The origin-binding domain of the herpes simplex virus type 1 UL9 protein is not required for DNA helicase activity

Adrian P. Abbotts and Nigel D. Stow*

MRC Virology Unit, Institute of Virology, Church Street, Glasgow G11 5JR, UK

The UL9 protein of herpes simplex virus type 1 binds to specific sequences within the viral origins of DNA replication and also functions as a DNA helicase. The C-terminal 317 amino acids of the 851 residue protein specify sequence-specific binding to the viral origins and the N-terminal 400 contain several motifs characteristic of many DNA and RNA helicases. To investigate whether the origin-binding domain is required for helicase function we have expressed a truncated version comprising amino acids 1–535 of UL9 using a recombinant baculovirus. Extracts were prepared from cells infected with the recombinant virus and chromatographed over ATP-agarose. DNA helicase, DNA-dependent ATPase and a novel single-stranded DNA-binding activity were present in fractions containing the truncated UL9 protein but not in corresponding gradient fractions from a control virus infection. These results indicate that the DNA helicase function of UL9 does not require the presence of the origin-binding domain and suggest that an interaction between the N-terminal domain and distorted or partially single-stranded regions of DNA may play a role in unwinding the origin region.

Gene UL9 of herpes simplex virus type 1 (HSV-1) encodes one of the seven viral proteins which perform direct and essential roles in viral DNA synthesis (reviewed by Challberg, 1991). The UL9 protein comprises 851 amino acids and has two important activities which are likely to contribute during viral genome replication. Firstly, UL9 is a sequence-specific DNA-binding protein which interacts with defined sites within the viral origins of replication. These sequences are recognized in double-stranded DNA with the C-terminal 317 amino acids being sufficient for binding (Olivo et al., 1988; Koff & Tegtmeyer, 1988; Weir et al., 1989). Secondly, UL9 exhibits DNA helicase and associated DNA-dependent ATPase activities (Fierer & Challberg, 1992; Boehmer et al., 1993; Dodson & Lehman, 1993), consistent with the presence of several motifs characteristic of a superfamily of DNA and RNA helicases within the N-terminal 400 amino acids (Gorbalenya & Koonin, 1993). Genetic evidence suggests that both functions are necessary for genome replication (Martinez et al., 1992; Stow et al., 1993) and that UL9 may participate in the initiation of DNA synthesis by first binding to and then subsequently facilitating the unwinding of the origin regions. Although UL9 bound to an HSV-1 origin is able to distort the DNA duplex, energy-dependent, sequence-specific, DNA unwinding has not been demonstrated (Koff et al., 1991; Fierer & Challberg, 1992).

To investigate whether the DNA helicase activity of UL9 is independent of its origin-binding function we have characterized a truncated UL9 molecule expressed by a recombinant baculovirus which comprises only the N-terminal 535 amino acids but retains all the conserved helicase motifs. The recombinant baculoviruses AcUL9 and AcUL9NT express full-length UL9 and amino acids 1–535 respectively (Stow, 1992; McLean et al., 1994). A recombinant, AcRP23lacZ (Possee & Howard, 1987), which expresses β-galactosidase was employed as a control. Spodoptera frugiperda (Sf) cells were propagated and virus stocks prepared as previously described (Stow, 1992).

Nuclear extracts were prepared from approximately 2 × 10^8 Sf cells infected with AcUL9, AcUL9NT or AcRP23lacZ for 72 h as described by Calder & Stow (1990) and diluted to 300 mM-NaCl using Buffer A (20 mM-HEPES–NaOH pH 7.6, 10% glycerol, 1 mM-DTT, 1 mM-PMSF) supplemented with 0.1% NP40. The diluted nuclear extract was applied to a 4 ml Sepharose Fast Flow (Pharmacia) column pre-equilibrated with Buffer B (20 mM-HEPES–NaOH pH 7.6, 10% glycerol, 1 mM-EDTA, 1 mM-DTT, 1 mM-PMSF) containing 300 mM-NaCl and 0.1% NP40. The columns were washed with 16 ml Buffer B containing 300 mM-NaCl and 0.1% NP40. The eluate was retained, further diluted to 250 mM-NaCl using Buffer A and
Fig. 1. SDS-PAGE analysis of ATP-agarose column fractions. The SP Sepharose flow-through (E) and ATP-agarose column fractions (1–14) obtained from cells infected with AcRP23lacZ (lacZ), AcUL9NT (UL9NT) or AcUL9 (UL9) were analysed on 9% polyacrylamide gels stained with Coomassie blue. Lane M, protein molecular mass markers (205, 116, 97.4, 66, 45 and 29 kDa). *, UL9NT and UL9 proteins; ●, an example of an AcNPV or host protein present in each extract.

applied directly onto a 1.5 ml ATP-agarose (Sigma) column pre-equilibrated with Buffer B containing 250 mM-NaCl. The column was washed with 6 ml Buffer B containing 250 mM-NaCl and proteins were eluted with a 14 ml gradient containing 250–1250 mM-NaCl in Buffer B. Fractions of 1 ml were collected and stored at −70 °C.

DNA helicase assays were performed with a substrate consisting of a labelled 45 base oligonucleotide annealed to M13mp18 single-stranded DNA and containing a 23
base 3’ tail as previously described (Calder & Stow, 1990) except that reactions contained 5 µl column fraction in a final volume of 50 µl and were incubated for 1.5 h at 37 °C. The oligonucleotide lacks sequences corresponding to the UL9 origin-binding sites.

DNA-dependent ATPase activity was assayed in 50 µl reactions containing 5 µl enzyme fraction, 40 mM-HEPES-NaOH pH 7.5, 3 mM-MgCl₂, 100 µg/ml BSA, 10% glycerol, 0.03 mM-ATP, 0.1 µg activated calf thymus DNA (Sigma) and 0.9 µCi [γ-³²P]ATP (Dodson & Lehman, 1993; Calder & Stow, 1990). After incubation at 37 °C for 1.5 h, 150 µl activated charcoal in 50 mM-HCl, 5 mM-H₃PO₄ was added, the mixes vortexed, allowed to stand for 2 min and centrifuged at 13 000 g for 1 min. Inorganic phosphate liberated during the reaction was detected by counting the radioactivity in 100 µl of the supernatant and the amount of ATP hydrolysed was determined.

Single-stranded DNA-binding activity was determined in a gel retardation assay. Mixtures (30 µl) containing 20 mM-HEPES-NaOH pH 7.6, 5 mM-MgCl₂, 1 mM-DTT, 10% glycerol (Buffer M), 5 µl column fraction and 125 pg of a ³²P 3’-end-labelled 45 base oligonucleotide (see above) were incubated for 20 min at 18 °C; 6 µl running dye [1 × TBE (90 mM-Tris base, 90 mM-boric acid, 10 mM-EDTA), 10 mM-DTT, 25% glycerol, 0.05% bromophenol blue] was added and complexes were resolved by electrophoresis through a 10% non-denaturing polyacrylamide gel in 1 × TBE. To verify that complexes contained UL9-related protein, 5 µl of a monoclonal antibody against the UL9 protein (13938; McLean et al., 1994) was added to similar reactions and incubation continued at 4 °C overnight. To collect the immune complexes, 20 µl of a 6:6% (v/v) preparation of fixed Staphylococcus aureus cells (Pansorbin, Calbiochem) in Buffer M was added and mixing continued for 1 h. Finally, each reaction tube was supplemented with 100 µl Buffer M, vortexed and the S. aureus cells were pelleted by centrifugation at 13000 g for 1 min; ³²P-labelled single-stranded DNA in the pellet was determined in a liquid scintillation counter.

Fig. 1 shows the results of ATP-agarose chromatography of extracts from Sf cells infected with AcUL9, AcUL9NT or the control virus, AcRP23lacZ. Several proteins, presumably representing host- or AcNPV-encoded species, were eluted from a NaCl gradient from all three extracts, but additional major proteins corresponding in size to full-length UL9 and the truncated form, UL9NT, were also detected in the appropriate extracts. The identities of UL9 and UL9NT were confirmed and their relative yields compared by electroblotting various dilutions of column fractions and detecting the proteins with monoclonal antibody 13938. The molar amount of UL9NT in peak fractions was generally 4–8 times greater than UL9, reflecting both its higher expression level and also the loss of a proportion of the full-length protein during SP Sepharose chromatography. The binding of UL9NT to ATP-agarose is consistent with the presence of a functional NTP-binding site as predicted from its amino acid sequence (helicase motifs I and II; Gorbalenya & Koontz, 1993).

To determine whether UL9NT retained helicase activity, fractions from columns were assayed. In agreement with previous results, Fig. 2(a) shows that fractions containing full-length UL9 protein exhibited DNA helicase activity significantly greater than corresponding fractions from the AcRP23lacZ gradient. Moreover, a similar peak of DNA helicase activity was present in the fractions containing UL9NT protein. Dilution of the UL9NT fractions showed that their increased activity in comparison with the corresponding UL9 fractions correlated closely with the increased amount of UL9-related protein present (data not shown). Fig. 2(b) shows the results of ATPase assays performed on the AcUL9NT and AcRP23lacZ column fractions in the presence of activated DNA. Greatly increased ATPase activity was present in fractions containing UL9NT, and further experiments showed that this activity was reduced 5–10-fold when activated DNA was omitted from the reaction.

Since DNA helicase and associated DNA-dependent ATPase activities depend on the ability of a protein to interact with single-stranded DNA, we also assayed column fractions for single-stranded DNA-binding activity. Fig. 3(a) shows an experiment in which binding to the labelled 45 base single-stranded oligonucleotide was assessed by gel retardation. An activity which resulted in the generation of complexes exhibiting about one-third the mobility of the free probe was present in fractions 6–12 of both the AcRP23lacZ and UL9NT gradients (labelled B). An additional activity resulting in the formation of a much slower migrating complex (A) was detected only with the UL9NT fractions, suggesting that this might be due to binding of the truncated UL9 protein. To confirm the presence of UL9NT protein in these fractions, protein–DNA complexes were immunoprecipitated with monoclonal antibody 13938, which recognizes the truncated UL9 protein. The counts present in immune complexes were determined following binding to fixed S. aureus cells. Fig. 3(c) shows that although fractions from both gradients contained single-stranded DNA-binding activity, labelled complexes were immunoprecipitated only from fractions containing UL9NT protein. Addition of 0.25 µg unlabelled single-stranded circular M13mp18 DNA (Gibco BRL) to the binding reactions prevented the formation of retarded complexes A and B (Fig. 3b) and reduced the counts in the immune complexes formed with fractions containing UL9NT to
Fig. 2. DNA helicase and ATPase activities of ATP-agarose column fractions. (a) Fractions 4–10 from each of the gradients shown in Fig. 1 were assayed for DNA helicase activity. The reaction products were analysed on a 10% non-denaturing polyacrylamide gel. Marker lanes contain the starting undenatured helicase substrate (S) or the same substrate following heat denaturation (BS) to displace the 45 base labelled oligonucleotide (O). (b) The AcRP23lacZ and AcUL9NT ATP-agarose column fractions shown in Fig. 1 were analysed for ATPase activity in the presence of activated calf thymus DNA. □, AcUL9NT fractions; ●, AcRP23lacZ fractions.

The background levels observed for the corresponding fractions of the AcRP23lacZ gradient. The DNA-binding activity therefore appears to result from a genuine interaction with single-stranded regions of DNA as opposed to ends of molecules.

The presence of DNA-dependent ATPase, DNA helicase and single-stranded DNA-binding activities in fractions containing UL9NT protein but not in corresponding fractions from a control gradient strongly suggests that UL9NT is directly responsible for these activities. The possibility exists that a contaminating protein that sticks to UL9NT might be involved but it would have to remain associated at NaCl concentrations up to 600 mM. Moreover, all three activities co-fractionated with UL9NT when an extract was purified sequentially on ATP-agarose, SP Sepharose and heparin-agarose. This procedure yielded greater than 90% pure UL9NT and has the advantage of not relying upon ATP-binding for the final stage (data not shown).

The above results therefore indicate that the C-terminal sequence-specific origin-binding domain of UL9 is dispensable for both its DNA helicase and single-stranded DNA-binding activities. Although the helicase assay utilizes model substrates, this finding may nevertheless be relevant to the way in which UL9 functions at viral replication origins. Previous studies have indicated that sequence-specific binding of UL9 to the viral replication origins results in the formation of higher-
Fig. 3. Single-stranded DNA-binding activity of AcRP23\textit{lacZ} and AcUL9NT ATP–agarose column fractions. (a) Fractions shown in Fig. 1 were incubated with a 45 base single-stranded DNA probe and retarded complexes resolved on 10% non-denaturing polyacrylamide gels. P, indicates free probe and A and B retarded complexes. Fractions 1–5 contain significant levels of an activity which degrades the labelled probe. (b) Fraction 7 from each of the two gradients was tested for DNA-binding activity in the presence or absence of 0.25 μg M13mp18 single-stranded DNA. Lanes 1 and 2, UL9NT gradient fraction without and with M13mp18 DNA, respectively; lanes 3 and 4, AcRP23\textit{lacZ} gradient fraction without and with M13mp18 DNA, respectively; lane 5, no added extract. (c) Complexes formed as in (a) were immunoprecipitated with monoclonal antibody 13938 and the radioactivity determined in a liquid scintillation counter. ■, AcUL9NT fractions; ○, AcRP23\textit{lacZ} fractions.
order nucleoprotein structures (Rabkin & Hanlon, 1991) and that distortion and wrapping of the DNA occurs under these conditions (Elias et al., 1990; Koff et al., 1991; Hazuda et al., 1992; Fierer & Challberg, 1992). The helicase-associated DNA-binding activity of UL9 molecules within the nucleoprotein complex may then allow interaction with such regions of DNA and subsequent energy-dependent unwinding of the DNA duplex. Since origin-specific unwinding by UL9 has not been demonstrated it is likely that other viral and/or host proteins participate in modifying the DNA conformation of the origin region to promote UL9 helicase activity. One such candidate, the viral single-stranded DNA-binding protein, ICP8, may contribute to helix destabilization and is able both to interact with UL9 and to stimulate its helicase activity (Fierer et al., 1992; Boehmer et al., 1993; Boehmer & Lehman, 1993). A further specific interaction of origin-bound UL9 with the UL8 component of the viral helicase–primase complex is possibly also important for initial unwinding of the origin region. Interestingly, the interaction with UL8 involves sequences which lie within the functional helicase domain we have identified (McLean et al., 1994), whilst sequences at the C terminus of UL9 that are dispensable for origin binding contribute to the interaction with, and stimulation of helicase activity by, ICP8 (Boehmer et al., 1994).

Finally, our data are consistent with the sequence-specific origin-binding and DNA helicase activities of UL9 being independent functions carried out by distinct domains of the protein. That these domains may even have separate evolutionary origins is suggested by the observation that whilst the N-terminal helicase domain shares homology with a large number of putative helicases from a variety of eukaryotes, prokaryotes and viruses, sequences with significant homology to the origin-binding region have been described only in other members of the alphaherpesvirus subfamily. Distinct additional domains have also been identified flanking a core helicase domain in a number of other proteins and these have been associated with endonuclease, primase or protease activities (reviewed by Gorbalenyva & Koonin, 1993).

We thank Gordon McLean and Vivien Mautner for constructive discussions and are grateful to Andrew Davison for helpful comments on the manuscript.

References


(Received 27 March 1995; Accepted 11 September 1995)