepharose column was removed. The heparin-Sepharose column was eluted with a 20-mL gradient from 100 to 800 mM NaCl in Buffer A at 10 ml/h, and 700-μl fractions were collected. After removal of analytical samples from selected fractions, all fractions were frozen in liquid nitrogen and stored at −80 °C. Conductivity measurements were recorded for the analytical samples, which were then prepared for SDS-PAGE using a pyrogallol red-molydate precipitation reagent as described (43). UL9/N protein eluted at 250–650 mM NaCl, as determined by conductivity measurements. Densitometry of peak fraction 18 (described below) showed that the full-length UL9/N protein and its degradation products comprised 26 and 23% of the total protein, respectively.

Mock Purification of UL9/N Protein—As a negative control, the above procedures for expression and purification of UL9/N protein were performed using a recombinant baculovirus strain expressing UL8 protein (AcNPV/UL8/H) in place of AcNPV/UL9/N.

Denaturing Polyacrylamide Gel Electrophoresis and Immunodetection—The protein samples were subjected to SDS-PAGE on identical 10% polyacrylamide gels that were stained with Coomassie Brilliant Blue or transferred to nitrocellulose for immunoblotting. The nitrocellulose was probed with UL9 antiserum followed by protein A-horseradish peroxidase conjugate; 4-chloro-1-naphthol was used as the substrate (44). (Because a significant loss of UL9/N activity was observed throughout the purification procedure (data not shown), despite the presence of multiple protease inhibitors. The full-length UL9/N protein and its degradation products comprised approximately 49% of the total protein. As a control, an identical purification procedure was carried out on cytosolic extracts from cells infected with a baculovirus expressing a different HSV-1 protein (UL8). Column fractions from this procedure yielded no major 60-kDa band visible by Coomassie staining and no bands visible by immunodetection with anti-UL9 antiserum (data not shown).

Adenosine Triphosphatase Activity of UL9/N—The UL9/N column fractions were assayed for ATPase activity in the presence and absence of 800 nM (dT)22. Both activities were found to copurify with the immunoreactive 60-kDa polypeptide, peaking in fractions 14–18 (Fig. 6a). The addition of (dT)22 was found to normalized relative to this standard. For UL9 assays, the standard reaction contained 572 nm Box/DS. For UL9/N assays, the standard reaction contained 800 nM (dT)22. For each DNA substrate, the normalized data from 3 to 6 replicate series were combined and then fit to the Michaelis-Menten equation using the curve fit feature of KaleidoGraph 3.1b (Synergy Software). The standard errors for the curve fit parameters and the averages and standard deviations for the data were also calculated by KaleidoGraph. Assays of UL9/N or UL8 column fractions were as described above for UL9/N except that the reaction mixtures were made up with or without DNA, and reactions were initiated by the addition of 0.5 μl of a column fraction. Reactions were performed in duplicate in both the presence and absence of 800 nM (dT)22.
Menten equation. The resulting kinetic parameters described under “Experimental Procedures.” V37082

varying amounts of DNA in the presence of 2 mM ATP or 10°C. The DNA-dependent formation of inorganic phosphate was determined as ATP hydrolyzed per nmol of UL9/N polypeptide.

The formation of inorganic phosphate upon incubation of purified recombinant UL9 in the presence of DNA and 2 mM ATP was determined as described under “Experimental Procedures.” Each data point represents the average of 3–6 replicates; error bars represent 1 S.D. of the data. A relative maximum velocity of 1 (obtained with 572 nM BoxI/DS DNA) corresponds to 2.4 ± 0.1 × 10³ nmol of ATP hydrolyzed per nmol of enzyme. a, duplex DNAs: BoxI/DS (●), Mut/DS (○), and Ran/DS (△); b, single-stranded DNAs: BoxI/U (●), Mut/U (○), Ran/U (△), BoxI/L (○), Mut/L (○), and Ran/L (△); c, relative efficiency of activation (relative $V_{max}/K_d$(app)) for each of the DNAs shown in a and b. Gray bar, upper strands; black bar, lower strands; cross-hatched bar, duplex DNAs. A relative activation efficiency of 1 corresponds to 0.10 pmol of ATP hydrolyzed per pmol of enzyme; nM DNA).

| DNA      | Strand(s) | Relative $V_{max}$ | $K_d$(app) |
|----------|------------|---------------------|-------------|
| Box I    | BoxI/DS    | 1.18 ± 0.06         | 29 ± 4.8    |
|          | BoxIU      | 1.09 ± 0.09         | 740 ± 100   |
|          | BoxI/L     | 0.27 ± 0.03         | 260 ± 60    |
| Mutant   | Mut/DS     | 0.76 ± 0.16         | 690 ± 210   |
|          | Mut/U      | 1.08 ± 0.24         | 1480 ± 460  |
|          | Mut/L      | 0.61 ± 0.05         | 620 ± 100   |
| Random   | Ran/DS     | 0.49 ± 0.09         | 240 ± 85    |
|          | Ran/U      | 1.31 ± 0.18         | 800 ± 180   |
|          | Ran/L      | 0.34 ± 0.08         | 420 ± 210   |

The single-stranded and duplex mutant DNAs yielded the highest relative activities overall, with $V_{max}$ ranging from 0.97 to 1.10 (Fig. 7, a and b). In contrast, the single-stranded and duplex Box I DNA yielded the lowest activities overall, with $V_{max}$ ranging from 0.33 to 0.47. The single-stranded and duplex random DNAs were intermediate in activity but displayed the highest apparent efficiencies (with $K_d$(app) ranging from 36 to 49 nM), yielding the highest activation efficiencies overall (Fig. 7c). The apparent efficiencies of the mutant DNAs were lowest, with $K_d$(app) ranging from 110 nM (for Mut/Ds) to 270 nM (for Mut/U and Mut/L), whereas the apparent efficiencies of the Box I DNAs were intermediate, with $K_d$(app) ranging from 73 (for BoxI/DS) to 123 nM (for BoxI/U). Single-stranded and duplex random DNAs exhibited very little difference in their activation efficiencies. In contrast, the higher affinity observed for the duplex Box I and mutant DNAs

stimulate the ATPase activity of the peak fractions by 10–16-fold. Quantitation of the amount of full-length UL9/N in fraction 18 by densitometry allowed the DNA-dependent ATPase activity of UL9/N to be estimated at 3.6 ± 1.5 × 10⁴ pmol of ATP hydrolyzed per pmol of UL9/N polypeptide; the specific activity of UL9/N is thus 15-fold higher on a molar basis than that of UL9. UL9/N appeared to be extremely labile; in addition to the degradation described above, a significant loss of ATPase activity was observed upon incubation of the column fractions at 30 or 37°C (data not shown). The control UL8 column fractions yielded no detectable ATPase activity in the presence or absence of DNA (Fig. 6b).

Duplex Box I DNA Is a Poor Activator of UL9/N ATPase Activity—The results of UL9/N ATPase activity assays carried out in the presence of various DNAs are summarized in Fig. 7 and Table II. The single-stranded and duplex mutant DNAs yielded the highest relative activities overall, with $V_{max}$ ranging from 0.33 to 0.47. The single-stranded and duplex random DNAs were intermediate in activity but displayed the highest apparent efficiencies (with $K_d$(app) ranging from 36 to 49 nM), yielding the highest activation efficiencies overall (Fig. 7c). The apparent efficiencies of the mutant DNAs were lowest, with $K_d$(app) ranging from 110 nM (for Mut/Ds) to 270 nM (for Mut/U and Mut/L), whereas the apparent efficiencies of the Box I DNAs were intermediate, with $K_d$(app) ranging from 73 (for BoxI/DS) to 123 nM (for BoxI/U). Single-stranded and duplex random DNAs exhibited very little difference in their activation efficiencies. In contrast, the higher affinity observed for the duplex Box I and mutant DNAs...
compared with their single-stranded forms resulted in a doubling of the activation efficiencies of the duplex forms compared with the ssDNAs. Overall, the activation efficiencies for the random DNAs were about 2.5-fold higher than those for the other duplex DNAs (BoxI/DS and Mut/DS), and about 5-fold higher than for the Box I and Mut ssDNAs. Thus, the best ATPase coeffector for UL9, BoxI/DS, is a poor coeffector for UL9/N, as shown by a comparison of the results shown in Figs. 4 and 7.

**DISCUSSION**

As the initiator of replication at the HSV-1 replication origin, UL9 must accomplish two distinct tasks. First, it must accurately locate the replication origin, and second, it must initiate replication at this site. Clearly, UL9 can locate the replication origin by virtue of its high affinities for Boxes I and Box II. There is much evidence to indicate that cooperative binding of UL9 at Boxes I and II results in a distortion of the ATR structure in which the ATR structure is distorted (13, 15, 18, 23–26). The role of UL9 in the subsequent events in the initiation process is not well understood, however. Are additional molecules of UL9 recruited to the distorted ATR to act as helicases, as suggested by the finding that UL9 acts as a stoichiometric helicase (45)? If additional UL9 molecules are not required, what is the signal for the origin-bound dimers to switch from a binding mode to an unwinding mode? Formation of the complex might serve as the signal for the bound UL9 molecules to become active helicases or, alternatively, might serve as the signal for additional UL9 molecules to begin unwinding the DNA at the origin. In this report we have demonstrated that UL9 ATPase activity is specifically activated by duplex Box I DNA. This specific activation of ATPase activity by Box I, which is not a substrate for helicase activity (26), indicates that UL9 dimers bound to the origin may be enzymatically functional as ATPases but not as helicases. We speculate that this phenomenon may be part of a regulatory mechanism for controlling UL9 unwinding activity at the origin, as described below.

We examined several duplex and single-stranded 22-mer DNAs for their ability to act as coeffectors of the DNA-stimulated ATPase activity of UL9. We examined upper and lower strands of Box I as well as the Box I duplex. We also examined the component single strands and duplex forms of two variants of Box I as follows: (a) a mutant form, in which 4 base changes were made at the sites that are known to be critical for high affinity binding of UL9 to Box I (15, 17), and (b) a random form, in which the base composition remained the same but the sequence was randomized. Of all the DNAs examined, BoxI/DS was by far the most effective at stimulating ATPase activity of UL9, displaying a relative activation efficiency at least 20-fold greater than any of the other duplex or ssDNAs (Fig. 4). One salient feature of this potent stimulatory activity is that the apparent affinity of UL9 for BoxI/DS is much higher (by 9–51-fold) than for the other DNAs examined. Double-stranded Box I acts therefore as both the preferred ligand for UL9 and as its preferred coeffector for ATPase activity.

We also examined the above DNAs for their ability to stimulate the ATPase activity of the isolated N-domain of UL9 (UL9/N). This protein, which consists of amino acids 1–536 of UL9 and contains all of the helicase and ATP-binding site...
motifs, was found to be extremely labile when partially purified from recombinant baculovirus-infected cells. The extreme lability of UL9/N prohibited its further purification but was not unexpected, as removal of a large domain of a globular protein is likely to expose a previously solvent-protected hydrophobic interface. We found that UL9/N is a weak DNA-independent ATPase and that its ATPase activity is increased 10–16-fold upon addition of 800 nM (dT)22. This result is consistent with a previous report that UL9/N is a DNA-stimulated ATPase and ATP-dependent helicase, with an approximately 5–10-fold increase in ATPase activity in the presence of 2 μg/ml activated calf thymus DNA (36).

The above result demonstrates that removal of the C-terminal domain of UL9 does not eliminate the ability of the enzyme to act as a DNA-stimulated ATPase. It does, however, dramatically alter the specificity characteristics of this stimulation. Whereas BoxI/DS is a specific and potent coeffect for UL9 ATPase activity, it is a very poor coeffect for UL9/N ATPase activity. In fact, the duplex and single-stranded Box I and mutant sequences are all poor coeffectors in terms of activation efficiencies, with the single-stranded sequences being approximately half as effective as the duplex DNAs. Interestingly, the most potent activators of UL9/N ATPase activity, in terms of both apparent affinity and activation efficiency, are the single-stranded and duplex random DNAs. These sequences exhibit apparent affinities for UL9/N (Kd(app) = 36–49 nM) of the same order of magnitude as that of BoxI/DS for UL9 (Kd(app) = 29 nM). The overall pattern of activation is quite different from that of UL9; UL9/N is activated most effectively by those DNAs (Ran/DS, Ran/U, and Ran/L) that least resemble Box I in sequence. UL9/N also exhibits a much higher DNA-dependent
ATPase activity than UL9, even at a reduced assay temperature. By using our most conservative estimates, we find that UL9/N has a molar specific activity (obtained at 30 °C with (d)T$_{22}$) of 2.5–15-fold greater than that of UL9 (obtained at 37 °C with BoxI/DS).

These results demonstrate that UL9 has two discrete DNA-binding sites with different binding specificities. One site, located on the C-domain of each monomer, is required for the Box I-specific stimulation of the ATPase activity of the full-length enzyme, UL9/N, upon binding of duplex or ssDNAs that do not resemble Box I. The second site, located on the N-domain, mediates the activation of the truncated enzyme, UL9/N, upon binding to Box I DNA. This binding results in a conformational change in UL9/N that enables it to hydrolyze ATP efficiently. The conformational change required for ATPase activity is not effectively promoted by non-Box I DNA.

ATPase activity of UL9/N is likely to be similar to the active conformation of the N-domain in full-length UL9.

replication initiates at the correct site on the genome. Second, the preference of the N-domain for non-Box I ligands may assist the UL9 molecule in binding to the origin in the correct orientation (i.e. with Box I bound to the C-domain) rather than in the opposite, nonfunctional orientation (i.e. with Box I bound to the N-domain). The increased activity of UL9/N relative to UL9 suggests that the presence of the C-domain inhibits the enzymatic activity of the N-domain. This proposition is consistent with that of Simonsson et al. (16) who have previously suggested that the C-domain actively controls helicase activity and is not simply a passive anchor to DNA.

Integrating previous models (16, 18, 25, 26) with our results, we propose the tentative model shown in Fig. 8. In this model, the C-domain of UL9 acts to regulate the potential ATPase activity of the N-domain to ensure accurate and efficient replication. In this scheme, UL9 is a poor ATPase in the absence of DNA because the N-domain is trapped in an enzymatically incompetent conformation (Fig. 8a). When the UL9 C-domain binds to Box I, the conformation of the enzyme changes to relieve the inhibition of the enzymatic activity of the N-domain (Fig. 8b). At this point ATP hydrolysis can occur, but the enzyme cannot translocate along the DNA because it is locked into place on the DNA by the tight association between the C-domain and the DNA. Since the enzyme cannot translocate, it cannot use the energy derived from ATP hydrolysis to unwind the DNA. At some point during subsequent assembly of the complete initiation complex, such as binding of ICPS to the C-domains, a conformational change occurs that disrupts the tight UL9-Box I interaction, thus allowing unwinding to begin. The most likely candidate for the trigger of this conformational change is the association of ICP8 with the C-domain of UL9, promoted by the availability of a ssDNA region that interacts with ICP8. Other possible events triggering this second conformational change could include the N-domain-mediated association of Box I- and Box II-bound UL9 dimers and/or binding of an as yet unidentified cellular factor to UL9.
In this model, high affinity binding of UL9 to the origin is inhibitory to the unwinding activity of UL9, and this inhibition can be relieved by the subsequent association of another replication factor (such as ICP8) with the origin-bound UL9. These predictions are supported by several results of Lee and Lehman (26, 46). First, they have observed that UL9 can specifically unwind a partial duplex Box I substrate only in the presence of ICP8 and that mutation of the DNA substrate to prevent high affinity binding of UL9 results in significant helicase activity in the absence of ICP8 (26). We surmise that in the case of the Box I substrate, the binding of ICP8 to UL9 triggers the conformational change in UL9 which disrupts its interaction with the DNA, thus allowing unwinding to occur. We surmise that in the case of the mutated substrate, the above-mentioned ICP8-mediated conformational change is not required for helicase activity because the enzyme is not locked into place on the DNA substrate. These interpretations are further supported by the recent observations of Lee and Lehman (46) that ICP8 weakens the interaction of UL9 with the partial duplex Box I substrate. Furthermore, they report that incubation of UL9 with an antibody that prevents association with ICP8 inhibits unwinding of the Box I substrate in the presence of ICP8, and that preincubation of UL9 with Box I before addition of the antibody increases the inhibitory effect (46). This result suggests that binding of UL9 to Box I causes a significant conformational change in the protein, as we have suggested here.

The enzymatic characteristics of UL9/N can be explained in terms of our model (Fig. 9). Like full-length UL9, UL9/N is a poor ATPase in the absence of bound DNA (Fig. 9a). Binding of non-Box I DNA is proposed to convert UL9/N to a more active conformation (Fig. 9b), just as binding of Box I DNA is proposed to convert UL9 to a more active conformation (Fig. 8b). The increased ATPase activity of UL9/N relative to the full-length enzyme may be due to greater accessibility of ligand-binding sites in the absence of the C-domain. Alternatively, UL9/N may assume an optimally active conformational state that is inaccessible to the full-length enzyme.

In this work we have shown that the ATPase activity of UL9 is specifically and dramatically stimulated by duplex DNA containing Box I and that the C-domain is responsible for this stimulation. We have integrated this result into a model for initiation that proposes that (a) the differential preferences of the C- and N-domains for Box I DNA aid in orienting the UL9 molecule at the origin, and (b) both the correct binding of UL9 to the origin and the association of an additional replication factor with UL9 are necessary to switch the enzyme to a conformation that can unwind the origin DNA. These stepwise changes in conformation could serve to coordinate the enzymatic activities of UL9 with the various associative events and structural changes involving UL9 during initiation. The possible advantage of ATP hydrolysis in the absence of unwinding activity is not clear; perhaps a stable initiation complex can only form if the UL9 molecules are in an enzymatically competent conformation. We are currently conducting experiments to examine further the requirements for enzyme activation, including the effects of Boxes II and III, and to identify additional factors and/or associative events required for helicase activity of Box I-bound UL9.

The specific activation of ATPase activity by binding of origin DNA has not thus far been observed for the OBPs of other eukaryotic viruses such as SV40 and bovine papilloma virus. In light of the possibility that the specific activation of UL9 by Box I may be an example of a general regulatory mechanism, it would be interesting to determine the effect of origin sequence binding on the enzymatic activities of the OBPs in these other viral systems.

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