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Vaccinia Virion Protein I8R Has both DNA and RNA Helicase Activities: Implications for Vaccinia Virus Transcription

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A nucleic acid-dependent ATPase was purified from vaccinia virions and shown to have both DNA:DNA and RNA:RNA helicase activities. This is only the third helicase to be identified that can unwind both DNA and RNA duplexes. The DNA helicase activity copurified with nucleoside triphosphate phosphohydrolase II (NPHII), an RNA helicase encoded by gene I8R (S. Shuman, Proc. Natl. Acad. Sci. USA 89:10935–10939, 1992). Immunodepletion with two antisera to NPHII and analysis of recombinant NPHII protein (C. H. Gross and S. Shuman, J. Virol. 69:4727–4736, 1995) confirmed that the DNA helicase activity was encoded by the I8R gene. The I8R DNA helicase unwound DNA in a 3′-to-5′ direction only, unwound duplexes of 35 bp but not 45 bp, and could be stimulated to unwind longer duplexes by the *Escherichia coli* single-stranded DNA-binding protein. DNA helicase activity was not stimulated by salt and was sensitive to 100 mM NaCl or KCl. The I8R protein has amino acid similarity to human RNA helicase A and to nuclear DNA helicase II, a bovine DNA and RNA helicase. On the basis of the phenotype of I8R temperature-sensitive mutants, it was suggested that the I8R protein is not required for DNA replication but might aid in the extrusion of early mRNA from the virus core. The DNA helicase activity of the I8R protein allows another interpretation of the mutant phenotype, namely, that the I8R DNA helicase activity is required for initiation of early transcription from within vaccinia virions.

DNA helicases are enzymes that use the energy derived from cleavage of the β-γ bond of nucleoside triphosphates (NTPs) to unwind double-stranded DNA. The involvement of DNA helicases in DNA replication and recombination is well-known (21), but more recently, evidence of a role for DNA helicases in transcription has been presented (4). Two DNA helicases, ERCC2 and ERCC3, have been identified in eukaryotic transcription initiation factor TFIIH, and comparable proteins are encoded by *Saccharomyces cerevisiae* genes *RAD3* and *RAD25* (8). A mutation in the Walker A nucleotide binding motif of the *RAD25* gene is lethal, suggesting that the helicase activity of RAD25 is essential for transcription by RNA polymerase II (27). DNA helicases are thought to stimulate DNA melting at the promoter and to facilitate promoter clearance by the transcriptional complex but are not considered necessary for unwinding of the template during transcriptional elongation.

Several virus proteins with DNA helicase activity have been identified, and these proteins function in DNA replication and/or transcription. For example, the herpes simplex virus type 1 UL9 protein (12) and the bovine papillomavirus E1 protein (32) function in DNA replication, while the simian virus 40 (SV40) large T antigen (9) and the minute virus of mice NS-1 protein (17) are involved in both DNA replication and transcriptional regulation. In the latter case, it was shown by mutagenesis of the NS-1 gene that the DNA helicase and transcriptional activities of the NS-1 protein are separable (17). Vaccinia virus, the prototype poxvirus, differs from most DNA viruses in that it replicates in the cytoplasm and consequently encodes many of its own transcriptional and DNA replicative enzymes (22). It is likely, therefore, that vaccinia virus encodes a DNA helicase(s) which might be essential for one or more of the processes of DNA replication, repair, and recombination or for transcription of the viral genome.

Vaccinia virions contain a fully functional transcription system (23). By using purified virion proteins and plasmid templates containing vaccinia virus early promoters, early viral transcription can be demonstrated with a seven-subunit RNA polymerase, an RNA polymerase-associated protein (RAP94), a two-subunit early transcription factor (vETF), and a two-subunit termination factor (which is also a capping enzyme) (1). Transcription is initiated by sequential binding of vETF (20) and the viral RNA polymerase (vRNAPol)-RAP94 complex to the early promoter in the absence of NTPs (1). Promoter clearance then occurs in a step that requires ATP hydrolysis, which may be wholly or partly explained by the activity of vETF (20). A committed ternary complex is formed following synthesis of 10 or more phosphodiester bonds by vRNAPol (16). Although the amino acid sequences of the small subunit of vETF (encoded by gene D6R) and superfamily II helicases show similarity (14), an attempt to demonstrate DNA helicase activity for vETF failed (20). Moreover, none of the other vaccinia virus transcriptional proteins described above have amino acid sequence similarity to helicases, suggesting that transcription in the vaccinia virus system might not require a DNA helicase.

Four vaccinia virus genes (A18R, D6R, D11L, and I8R) encode proteins which have helicase motifs (14, 18), and each of these is found in vaccinia virions (23, 28, 30). Three have been purified from vaccinia virions and shown to have nucleic acid-dependent nucleoside triphosphatase (NTPase) activity (3, 26). The fourth, A18R, has been expressed as a recombinant protein and is also a DNA-dependent NTPase (2). Unlike that of the other three proteins, the NTPase activity of nucleoside triphosphate phosphohydrolase II (NPHII), the I8R gene product (28), is stimulated by both DNA and RNA (25). Subsequently, it was reported that the I8R enzyme had a 3′-

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to-5′ RNA helicase activity and that it bound DNA but was inactive in an assay for DNA helicase activity (28, 29).

To determine whether the vaccinia virus proteins which have helicase motifs possess DNA helicase activity, we purified DNA-dependent NTPases from vaccinia virions and tested them for DNA helicase activity. We report here that I8R protein has 3′-to-5′ DNA helicase activity in addition to RNA helicase activity. The implications for the role of the I8R enzyme in vaccinia virus transcription are discussed.

MATERIALS AND METHODS

Purification of virion ATPases. Intracellular mature vaccinia virions were purified from infected HeLa spinner cultures, and a soluble enzyme fraction was prepared as described previously (26). Briefly, virus particles were treated sequentially with 0.5% Nonidet P-40 and 0.1% sodium deoxycholate to solubilize virion enzymes. The soluble enzyme fraction was applied sequentially to two DE-52 cellulose columns equilibrated in buffer A (50 mM Tris [pH 8.4], 0.1% Triton X-100, 3 mM dithiothreitol [DTT], 0.1 mM EDTA, 10% [vol/vol] glycerol) plus 200 or 50 mM NaCl, respectively, for the first and second columns. The flowthrough from the second column contained the majority of virion enzymes but not vRNAPol. This fraction was applied to a single-stranded-DNA (ssDNA)–cellulose column (Sigma) equilibrated in buffer A plus 50 mM NaCl. The column was developed with 20-ml volumes of increasing concentrations of NaCl (100, 150, 200, 250, 300, and 400 mM in buffer A), and fractions were assayed for DNA-dependent ATPase activity. Four peaks of DNA-dependent ATPase activity were identified, each of which was further purified by fast protein liquid chromatography (FPLC) (Pharmacia). Fractions containing I8R protein (peak 3; buffer A plus 300 mM NaCl) were pooled, diluted threefold with buffer B (50 mM Tris-HCl [pH 8], 2.5 mM DTT, 1 mM EDTA, 0.01% Nonidet P-40, 10% [vol/vol] glycerol), and applied to a MonoS HR 5/5 (Pharmacia) column which was developed with a 10-ml linear gradient of 0.1 to 1 M NaCl in buffer B.

Helicase substrates. The substrate used to detect DNA helicase activity was made by annealing a 5′-, ^{32}P-labeled 68-mer oligonucleotide (6) to ssM13mp18 DNA such that there was a 23-bp duplex region and 3′ and 5′ ss tails of 23 and 22 nucleotides, respectively. The oligonucleotides used to investigate the length of duplex unwound were 45, 35, or 25 nucleotides long; each was completely complementary to ssM13mp18, and they were identical at their 5′ ends. The sequence of the 45-mer is 5′TGCAGGTCGACTCTAGAGGATCCCCGGGTACCGAGCTCGAATTCG3′. Hybrids were made in a fashion identical to that described for the 68-mer–M13mp18 substrate. The substrate employed to detect 3′-to-5′ helicase activity was made by labeling the 45-mer oligonucleotide at the 5′ end with [γ-^{32}P]ATP by using polynucleotide kinase prior to annealing the oligonucleotide to ssM13mp18. The substrate employed to detect 5′-to-3′ helicase activity was made by labeling the same 45-mer oligonucleotide at the 3′ end with [α-^{32}P]TTP by using Klenow enzyme after annealing the oligonucleotide to ssM13mp18. This process adds one extra base pair to the duplex region. The latter two substrates were then digested with *Sma*I. DNA helicase substrates were separated from unannealed oligonucleotides with a Bio-Gel A5m (Bio-Rad) column.

The RNA helicase template was constructed from plasmids encoding a 130-nucleotide mutant ribozyme (5) and its 600-nucleotide target substrate (a generous gift of S. Thompson and W. James, Sir William Dunn School of Pathology, University of Oxford). The resultant substrate had 5′ and 3′ unannealed regions on both RNA molecules and a 43-bp duplex region. RNA was transcribed with T7 RNA polymerase in the presence of [α-^{32}P]UTP and purified by ammonium acetate precipitation. RNA molecules were annealed in a high-salt buffer (40 mM Tris-HCl [pH 7.5], 1 mM EDTA, 0.5 M NaCl). Double-stranded RNA substrates were electrophoresed on 4% acrylamide gels and then eluted from gel slices with 0.5 M ammonium acetate–0.1% sodium dodecyl sulfate (SDS)–10 mM EDTA, extracted with phenol, and precipitated with ethanol.

Enzyme assays. ATPase assays were performed essentially as described previously (2), except that the reaction buffer consisted of 50 mM Tris-HCl (pH 8), 2 mM DTT, 2 mM $MgCl_2$, 1 mM ATP, and 8 to 12 μg of ssM13mp18 per ml. One unit of ATPase activity is defined as that amount of enzyme which hydrolyzes 1 nmol of ATP in 30 min at 37°C. Helicase reaction mixtures (40 μl) contained 20 mM Tris-HCl (pH 7.5), 3 mM $MgCl_2$, 5 mM DTT, and 10% (vol/vol) glycerol, plus (unless otherwise stated) 2.5 mM ATP, substrate, and enzyme. Reactions were stopped by the addition of 8 μl of 5× loading buffer (50% [vol/vol] sucrose, 2% SDS, 0.1% bromophenol blue in 1× Tris-borate-EDTA buffer. Samples were then electrophoresed on either 8% (DNA) or 4% (RNA) polyacrylamide gels in 1× Tris-borate-EDTA buffer. *Escherichia coli* ssDNA-binding protein (SSB) was obtained from Pharmacia.

Immunodepletion. Rabbit sera, raised against either NPHII or NPHI (24), were diluted 1:25 in 50 μl of buffer C (20 mM *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid [HEPES]-KOH [pH 7.9], 0.5 mM DTT, 0.2 mM EDTA, 50 mM KCl, 20% [vol/vol] glycerol, 0.01% Nonidet P-40). A rabbit antiserum (50 μl), raised against a bacterially expressed I8R gene product (i.e., recombinant NPHII) (11), was used undiluted. Antisera were incubated with 40 μl of protein A-Sepharose CL4B beads (Sigma) for 4 h at 4°C. The beads were collected by centrifugation and washed three times with buffer C. A 24-μl aliquot of protein I8R (NPHII) (FPLC fractions 35 and 38, as described below) and 3 μl of NPHI (see Fig. 1, lane 2) were diluted separately to 150 μl with buffer C plus 1 mM phenylmethylsulfonyl fluoride and 1 μg of leupeptin per ml. Diluted enzyme (50 μl) was either added to the anti-NPHI- or anti-NPHII-bearing beads and incubated for 3 h at 4°C or incubated directly. Bound enzyme was removed by centrifugation for 5 min at 6,500 rpm in a microcentrifuge. Supernatants were assayed for DNA-dependent ATPase and DNA helicase activities.

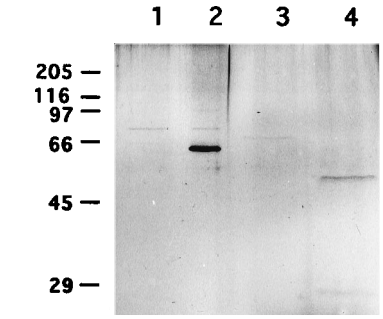

FIG. 1. Vaccinia virion DNA-dependent ATPases. Four peaks of ATPase activity were detected when vaccinia virion enzymes were separated on an ssDNA-cellulose column. Each of these four peaks was further purified by FPLC, and samples from the peak fractions of ATPase activity were electrophoresed on a 10% polyacrylamide gel, which was subsequently stained with the Silver Stain Plus kit (Sigma). Lane 1, 74 U of peak 1 ATPase (putative vETF); lane 2, 283 U (2.5 μl) of peak 2 ATPase (NPHI, encoded by gene D11L); lane 3, 74 U (10 μl of FPLC fraction 37) of peak 3 ATPase (NPHII, encoded by gene I8R); lane 4, 19 U of peak 4 ATPase (the A18R gene product). Molecular mass markers (Sigma) are shown in kilodaltons.

RESULTS

Vaccinia virion DNA-dependent ATPases. A protein fraction containing the known DNA-dependent ATPases was prepared as previously described (26). Separation of this fraction on an ssDNA-cellulose column identified four well-separated peaks of DNA-dependent ATPase activity. Each of these was further purified by FPLC, and the protein profiles of the peak fractions of ATPase activity from the FPLC columns are shown in Fig. 1. Peak 1 contained two proteins (Fig. 1, lane 1, and data not shown), which probably correspond to the two subunits of vETF (82 and 74 kDa). Peak 2 contained one major polypeptide of 63 kDa (Fig. 1, lane 2); this polypeptide was identified as NPHI (72 kDa), encoded by gene D11L, by immunoblotting with the anti-NPHI serum (data not shown). Peak 2 also had other minor polypeptides, two of which comigrated with the proteins of peak 1 and a third, 105-kDa peptide which possibly represented the large subunit of the capping enzyme. Peak 3 had one polypeptide of 66 kDa (Fig. 1, lane 3), which was identified as the product of the I8R gene (77 kDa) by immunoblotting with a specific antiserum (reference 11 and data not shown). Lower apparent molecular masses for the NPHI and I8R proteins have been observed previously after polyacrylamide gel electrophoresis (26, 28). Peak 4 had one major polypeptide of 55 kDa (Fig. 1, lane 4), which was recognized by an antiserum specific for the A18R gene product; the purification and activities of this protein will be described elsewhere.

I8R protein unwinds both DNA and RNA. Fractions from the MonoS FPLC purification of the I8R protein were tested for DNA-dependent ATPase, DNA helicase, and RNA helicase activities (Fig. 2). All three activities were observed in fractions 34 to 40, with the peak being in fractions 36 and 37, which contained the 66-kDa polypeptide described above. This copurification suggested either that these activities were all from the same enzyme or that other, undetectable proteins

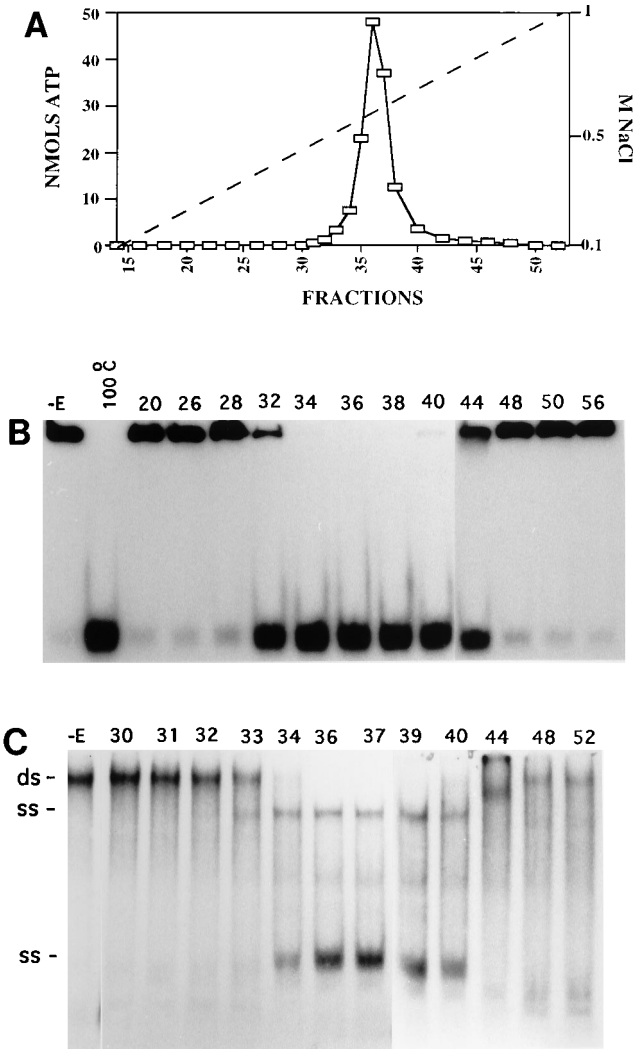

FIG. 2. Activity profile of I8R FPLC fractions. DNA-dependent ATPase (peak 3) was applied to a MonoS FPLC column, and protein was eluted with a 10-ml gradient of 0.1 to 1 M NaCl (indicated by the broken line [A]). Indicated fractions (250 μl) were tested for DNA-dependent ATPase activity (A), for DNA helicase activity with 0.3 fmol of the DNA helicase substrate (B), and for RNA helicase activity with 1.8 fmol of the RNA helicase substrate (C); 5, 5, and 3 μl of each fraction, respectively, were used. Reaction mixtures were incubated for 30 min at 37°C. Control reaction mixtures containing equivalent amounts of substrate were incubated either in the absence of enzyme (−E) or after heat denaturation for 3 min at 100°C (100°C). ds, double stranded.

were present. This DNA helicase activity could not be due to one of the other vaccinia virion ATPases, vETF, NPHI, or the A18R gene product, since these proteins were present in FPLC-purified fractions (for examples, see Fig. 1, lanes 1, 2, and 4) which lacked DNA helicase activity with the 68-mer–ssM13mp18 substrate (unpublished data). However, to address the possibility of a contaminating activity, the peak fractions shown in Fig. 2 were further purified on a Superdex 75 gel filtration column. A single peak of DNA-dependent ATPase activity was observed in fractions 31 to 34, and once again this peak coincided with peaks of DNA and RNA helicase activity (data not shown).

Shuman (28) previously showed that NPHII had RNA helicase activity and that this protein was encoded by the I8R gene. To confirm that NPHII was also responsible for the DNA helicase activity, an immunodepletion assay was performed.

TABLE 1. Immunodepletion of I8R and NPHI ATPase activities[a]

Protein	Antiserum added	ATPase activity[b]	Fold depletion[c]
I8R	None	30.5	
	NPHI	13.5	2.3
	NPHII	2	15.3
NPHI	None	240	
	NPHI	26	9.2
	NPHII	115	2.1

[a] Protein fractions were incubated with protein A-Sepharose-bound antibodies specific for NPHI or NPHII (the I8R gene product) for 3 h at 4°C, and bound protein was removed by centrifugation. Controls were incubated in the absence of protein A-Sepharose. ATPase activity was assayed in the presence of 0.4 μg of ssDNA for 30 min at 37°C.

[b] Units of ATPase activity in 50 μl of immunodepleted supernatant.

[c] Activity in the sample without antiserum divided by activity in the sample after immunodepletion.

Fractions obtained from the FPLC MonoS column containing the I8R protein were depleted of both ATPase (Table 1) and DNA helicase activities by the anti-NPHII serum (Fig. 3, lane 2) but not by the anti-NPHI serum (Fig. 3, lane 3). Conversely, the NPHI fraction was depleted of ATPase activity by the anti-NPHI serum but not by the anti-NPHII serum (Table 1). The approximately twofold depletions of ATPase activity (Table 1) and the different levels of DNA helicase activity (Fig. 3, lanes 1 and 3) were a result of sample dilution due to the addition of beads during immunodepletion. Lastly, a second antiserum to the I8R gene product, raised against bacterially expressed recombinant protein (11), also depleted the DNA helicase activity of a fraction containing NPHII protein (data not shown). We conclude that the DNA helicase activity was produced by the action of the NPHII protein, which is the I8R gene product.

To demonstrate that the NPHII protein alone was responsible for the DNA helicase activity, assays were performed with histidine-tagged recombinant NPHII proteins which had been purified by nickel affinity column chromatography and glycerol gradient centrifugation after expression in either a vaccinia virus (15) or a baculovirus (15a) expression system (Fig. 4). Both the vaccinia virus-derived (Fig. 4, lanes 3 to 5) and the baculovirus-derived (Fig. 4, lanes 8 to 10) recombinant NPHII proteins contained DNA helicase activity. No activity was observed in the absence of ATP (Fig. 4, lanes 6, 7, 11, and 12).

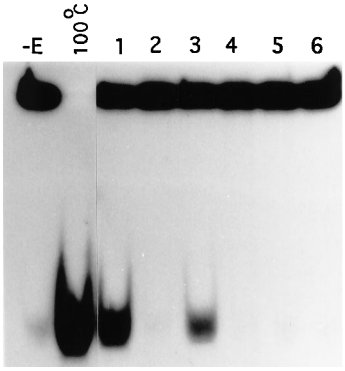

FIG. 3. Immunodepletion of DNA helicase activity. DNA helicase assays were performed with 5 μl of each immunodepleted supernatant and 1.2 fmol of DNA helicase substrate for 30 min at 37°C. Lane 1, I8R control; lane 2, I8R depleted with anti-NPHII serum; lane 3, I8R depleted with anti-NPHI serum; lane 4, NPHI depleted with anti-NPHII serum; lane 5, NPHI depleted with anti-NPHI serum; lane 6, NPHI control. Controls were as described for Fig. 2.

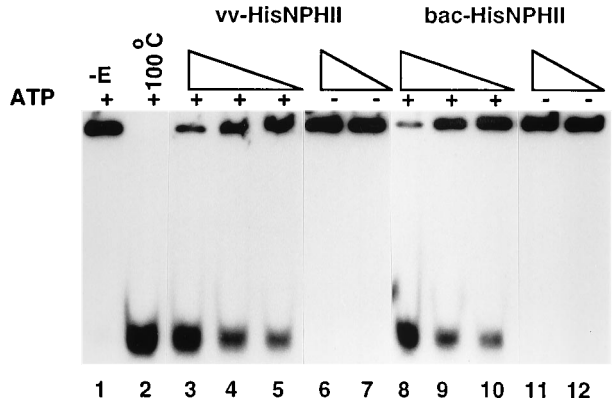

FIG. 4. Recombinant NPHII protein has DNA helicase activity. DNA helicase assays were performed with either a vaccinia virus-expressed (vv-HisNPHII) or a baculovirus-expressed (bac-HisNPHII) wild-type, histidine-tagged NPHII recombinant protein (15, 15a) and 0.5 fmol of DNA helicase substrate for 30 min at 37°C. Assays were performed either in the presence of 2.5 mM ATP with 2 μl (lanes 3 and 8), 0.6 μl (lanes 4 and 9), and 0.2 μl (lanes 5 and 10) of enzyme or in the absence of ATP with 2 μl (lanes 6 and 11) and 0.6 μl (lanes 7 and 12) of enzyme. Controls (lanes 1 and 2) were as described for Fig. 2.

The NPHII protein is therefore an ATP-dependent DNA helicase.

Substrates for DNA unwinding. The length of DNA duplex unwound by the I8R DNA helicase activity was tested by using three substrates with DNA duplex regions of 25, 35, and 45 bp (Fig. 5). NPHII unwound the 25- and 35-bp duplexes (Fig. 5, lanes 9 and 8, respectively) but not the 45-bp duplex (Fig. 5, lane 7). NPHII could be stimulated to unwind the 45-bp duplex by the addition of 0.5 μg of the *E. coli* SSB (Fig. 5, lane 10).

The I8R helicase unwinds RNA duplexes in a 3′-to-5′ direction (29). To determine if the I8R DNA helicase activity had similar directionality, blunt-ended templates with a 5′- or 3′-labeled oligonucleotide were tested as substrates (Fig. 6). The enzyme released only the 5′-labeled oligonucleotide, indicating that an ss region was required for loading of the helicase onto

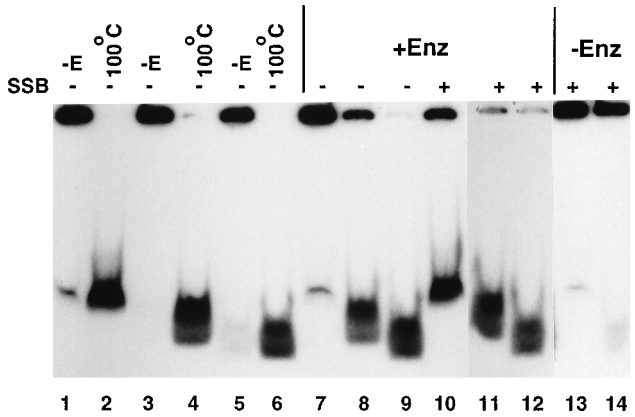

FIG. 5. Effects of DNA duplex length and the *E. coli* ssDNA-binding protein (SSB) on I8R DNA helicase activity. DNA helicase assays were performed for 30 min at 37°C with 3 U of I8R enzyme (+Enz) and 0.5 fmol of one of the following DNA substrates: for lanes 1, 2, 7, 10, and 13, a 45-mer–ssM13mp18 hybrid; for lanes 3, 4, 8, and 11, a 35-mer–ssM13mp18 hybrid; for lanes 5, 6, 9, 12, and 14, a 25-mer–ssM13mp18 hybrid. Where indicated, 0.5 μg of *E. coli* SSB was (+) or was not (−) added. Controls were as described for Fig. 2. An image from the PhosphorImager (Molecular Dynamics) scan of the gel is shown. −Enz, no enzyme was present.

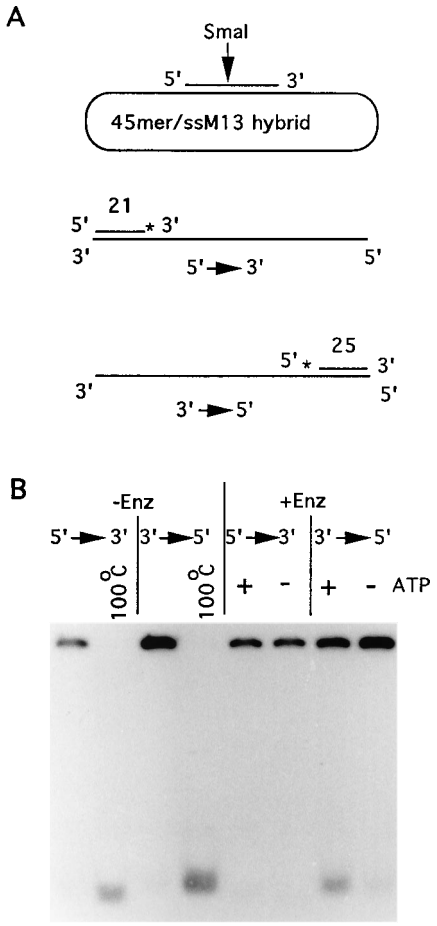

FIG. 6. Direction of DNA unwinding by the I8R helicase. (A) Substrates used to determine the direction of unwinding. The DNA hybrid from which the directional substrates were derived by *Sma*I digestion is shown at the top. The oligonucleotide of this hybrid was labeled at either the 5′ or the 3′ end as described in Materials and Methods. The middle diagram shows the substrate, derived from a 3′-labeled precursor, that can be unwound only by DNA helicases that move in a 5′-to-3′ direction. The bottom diagram shows the substrate, derived from a 5′-labeled precursor, that can be unwound only by DNA helicases that move in a 3′-to-5′ direction. Asterisks indicate the end of DNA labeled with ^{32}P. (B) Results of DNA helicase assays with substrates described in panel A. Control assays (−Enz) involved incubation for 30 min at 37°C in the absence of enzyme either with (100°C) or without heat denaturation. Test assays involved incubation for 30 min at 37°C with 3 U of NPHII enzyme (+Enz) in the presence (+) or absence (−) of 2.5 mM ATP.

DNA duplexes and that the direction of movement on DNA was 3′ to 5′ (Fig. 6).

Comparison of the requirements for DNA and RNA unwinding. DNA unwinding was dependent on an NTP cofactor (Fig. 6) and on the presence of a divalent cation (data not shown). The requirement of divalent cations for DNA unwinding was identical to that observed for RNA unwinding (28): Mg^{2+}, Mn^{2+}, and Co^{2+} were effective cofactors, whereas Ca^{2+}, Cu^{2+}, and Zn^{2+} were not (data not shown). RNA unwinding was stimulated by all of the NTPs and deoxynucleoside triphosphates (dNTPs) (Fig. 7B), as described previously (28). In contrast, DNA unwinding was stimulated most effectively by ATP, dATP, dGTP, and dCTP and less effectively by the other (d)NTPs (Fig. 7A). Shuman (29) observed that RNA unwinding by the I8R helicase was not stimulated by NaCl and was inhibited by NaCl concentrations higher than 100 mM. DNA unwinding also was not stimulated by NaCl or KCl, and con-

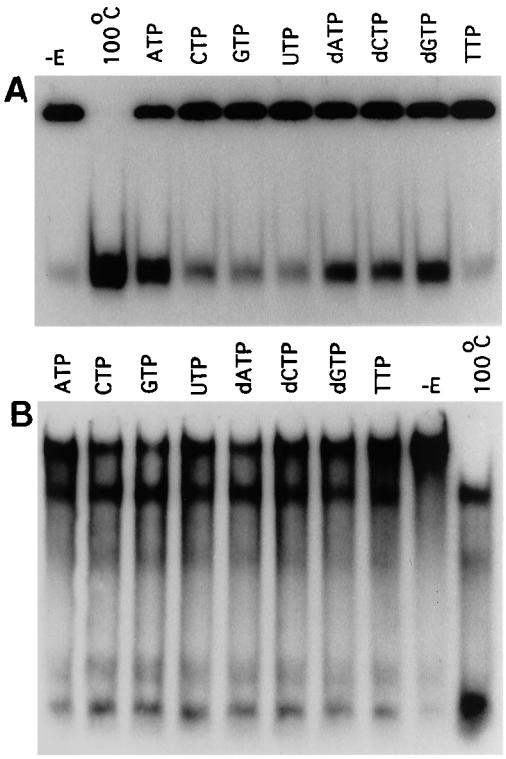

FIG. 7. Nucleotide requirements of helicase activity. DNA helicase assays (A) were performed with 3 U of I8R enzyme and 2 fmol of DNA helicase substrate for 30 min at 37°C. RNA helicase assays (B) were performed with 6 U of I8R enzyme and 1.8 fmol of RNA helicase substrate for 30 min at 37°C. NTPs and dNTPs were present at concentrations of 2.5 mM. Controls were as described for Fig. 2.

centrations of 100 mM NaCl or KCl or greater were inhibitory (Fig. 8). However, some activity was observed in 100 mM KCl (Fig. 8) (3.4-fold less than observed in the absence of KCl), which is a closer approximation of cytosolic conditions.

DISCUSSION

A DNA helicase has been purified from vaccinia virions. This activity copurified with DNA-dependent ATPase and RNA helicase activities over five column chromatography steps

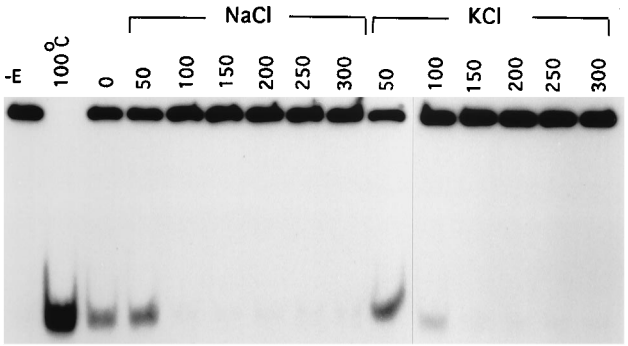

FIG. 8. Effect of salt on DNA helicase activity. Assays were performed with 1.5 U of I8R enzyme and 0.6 fmol of the DNA helicase substrate for 30 min at 37°C. NaCl or KCl was added at the indicated millimolar concentrations. The results of helicase assays were quantitated from gels with a PhosphorImager (Molecular Dynamics). Controls were as described for Fig. 2.

and was inhibited by antisera specific for NPHII. Recombinant NPHII proteins also had DNA helicase activity on oligonucleotide-ssM13mp18 templates. Thus, NPHII, the I8R gene product, is both a DNA:DNA and an RNA:RNA helicase.

Previously, Shuman (28, 29) had shown that the I8R protein had RNA helicase activity but was unable to unwind a DNA:DNA duplex that consisted of a 38-mer oligonucleotide annealed to a 98-mer oligonucleotide; this substrate had a 29-bp duplex region, two 5′ tails of 9 and 28 nucleotides, and a 3′ tail of 41 nucleotides (this is the same template that was used to test the DNA helicase activity of RNA helicase A [19]; see below). We observed, with oligonucleotide-ssM13mp18 duplexes, that the I8R protein could unwind a DNA:DNA duplex region of 35 bp but not one of 45 bp unless the protein was stimulated by *E. coli* SSB. It therefore seems likely, considering that the helicase unwinds in a 3′-to-5′ direction, that a 3′ tail of more than 41 nucleotides may be required to demonstrate DNA helicase activity with the I8R protein. Interestingly, the I8R protein can unwind an RNA:RNA duplex with a 3′ tail of 41 nucleotides (28), which suggests that there may be an intrinsic difference between DNA:DNA and RNA:RNA unwinding by this enzyme.

Only two other RNA:RNA and DNA:DNA helicases have been described, SV40 large T antigen and nuclear DNA helicase II (NDHII) (9, 33). The I8R helicase shares with these helicases the direction of unwinding and a propensity to use all of the (d)NTPs as cofactors. SV40 T antigen, the major SV40 replicative helicase, has no sequence similarity to the I8R gene product and does not have the helicase motifs typical of the helicase superfamily proteins (14). NDHII was purified from calf thymus tissue and has a molecular mass of 130 kDa (33). Zhang and Grosse (33) have speculated that NDHII is the bovine homolog of RNA helicase A, which is a 130-kDa polypeptide that was purified from the nuclei of HeLa cells and shown to have RNA (3′-to-5′) helicase activity but not DNA helicase activity (19). Figure 9 shows a comparison of the amino acid sequences of NDHII, RNA helicase A, vaccinia virus I8R, and a related protein from fowlpox virus. Within the six domains characteristic of the helicase II superfamily (14), the sequences have considerable identity and similarity. In contrast, comparisons of the amino acid sequences of I8R and other known helicases, such as RAD25 and RAD3, show a lower degree of similarity within the helicase domains (data not shown). Thus, the proteins shown in Fig. 9 may represent a new subgroup of helicases which possess both DNA:DNA and RNA:RNA helicase activity. The very high degree of similarity between NDHII and RNA helicase A suggests that the latter protein may be the human equivalent of the bovine protein and thus may also have DNA helicase activity. Although RNA helicase A was reported not to have DNA helicase activity (19), the same assay (see above) also failed to detect DNA helicase activity for I8R, and therefore it is quite possible that RNA helicase A is a DNA helicase. The significance of the functional and sequence similarities between the I8R enzyme and NDHII or RNA helicase A is unclear, as little is known about the biological functions of these proteins.

Temperature-sensitive (*ts*) mutants of the I8R gene exhibit normal early and late protein synthesis, DNA replication, and virion morphogenesis at the nonpermissive temperature (10, 11). The virions formed are, however, noninfectious. This phenotype suggests that I8R DNA helicase activity is not required for DNA replication, although the enzymatic activities of the *ts* I8R proteins have not been analyzed (10). Instead, the phenotype implies a defect in an early stage of virus replication, such as early transcription. On the basis of this phenotype and the RNA helicase activity of the enzyme, Shuman (28, 29) specu-

FIG. 9. The poxvirus I8R protein is related to two eukaryotic proteins, NDHII and RNA helicase A. The amino acid sequences of the I8R gene from the vaccinia virus Copenhagen strain (I8R VV) (GenBank accession number D42511), a related protein from fowlpox virus (I8R FPV) (GenBank accession number G48563), the bovine NDHII gene (NDHII) (GenBank accession number S49822), and the HeLa RNA helicase A gene (Hel A) (GenBank accession number Q08211) were aligned with the program Pileup (7). Identical or physicochemically similar amino acids (using the following groups: L, V, I, and M; G and A; S and T; K and R; D, E, N, and Q; and F, Y, and W) among three of the four sequences are shown in uppercase letters, while nonmatching amino acids are shown in lowercase letters. In regions where the four sequences are identical or similar, a consensus is shown, with characters defined as follows: uppercase letters, identical amino acids; lowercase letters, three identical amino acids and one similar amino acid; !, physicochemically similar amino acids (as above); +, hydrophobic amino acids (I, L, V, M, F, Y, and W); and o, charged or polar amino acids (S, T, D, E, N, Q, K, and R). The six helicase domains, as defined by Gorbalenya et al. (14), are underlined and indicated by Roman numerals.

lated that the I8R gene product was responsible for the extrusion of mRNA molecules from the virus core during early transcription. The observation that the I8R enzyme has DNA helicase activity permits an alternative, but not mutually exclusive, interpretation of the mutant phenotype, which is that the I8R enzyme is required for efficient initiation of early transcription within the virus core. This theory predicts an intimate association of I8R protein and vRNAPol, and it has been observed that I8R ATPase activity is absent from mutant virions that lack vRNAPol (34). However, the phenotype of the I8R mutants also suggests that the DNA helicase activity of the I8R protein is not needed for intermediate or late viral transcription (10, 11). A difference between the requirements for early transcription and those for intermediate or late transcription might be explained by the physical constraint of the template within viral particles, such that melting of the DNA strands during early transcription initiation may require the stimulation of a DNA helicase activity. Whether the DNA helicase activity, the RNA helicase activity, or both activities of the I8R protein are essential for early transcription may be determined from a more detailed analysis of transcription with virions containing the I8R *ts* proteins.

Another putative vaccinia virus DNA helicase has also been implicated in early transcription. Virions containing a *ts* A18R protein have a reduced level of activity in core transcription assays (30). The A18R protein has amino acid similarity to the DNA helicases ERCC3 and RAD25; thus, it has been suggested that the A18R protein is a DNA helicase which is essential for early transcription. Consequently, it is possible that more than one protein with DNA helicase activity (A18R and I8R) may be involved in early transcription.

Early transcription may be reconstituted in vitro with a plasmid template and purified vaccinia virus proteins but without the I8R helicase (see the introduction). This situation may be analogous to that of transcription by RNA polymerase II, which can occur in the absence of the TFIIH DNA helicase on supercoiled plasmids (13) but not (i) on relaxed plasmids or (ii) in the presence of a repressor of basal transcription such as the DNA-binding protein HMG2 (31). Thus, the reconstituted vaccinia virus transcription system may have identified only a basal transcription apparatus, and the addition of other levels of complexity to this system may lead to the identification of a function for the I8R DNA helicase activity in vaccinia virus transcription.

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