

Vaccinia Virus Gene A18R Encodes an Essential DNA Helicase

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The vaccinia virus A18R protein is a DNA-dependent ATPase that contains the canonical sequence motifs associated with the DEXH group of DNA and RNA helicases. Investigation of A18R protein function during infection indicated it functions in the early and late phases of vaccinia virus transcription. The A18R protein shares sequence similarity with the mammalian DNA helicase ERCC3. The ERCC3 protein has a dual function: it is a component of the transcription factor TFIIH and is an essential participant in the cellular nucleotide excision repair pathway. Here we present evidence that the A18R protein is a DNA helicase that unwinds duplex DNA in a 3'-to-5' direction. The A18R helicase was inactive on RNA-DNA and RNA-RNA hybrids. The A18R unwinding activity was most efficient on DNA substrates with lengths of 20 nucleotides or less, and its unwinding activity was not stimulated by the addition of *Escherichia coli* single-strand-binding protein (SSB), the bacteriophage T4 gene 32 SSB, or the vaccinia virus I3L protein, a putative SSB. We have used an electrophoretic gel mobility shift assay to show that the A18R protein forms a stable complex with single-stranded DNA, and to a lesser extent RNA, in a reaction that does not require ATP.

Vaccinia virus, the prototypic member of the orthopoxvirus family, contains a 192-kb double-stranded DNA (dsDNA) genome with the coding potential for at least 200 open reading frames (25). The virus replicates in the cell, with limited contribution from the host nucleus (44 [reviewed in references 36, 38, and 58]). Because of its replicative and transcriptional autonomy within the cell, vaccinia virus encodes most if not all of the proteins required for viral genome replication, recombination, repair, and transcription. Vaccinia virus-encoded proteins often share significant sequence homology with the functionally equivalent cellular protein (e.g., references 6 and 43). Thus, vaccinia virus provides a useful model system for the biochemical and genetic investigation of RNA and DNA metabolism.

Vaccinia virus transcription occurs in a temporally regulated cascade that is divided into three classes: early, intermediate, and late (3 [reviewed in reference 37]). In this manner, the transcription factors encoded by the early transcribed genes activate transcription of the intermediate genes (or in a single instance a viral late gene or genes [29]), which encode the transcription factors responsible for activation of late gene transcription. Expression of intermediate and late genes is prevented by the inhibition of viral DNA replication, suggesting an intimate association between viral gene expression and DNA replication. Late during infection, the multisubunit viral RNA polymerase, the heterodimeric early transcription factor (vETF), and additional proteins necessary for early transcription are packaged in the virion. The infectious cycle is completed with virion entry into the host cell and subsequent initiation of viral early RNA synthesis. Biochemical characterization of vaccinia virus transcription *in vitro* has identified the minimal essential viral *cis*- and *trans*-acting factors (references 1, 2, 13, 14, 22, and 33 and references therein) and has implicated involvement of yet unidentified host proteins in vaccinia virus gene expression (44, 60).

Genetic investigation has identified several vaccinia virus genes not identified by biochemical methods that when mutated, alter the regulated program of gene expression (reviewed in reference 11). For one such gene, A18R, the initial characterization identified a complex phenotype which suggested A18R protein involvement in the early and late phases of viral transcription (39). The A18R protein is synthesized throughout infection and is packaged in the virion core particle (40, 54). A comparison *in vitro* of virion early transcription between wild-type vaccinia virus and mutant Cts23 virus, which synthesizes a thermolabile A18R protein, showed an overall decrease in the amount of RNA synthesized by the mutant virus (54). Since the wild-type virus and Cts23 virus contained equivalent amounts of individual proteins, including RNA polymerase and A18R protein, the transcription deficiency was associated with the defective A18R protein. In addition, A18R protein involvement in vaccinia virus late transcription was observed when Cts23 virus-infected cells maintained under nonpermissive conditions showed a striking alteration in the late transcription pattern indicative of unregulated transcription from regions of the genome which would be normally transcriptionally silent (4). A direct consequence of the loss of transcriptional regulation was the increased synthesis of complementary viral RNAs and accumulation of viral dsRNA, a potent activator of the interferon-inducible cellular endoribonucleolytic ppp(A2'p)*n*A (2-5A) pathway. Ultimately the viral and cellular RNAs were degraded by the cellular 2-5A-activated RNase L enzyme, leading to abortion of the infection (4, 8, 39, 40). The evidence did not, however, completely eliminate the possibility that the A18R transcription defect was a result of A18R protein involvement in vaccinia virus DNA metabolism (see Discussion).

A18R protein contains the conserved motifs associated with nucleoside phosphohydrolase enzymes and with the helicase superfamily II, specifically the DEXH family of DNA and RNA helicases (19, 28). The most significant homology existed between the A18R protein and the mammalian ERCC3 protein and the yeast homolog of ERCC3, RAD25/SSL2 (28). ERCC3 and RAD25 have ATPase and DNA helicase activity and function in cellular transcription as components of the mammalian RNA polymerase II transcription complex TFIIH

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(15, 34, 47, 61) and the analogous yeast complex factor B, respectively (16, 20, 42). In addition, the ERCC3 and RAD25 proteins function in the repair of damaged DNA as members of the DNA nucleotide excision repair (NER) pathway (21, 57, 59). Significantly, the loss of ERCC3 protein function in transcription or NER results in the extreme UV sensitivity or neurological disorders associated with the human hereditary conditions xeroderma pigmentosum, Cockayne's syndrome, and trichothiodystrophy (reviewed in reference 23). Collectively, a growing body of evidence demonstrates that in mammalian cells, yeast cells, and bacteria, a number of proteins originally associated with the cellular basal transcription machinery also function in DNA repair, suggesting a tight coupling between transcription and DNA repair (7, 16, 23, 49).

To date, the similarity between the A18R protein and the ERCC3 and RAD25 proteins has relied upon shared amino acid sequence, their involvement in transcription, and, more recently, the identification of the A18R protein as a DNA-dependent ATPase (5). We present here evidence that like ERCC3, the A18R protein is a DNA helicase that unwinds DNA in a 3'-to-5' direction. In addition, we show that the A18R protein interacts with DNA and to a lesser extent with RNA to form a stable complex that is detectable in an electrophoretic mobility shift assay.

MATERIALS AND METHODS

Cells and virus. BSC40 cells; the recombinant vaccinia virus vTF7.3, which expresses the bacteriophage T7 RNA polymerase (17); the recombinant vaccinia virus VVTMHisA18, which contains a polyhistidine-A18R gene fusion driven by a T7 RNA polymerase promoter (5); and the conditions for their growth, infection, and plaque titration have been described previously (9, 10).

Purification of the His-A18R protein. The amino-terminal polyhistidine-A18R fusion protein (His-A18R) was purified from BSC40 cells coinfecting with the recombinant vaccinia viruses vTF7.3 and VVTMHisA18 as described previously (5). Purification of the His-A18R protein by His-bind resin chromatography (Novagen) resulted in copurification of the His-A18R DNA-dependent ATPase (57.5 kDa) and a presumed His-A18R amino-terminal fragment with a size of 30 kDa shown previously to lack helicase activity (5). The fractions containing His-A18R (300 μ g) were dialyzed for 24 h at 4°C against four changes (500 ml) of 40 mM Tris (pH 8)–20 mM KCl–40% glycerol (5) and were used without further purification to assay helicase activity unless stated otherwise. The enzyme was stored at –20°C, where it was stable for at least 9 months. In order to independently assay the 57.5- and 30-kDa polypeptides for helicase activity, the A18R protein from the peak His-bind column fractions (150 μ g) was centrifuged in a 15 to 30% linear glycerol gradient made in 40 mM Tris (pH 8)–20 mM KCl–1 mM dithiothreitol–0.1% Nonidet P-40 for 66 h in an SW41 rotor (Beckman) at 35,000 rpm and 4°C. Gradient fractions (\approx 250 μ l) were collected dropwise and analyzed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE [10% polyacrylamide]) (45) followed by silver staining (Bio-Rad). The fractions were stored at –70°C.

Preparation of helicase substrates. The following DNA oligonucleotides were annealed to bacteriophage M13mp18 single-stranded DNA (ssDNA): 17 nucleotides (nt), GTTTTCCAGTCACGAC (universal sequencing primer [New England Biolabs]) (Fig. 1 to 3); 20 nt, TCGACTCTAGAGGATCCCCG (Fig. 4, lanes 1 to 3); 25 nt, TCGACTCTAGAGGATCCCCGGGTAC (Fig. 4, lanes 4 to 6); 30 nt, TCGACTCTAGAGGATCCCCGGGTACCGAGC (Fig. 4, lanes 7 to 9); 68 nt, GTTATTGCATGAAAGCCCGGCTGACTCTAGAGGATCCCCGGGTACCGTTATTGCATGAAAGCCCGGCTG (Fig. 4, lanes 10 to 12 [12]); 47 nt, TGCCTGCAGGTCGACTCTAGAGGATCCCCGGGTACCGAGCTCGAATT (Fig. 5, lanes 1 to 4); and 49 nt, TCGACTCTAGAGGATCCCCGGGTACCGAGCTCGAATTCGTAATCATGGT (Fig. 5, lanes 5 to 8). Nucleotides complementary to M13 are underlined. The following DNA oligonucleotide is complementary to pGEM3ZF(+) ssDNA (Promega): 20 nt, GGCGAATTCGAGCTCGGTAC (Fig. 6, lanes 1 to 4). The analogous RNA oligonucleotide was synthesized *in vitro* by T7 RNA polymerase transcription of *Aval*-linearized pGEM3ZF(+) plasmid DNA as described previously (31). The RNA was annealed to pGEM3ZF(+) ssDNA (Fig. 6, lanes 5 to 8) or annealed to a complementary *in vitro*-synthesized RNA made by SP6 RNA polymerase transcription of *Pvu*II-digested pGEM3ZF(+) plasmid DNA (Fig. 6, lanes 9 to 12). A T7 RNA polymerase-synthesized transcript identical to the first 16 nt of the RNA 20-mer described above and complementary to pGEM3ZF(+) ssDNA (Fig. 6, lanes 17 to 20) was synthesized from *Kpn*I-linearized pGEM3ZF(+) plasmid made blunt with Klenow enzyme according to the manufacturer's recommendations (Promega) (Fig. 6, lanes 21 to 24). SP6 RNA polymerase transcription of *Acc*I-linearized pGEM3ZF(+) plasmid produced a 29-nt transcript capable of form-

ing a 17-nt duplex with M13 ssDNA (Fig. 6, lanes 13 to 16). pGEM3ZF(+) ssDNA was produced with the helper bacteriophage M13K07 and bacterial strain JM101 according to standard procedures (45).

DNA helicase substrates were prepared by phosphorylation of 10 pmol of oligonucleotide with 50 μ Ci of [γ -³²P]ATP (6,000 Ci/mmol [Amersham]) and 10 U of T4 polynucleotide kinase (New England Biolabs) in a 25- μ l volume. The amount of reaction mixture was increased to 100 μ l and made 10 mM Tris (pH 7.5)–1 mM EDTA–100 mM NaCl, and M13 ssDNA or pGEM3ZF(+) ssDNA (400 fmol and 1.1 pmol, respectively [1 μ g]) was added. The mixture was heated to 100°C for 5 min and transferred to a 68°C water bath, and the bath was allowed to cool to room temperature overnight. Unincorporated nucleotides were removed by G-50 spin column chromatography (Sigma), and the annealed duplexes were isolated by Bio-Gel A5-M column chromatography (Bio-Rad) in 10 mM Tris (pH 8)–1 mM EDTA–100 mM NaCl. Phosphorylated DNA oligonucleotides greater than 40 nt were purified by 10% polyacrylamide gel electrophoresis with 50% urea as described for RNA prior to annealing (31).

For the determination of His-A18R helicase directionality, the oligonucleotide was radiolabeled with T4 polynucleotide kinase and annealed to M13 ssDNA as described above and the duplex DNA was precipitated with ethanol. The DNA was then resuspended in 10 mM Tris-HCl (pH 7.5)–5 mM MgCl₂–7.5 mM dithiothreitol and incubated with 100 μ Ci of [α -³²P]dCTP (6,000 Ci/mmol) and 5 U of Klenow enzyme (New England Biolabs) for 30 min at 37°C prior to G-50 spin column chromatography and purification through Bio-Gel A5-M equilibrated in *Sma*I restriction enzyme buffer (20 mM Tris-acetate, 10 mM magnesium acetate, 50 mM potassium acetate, 1 mM dithiothreitol [pH 7.9, 25°C]; New England Biolabs). The purified duplexes were linearized with *Sma*I enzyme for 60 min at 25°C and passed through a G-50 spin column equilibrated in helicase buffer prior to use in the helicase assay. The resulting linearized M13 DNA contained blunt-ended duplex termini separated by approximately 7.2 kb of M13 ssDNA (Fig. 5).

RNA helicase substrates were prepared as described previously (31). Briefly, *in vitro*-synthesized RNA transcripts were purified by 10% PAGE with 50% urea. The RNA was then incubated with its complementary RNA or DNA in 50 mM Tris (pH 7.5)–0.5 M NaCl–0.1% SDS–1 mM EDTA at 100°C for 5 min and transferred to a 68°C water bath, and the bath was allowed to cool to room temperature overnight. The annealed duplex was then purified by native 8% PAGE and resuspended in helicase buffer.

Helicase assay and ATPase assay. Helicase reaction mixtures (25 μ l) contained 40 mM Tris (pH 8), 50 mM KCl, 8 mM MgCl₂, 8 mM ATP, 2 mM dithiothreitol, 0.5 fmol of helicase substrate (1,600 dpm), and 350 fmol (20 ng) of purified His-A18R protein unless indicated otherwise (standard reaction conditions). Reaction mixtures were incubated at 37°C for 30 min before the addition of 5 μ l of stop buffer (24) and were analyzed by native 8% PAGE. The dried gels were autoradiographed, and the percentage of oligonucleotide displaced was determined by PhosphorImager analysis (Molecular Dynamics). The *Escherichia coli* single-strand-binding protein (SSB) and the bacteriophage T4 gene 32 SSB were purchased from Pharmacia. ATPase assays were done by the method of Lanzetta et al. (30) as described previously (5). Briefly, the ATPase assay was done in a 50- μ l volume containing 40 mM Tris (pH 8), 100 mM KCl, 4 mM MgCl₂, 4 mM ATP, 2 mM dithiothreitol, 200 fmol of M13 ssDNA, and 1.2 pmol of His-A18R protein. Reaction mixtures were mixed on ice, incubated at 37°C for 15 min, and terminated with the addition of 5 μ l of 0.5 M EDTA. The release of P_i was determined at an optical density of 600 nm.

Electrophoretic mobility shift analysis. Oligonucleotides used for the electrophoretic mobility shift analysis were radiolabeled and purified as described above. Each reaction mixture (10 μ l) contained 40 mM Tris (pH 8), 50 mM KCl, 4 mM MgCl₂, and 10% glycerol. Enzyme and ATP were added as indicated in the figure. The reaction mixtures were incubated at 37°C for 15 min and analyzed by 6% nondenaturing PAGE with 0.25 \times Tris-borate-EDTA. The gels were prerun at 100 V for 2 h, and the samples were analyzed at 150 V. The electrophoresis buffer was recirculated. Gels were vacuum dried prior to autoradiography.

RESULTS

Identification of His-A18R helicase activity. The expression of His-A18R protein in vaccinia virus-infected cells and subsequent purification by Ni²⁺ affinity chromatography were described previously and resulted in a copurification of the 57.5-kDa His-A18R DNA-dependent ATPase and an additional \approx 30-kDa polypeptide that is presumed to be a His-A18R amino-terminal fragment. The 30-kDa polypeptide does not contain ATPase activity (5). Although it was unlikely the 30-kDa polypeptide had helicase activity, the 57.5- and 30-kDa polypeptides were independently tested for helicase activity. The polypeptides were purified by Ni²⁺ chromatography and separated by differential centrifugation in a linear 15 to 30% glycerol gradient. Individual gradient fractions were analyzed by SDS-PAGE and tested for helicase activity and ATPase

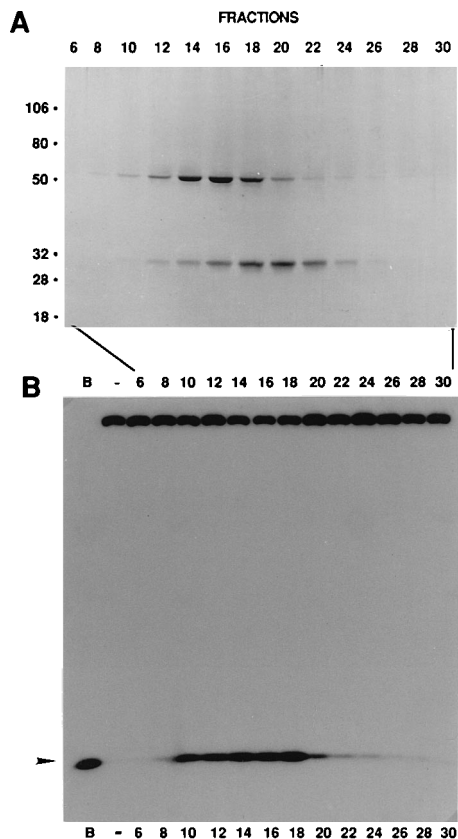


FIG. 1. DNA-dependent ATPase activity and DNA helicase activity copurify with the His-A18R protein. The His-A18R protein was expressed in vaccinia virus-infected cells and purified by Ni^{2+} affinity chromatography and glycerol gradient sedimentation as described in Materials and Methods. The fractions were collected from the gradient bottom and analyzed by SDS-PAGE (10% polyacrylamide) followed by silver staining (A). Gradient fractions are labeled (6 = bottom). Molecular mass markers are indicated in kilodaltons on the ordinate. The same fractions were analyzed for DNA helicase activity (B) and DNA-dependent ATPase activity (C). The helicase reactions shown in panel B were analyzed by 8% nondenaturing PAGE and visualized by autoradiography. Lane B contains substrate boiled for 4 min and analyzed directly, and a reaction mixture lacking A18R protein is indicated by a α . The position of the nonannealed oligonucleotide removed by boiling or helicase activity is indicated by an arrowhead. The percentage of oligonucleotide unwound in panel B was quantified by Phosphor-Imager analysis and is presented in panel C (left ordinate). ATPase activity was determined by colorimetric detection of the hydrolyzed phosphate (4, 30) and is presented in panel C (right ordinate) as nanomoles of phosphate.

activity (Fig. 1). The helicase assay tested the ability of the His-A18R protein to unwind a DNA duplex formed between an M13 ssDNA and a radiolabeled 17-nt DNA oligonucleotide. The unwound 17-mer was identified by its increased elec-

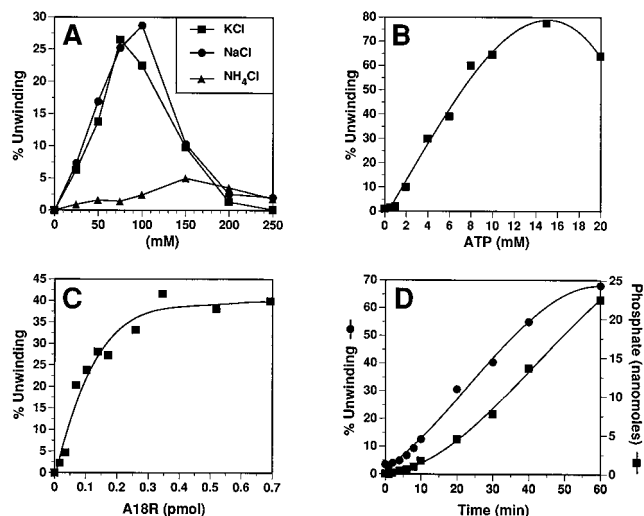


FIG. 2. Characterization of His-A18R helicase activity. (A) His-A18R protein helicase activity was analyzed in a reaction mixture containing 40 mM Tris (pH 8), 2 mM dithiothreitol, and 0.5 fmol of substrate and supplemented with 8 mM MgCl_2 , 8 mM ATP, 350 fmol of enzyme, and the indicated amount of KCl, NaCl, and NH_4Cl . (B) The helicase reaction mixture described for panel A was supplemented with 50 mM KCl and the indicated equivalent concentrations of MgCl_2 and ATP. (C) His-A18R helicase activity was analyzed in a reaction mixture containing 40 mM Tris (pH 8), 50 mM KCl, 8 mM MgCl_2 , 8 mM ATP, 2 mM dithiothreitol, and 0.5 fmol of substrate and supplemented with the indicated amount of A18R enzyme. (D) His-A18R DNA-dependent ATPase activity and helicase activity were measured at the times indicated in the figure under the standard reaction conditions described above (C) with 350 fmol of His-A18R protein. Aliquots were removed, and the reactions were stopped and assayed as described in Materials and Methods.

trophoretic mobility on nondenaturing PAGE. As seen in Fig. 1, the His-A18R helicase activity and ATPase activity cosedimented with the glycerol gradient fractions containing the 57.5-kDa His-A18R protein (Fig. 1B and C, fractions 8 to 20). Since the 30-kDa polypeptide did not contribute to the observed helicase or ATPase activity, we have used the Ni^{2+} -purified His-A18R protein in subsequent experiments.

His-A18R helicase in vitro reaction optima. The reaction optima for His-A18R helicase activity were investigated. As seen in Fig. 2A the His-A18R helicase activity was stimulated by KCl and NaCl, but not NH_4Cl , when incubated under standard reaction conditions. Previous experiments had indicated that the His-A18R ATPase activity was optimal with equivalent concentrations of MgCl_2 and ATP; indeed, ATP in excess of MgCl_2 appeared to inhibit His-A18R ATPase activity (5). In Fig. 2B, the His-A18R helicase was incubated with increasing and equivalent amounts of MgCl_2 and ATP. The data show the corresponding increase in His-A18R helicase activity with increasing concentrations of MgCl_2 and ATP. Substitution of various mono- and divalent salts (8 mM salt with 8 mM ATP) for MgCl_2 resulted in variable helicase activity: MgSO_4 and MnCl_2 substituted with equal efficiency; $\text{Mg}(\text{CH}_3\text{O}_2)_2$ resulted in a 40% loss of activity; and CaCl_2 , CoCl_2 , NiCl_2 , ZnSO_4 , NiSO_4 , and $\text{NH}_4\text{C}_2\text{H}_3\text{O}_2$ resulted in a 95 to 100% loss of activity (data not shown). The data in Fig. 2C show the direct relationship between the His-A18R enzyme concentration and the increase in helicase activity. These experiments indicated that the His-A18R helicase and ATPase reaction optima were nearly identical (5). In order to confirm that the reaction conditions chosen to investigate the His-A18R helicase activity did not result in limiting the available helicase substrate and/or ATP during the course of the reaction, the His-A18R helicase

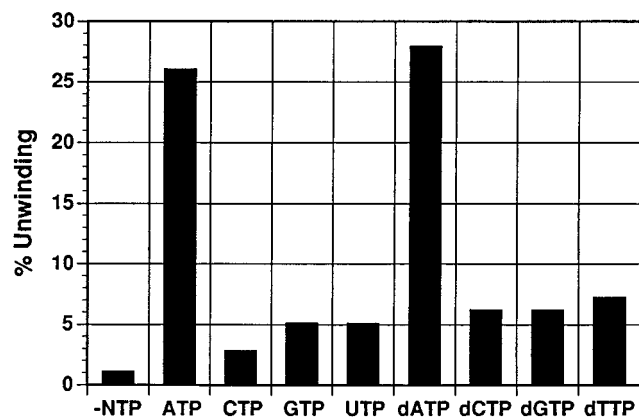


FIG. 3. His-A18R helicase unwinding activity with ribonucleotides and deoxyribonucleotides. His-A18R helicase activity was examined under standard reaction conditions with mixtures supplemented with 350 fmol of His-A18R protein and the indicated ribonucleotide or deoxyribonucleotide (8 mM). The reactions were analyzed by 8% nondenaturing PAGE (data not shown) and quantified by PhosphorImager analysis. NTP, nucleoside triphosphate.

and ATPase activities were tested under standard reaction conditions of 40 mM Tris (pH 8)–50 mM KCl–8 mM MgCl₂–8 mM ATP–2 mM dithiothreitol–0.5 fmol of substrate–350 fmol of enzyme at 37°C. The data in Fig. 2D show that under these conditions, the His-A18R helicase and ATPase activities remained linear for approximately 40 and 60 min, respectively. Therefore, neither helicase substrate nor ATP is in a limiting concentration during a standard 30-min reaction. The standard reaction conditions result in unwinding of approximately 40% of the helicase substrate (Fig. 2D) within 30 min and complete removal within 240 min (data not shown). We chose these conditions for our standard assay to permit the analysis and detection of increased His-A18R helicase activity in the presence of accessory protein factors (e.g., SSB protein, as described below). Since the kinetics of the His-A18R helicase interaction with the large region of ssM13 lacking oligonucleotide are as yet unknown and may effectively contribute to reducing the His-A18R helicase activity, we cannot arrive at an unequivocal His-A18R helicase specific activity.

The activity of the His-A18R helicase with individual ribonucleotides and deoxyribonucleotides was determined, and the data are presented in Fig. 3. The His-A18R helicase has an absolute requirement for nucleotide and shows the greatest activity with ATP and dATP but is moderately stimulated by the additional nucleotides tested. The His-A18R ATPase activity showed a similar preference for ATP and dATP (5).

His-A18R helicase duplex length requirement, direction of unwinding, and substrate specificity. It became apparent during our experiments that the His-A18R helicase inefficiently unwound oligonucleotides greater than approximately 20 nt in length. To directly test this observation, the His-A18R unwinding activity was investigated with 5'-coterminol oligonucleotides with lengths of 20, 25, and 30 nt annealed to M13 ssDNA. The data in Fig. 4 show the His-A18R helicase efficiently unwound the 20-nt duplex (lanes 1 to 3) but was incapable of unwinding the 25- and 30-nt substrates (compare lanes 1 to 3 with lanes 4 to 9). These data suggested that the His-A18R helicase was restricted to unwinding duplex regions less than 20 to 25 nt in length, but the data did not eliminate the possibility that the His-A18R helicase activity was compromised by the blunt-ended (non-tailed) oligonucleotides used in these assays. To address this possibility, we investigated the His-A18R helicase activity by using an oligonucleotide that pro-

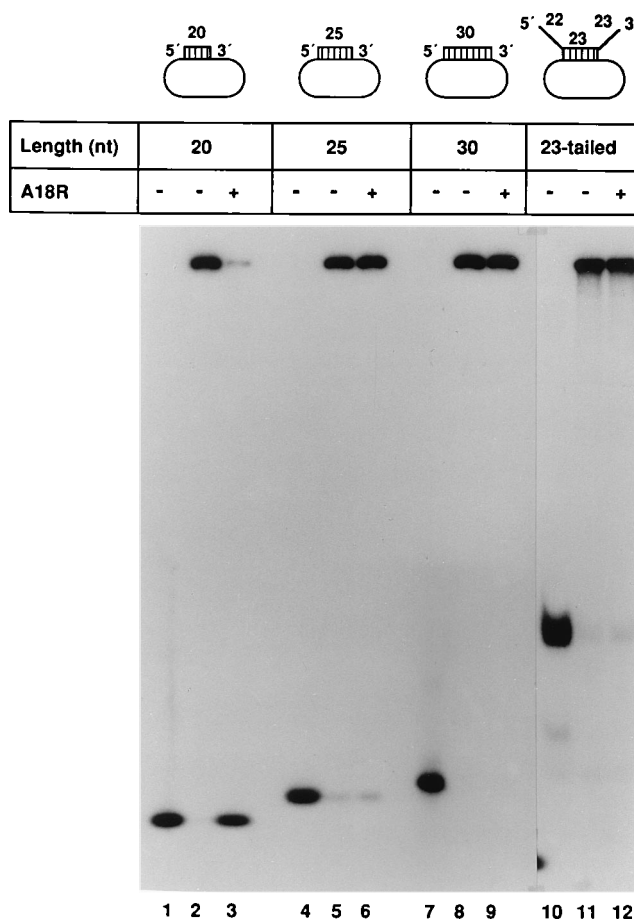


FIG. 4. Analysis of His-A18R helicase activity on oligonucleotides of increasing length. 5'-coterminol oligonucleotides of increasing length (20, 25, and 30 nt) in lanes 1 to 9 and an oligonucleotide that annealed to form a 23-nt duplex with 5' and 3' extensions in lanes 10 to 12 were annealed to M13 ssDNA and incubated under standard reaction conditions in the presence or absence of A18R protein (350 fmol). The reactions were analyzed by 8% nondenaturing PAGE followed by autoradiography. Lanes 1, 4, 7, and 10 were heat denatured prior to analysis. The M13-oligonucleotide substrates are diagrammed above the autoradiograph (not to scale), and the oligonucleotide 5' and 3' termini are indicated.

vided a 23-nt duplex with 5' and 3' nonhybridized extensions designed to simulate a DNA fork structure in vitro (12). The data in Fig. 4 (lanes 10 to 12) show that the His-A18R helicase was unable to unwind the forked DNA substrate. In summary, the His-A18R helicase does not require a forked DNA structure for activity and is unable to unwind in vitro DNA duplexes greater than 20 to 22 nt in length.

Helicases show a distinct polarity of DNA unwinding in vitro relative to the ssDNA surrounding the DNA duplex (32). The direction of A18R unwinding was analyzed with a linear M13 ssDNA containing a radiolabeled oligonucleotide at each terminus (diagrammed in Fig. 5). The preferential removal of either oligonucleotide by the His-A18R helicase defines the direction of helicase movement relative to the M13 ssDNA (32). To create the helicase substrate, a DNA oligonucleotide radiolabeled at the 5' and 3' termini that contained an internal *Sma*I restriction endonuclease site was annealed to M13 ssDNA. The duplex DNA was then digested with *Sma*I to produce a linearized M13 ssDNA containing oligonucleotides of asymmetric lengths at each terminus. In order to confirm the direction of the His-A18R helicase movement, we used two

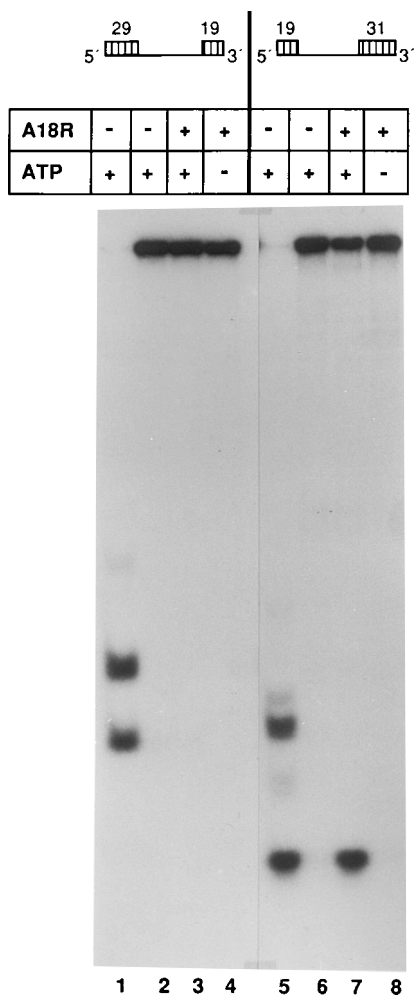


FIG. 5. Directionality of A18R helicase activity. Oligonucleotides designed to identify the polarity of A18R unwinding were 5' radiolabeled with T4 polynucleotide kinase and [γ - 32 P]ATP and annealed to M13 ssDNA. The DNA was ethanol precipitated, and the oligonucleotide 3' terminus was radiolabeled with Klenow enzyme and [α - 32 P]CTP. The M13-oligonucleotide complexes were then purified by Bio-Gel A5-M chromatography in *Sma*I restriction endonuclease buffer (New England Biolabs) and digested with *Sma*I restriction endonuclease to produce the helicase substrates depicted in the figure (not to scale). The sizes of the digested oligonucleotides are indicated in the diagram, and the M13 DNA 5' and 3' termini are indicated. The hybrids were then purified by G-50 spin column chromatography in helicase buffer and used directly in a helicase reaction mixture containing His-A18R and ATP as indicated in the figure. The oligonucleotides in lanes 1 to 4 and 5 to 8 were analyzed in different experiments, and therefore the relative migrations of the oligonucleotides between experiments are not directly comparable. The reaction mixtures in lanes 1 and 5 were heat denatured prior to analysis. The background bands in lanes 1 and 5 represent incomplete digestion products and do not affect the interpretation of the data.

independent oligonucleotides which when *Sma*I digested, would result in a 19-nt duplex of similar sequence at the M13 5' or 3' terminus. As seen in Fig. 5, the A18R helicase unwound the 19-nt duplex located at the M13 5' terminus (compare lanes 3 and 7) in an ATP-dependent manner (lane 8). These data indicated that the His-A18R protein translocated with 3'-to-5' directionality. Increasing the concentration of the A18R protein did not facilitate unwinding in the opposite direction (data not shown). The inability of the His-A18R helicase to unwind the 19-nt duplex at the M13 5' terminus in lane 3 is explained by the data in Fig. 4 showing that the helicase activity is unable to unwind a duplex greater than 20 to 22 nt in length.

Helicase enzyme activity is often stimulated *in vitro* with the addition of SSBs (50, 51). We investigated the ability of the *E. coli* SSB, the bacteriophage T4 gene 32 SSB, and the vaccinia virus I3L protein, which has *in vitro* ssDNA binding activity (42a), to stimulate His-A18R helicase activity. The addition of the SSBs to a standard helicase reaction mixture at concentrations up to 30 μ g/ml did not stimulate or inhibit A18R helicase unwinding of a 20-nt duplex. In addition, SSBs did not facilitate unwinding of a 30-nt duplex (oligonucleotides from Fig. 4 [data not shown]).

The ability of the His-A18R helicase to unwind DNA and RNA duplexes with identical nucleotide sequences was investigated with a 20-nt DNA-DNA duplex (Fig. 6, lanes 1 to 4), a comparable 20-nt RNA-DNA duplex (lanes 5 to 8), or the corresponding RNA-RNA duplex (lanes 9 to 12). The His-A18R helicase efficiently unwound the DNA-DNA hybrid in an ATP-dependent manner but failed to unwind either the RNA-DNA hybrid or the RNA-RNA hybrid. In order to confirm that the inability of the His-A18R enzyme to unwind RNA was not related to the length of the oligonucleotide, an RNA oligonucleotide containing the first 16 nt of the 20-mer used in lanes 5 to 8 was annealed to DNA (lanes 17 to 20) or RNA (lanes 21 to 24). Again, the His-A18R helicase failed to unwind the RNA duplexes. To test the His-A18R requirement for a forked RNA-DNA substrate, a 29-nt RNA oligonucleotide was annealed to DNA to form a 17-nt duplex with a 5' 12-nt extension (lanes 13 to 16). The His-A18R enzyme did not unwind this forked RNA-DNA substrate. In summary, the data show that *in vitro*, the His-A18R protein has DNA helicase activity but not RNA helicase activity.

Determination of His-A18R interaction with DNA and RNA *in vitro* by electrophoretic mobility shift analysis. We have shown previously that the virion-associated A18R protein was capable of binding ss- and dsDNA in a southwestern (DNA-protein) assay (54). The ability of the His-A18R protein to form a stable DNA-protein complex *in vitro* that is detectable by native gel electrophoresis was investigated. As seen in Fig. 7A, the addition of increasing amounts of His-A18R protein to a 68-nt DNA oligonucleotide resulted in a reduced electrophoretic mobility of the oligonucleotide (compare lanes 1 and 2 with lanes 3 to 9). At the highest His-A18R concentration, the protein and DNA formed a complex that was unable to migrate into the gel (lane 9). That the mobility shift was due to the His-A18R protein was confirmed when substitution of the His-A18R protein with bovine serum albumin (1.5 pmol) did not cause a DNA mobility shift (compare lanes 7 and 10). In addition, the His-A18R interaction with DNA could be inhibited by excess unlabeled oligonucleotide and excess poly(dI-dC) (data not shown). The A18R interaction with DNA was not affected by the addition of ATP (compare lanes 7 and 11). Since the His-A18R protein used in the electrophoretic mobility shift experiments was a mixture of the 57.5- and 30-kDa polypeptides, we investigated the ability of the individual polypeptides to bind DNA by using the glycerol gradient fractions shown in Fig. 1A. DNA binding activity was identified in gradient fractions 8 to 20 and was absent from fractions 22 to 30. These data indicate that the 57.5-kDa His-A18R ATPase and helicase protein was responsible for the mobility shift seen in Fig. 7A (data not shown).

His-A18R ATPase activity is not stimulated by ss- or dsRNA (5), and the enzyme does not possess RNA helicase activity (this paper). The data do not preclude the formation of a stable A18R-RNA complex. To address this possibility, we tested the ability of the His-A18R protein to form a complex detectable in an electrophoretic mobility shift assay. The His-A18R protein was allowed to interact with a 46-nt RNA oli-

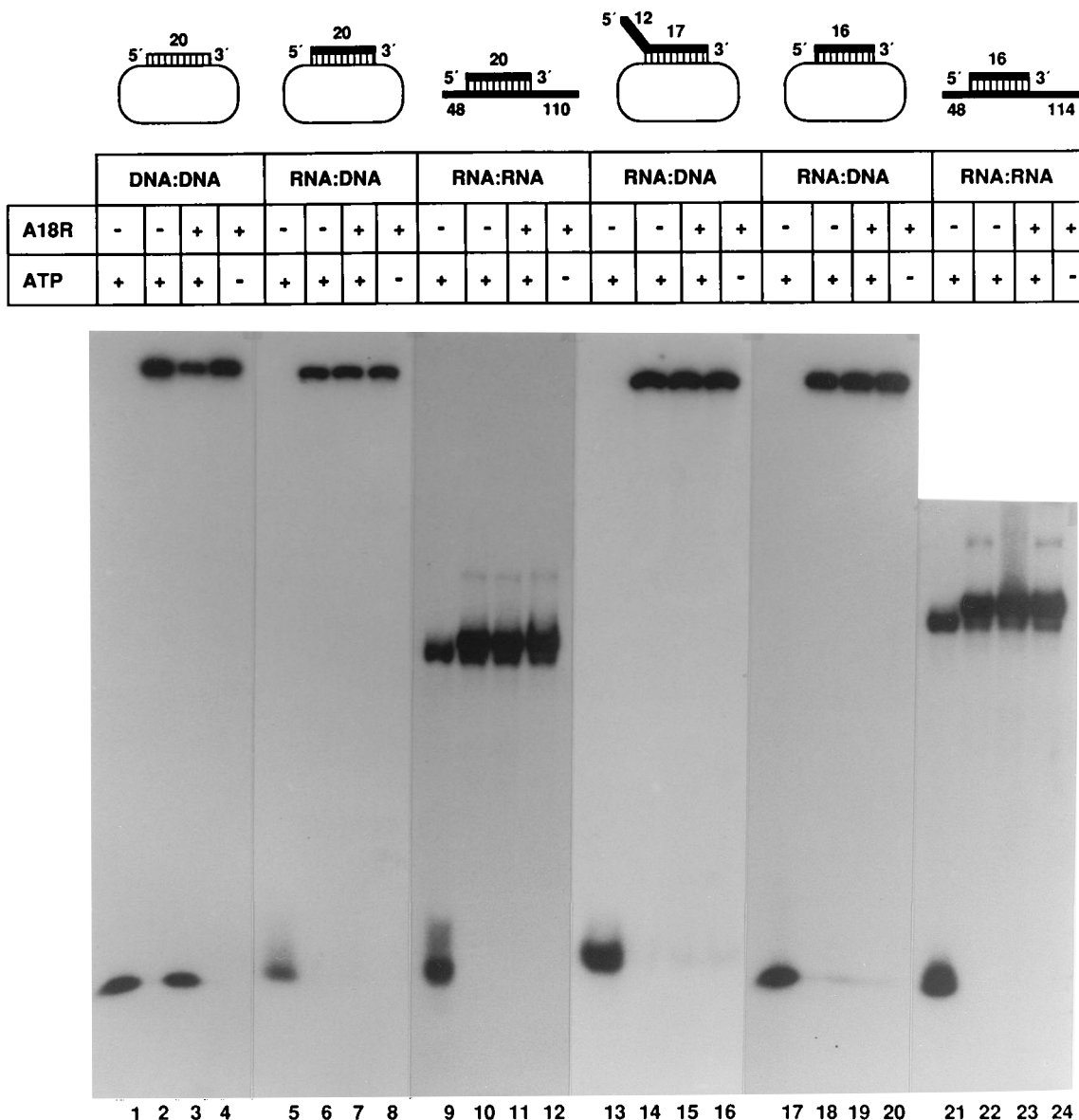


FIG. 6. Determination of His-A18R helicase substrate specificity. His-A18R unwinding activities were compared by using analogous DNA and RNA substrates. A 20-nt DNA oligonucleotide and its equivalent in vitro-synthesized RNA transcript were annealed to pGEM3ZF(+) ssDNA (lanes 1 to 4 and 5 to 8, respectively), or the RNA was annealed to an in vitro-synthesized complementary RNA (lanes 9 to 12). Similarly, an in vitro-synthesized RNA transcript containing the first 16 nt of the 20-mer used in lanes 1 to 12 was annealed to pGEM3ZF(+) DNA (lanes 17 to 20) or the RNA transcript used in lanes 9 to 12 (lanes 21 to 24). The RNA-DNA helicase substrate in lanes 13 to 16 is unique and contains a 12-nt 5' extension. The helicase substrates are diagrammed and use thickened lines to indicate RNA (not to scale). The DNA and RNA oligonucleotide length and 5' and 3' termini are indicated. The reaction mixtures in lanes 1, 5, 9, 13, 17, and 21 were heat denatured prior to gel electrophoresis.

gonucleotide, and for comparison, with a 47-nt DNA oligonucleotide. As seen in Fig. 7B, the His-A18R protein appeared to shift less of the input RNA compared with the DNA oligonucleotide in reactions that occurred independently of ATP and MgCl₂ (lanes 2, 4, 6, and 8). While the data are not quantitative, this experiment suggests that the mobility shift conditions used in this experiment permitted the formation of a significantly more stable His-A18R protein interaction with DNA than with RNA.

DISCUSSION

Identification of the vaccinia virus-encoded proteins required for viral DNA replication, recombination, repair, and

transcription is not complete, but by analogy with known viral, eukaryotic, and prokaryotic systems, helicases, which catalyze the vectorial unwinding of DNA and RNA, will play a fundamental role in these processes (reviewed in references 32 and 35). Sequence homology has identified several putative vaccinia virus-encoded NTPases and helicases (19, 28), and although numerous vaccinia virus proteins with NTPase activity have been identified, only the product of the I8R gene has been confirmed as an RNA helicase (52, 53). We have presented here evidence that the gene product of the vaccinia virus open reading frame A18R, in addition to having DNA-dependent ATPase activity, is a DNA helicase capable of unwinding a DNA-DNA duplex in a 3'-to-5' direction.

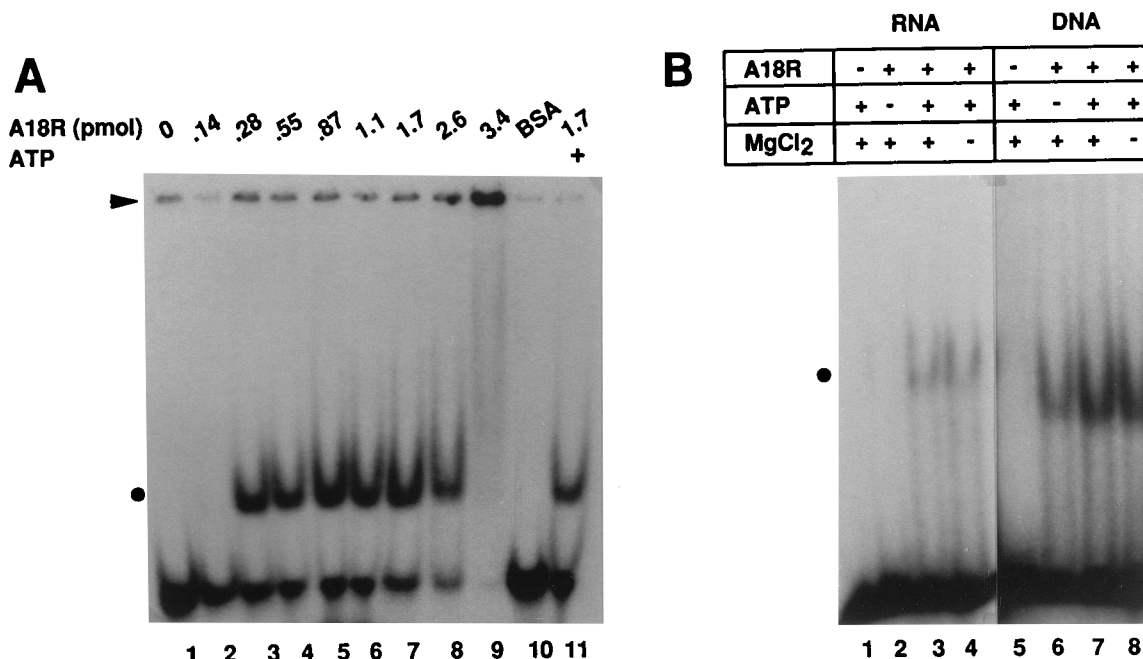


FIG. 7. Electrophoretic mobility shift analysis of the His-A18R interaction with DNA and RNA oligonucleotides. Increasing amounts of His-A18R protein were incubated with 12.5 fmol of a 5'-³²P-radiolabeled 68-nt oligonucleotide (Fig. 5, lanes 10 to 12) at 37°C for 15 min in buffer containing 40 mM Tris (pH 8), 50 mM KCl, 4 mM MgCl₂, 2 mM dithiothreitol, and 10% glycerol (A). Reactions were analyzed by 6% PAGE (0.25× Tris-borate EDTA) with recirculation of the buffer. The His-A18R mobility-shifted DNA is indicated by a solid circle, and the origin of sample loading is indicated by an arrowhead. In lane 10, the His-A18R protein was replaced with 1.5 pmol of bovine serum albumin (BSA). The reaction mixture in lane 11 contained 1.7 pmol of His-A18R protein and was supplemented with 4 mM ATP (indicated by a +). (B) His-A18R protein (1.0 pmol) was incubated with an in vitro-synthesized 46-nt ³²P-radiolabeled RNA oligonucleotide (lanes 1 to 4) or a 5'-³²P-radiolabeled 47-nt oligonucleotide (lanes 5 to 8) for 15 min at 37°C. The reaction conditions and analysis were as described for panel A, with the omission or addition of 4 mM MgCl₂ and 4 mM ATP as indicated. The autoradiographic exposure time of the DNA substrate was increased to compensate for the greater number of radiolabeled nucleotides in the RNA transcript.

The function of the A18R protein during vaccinia virus infection is not precisely known. Previous data obtained from experiments with a vaccinia virus expressing a thermolabile A18R protein strongly suggested the A18R protein was directly involved in the early and late phases of vaccinia virus transcription (4, 39, 40, 54). However, despite rigorous biochemical characterization of poxvirus transcription in vitro, evidence for involvement of A18R protein is lacking. This conundrum can be explained by three hypotheses.

First, the A18R protein interaction with the RNA polymerase (complex) is a transient event not detected by current biochemical approaches.

Second, the A18R protein is not essential in the assays currently used for identifying vaccinia virus transcription factors. As an example, the mammalian transcription factor TFIIF, which contains the ERCC3 helicase, the closest cellular homolog to A18R, is recruited to the polymerase complex to function during transcription at an intermediate step, termed "promoter clearance," that occurs between formation of the promoter open complex and transcription elongation (18). Interestingly, ERCC3 is not required for in vitro transcription of supercoiled DNA templates but is essential for transcription of linearized templates (18, 41). Analysis of vaccinia virus transcription factors in vitro has typically utilized supercoiled DNA templates, which may have overlooked a requirement for A18R protein in transcription. In comparisons of transcription from linear DNA and supercoiled DNA in a vaccinia virus-infected cell extract, one study showed that supercoiled and linear templates were transcribed with equal efficiency (62) while another showed that linearized templates were transcribed three- to fivefold less efficiently (48). The

difference in transcription activity in the latter study may represent a deficiency of a specific transcription factor(s). Likewise, vaccinia virus in vitro transcription systems prepared in a fashion that primarily assays initiation may not be sensitive to activities in elongation or termination. We conclude that involvement of the A18R protein in vaccinia virus transcription initiation, elongation, or termination cannot be ruled out.

Third, the data do not eliminate the possibility that the defect in transcription is due to the participation of A18R enzyme in vaccinia virus DNA replication, repair, or recombination. There is ample precedent in prokaryotes and eukaryotes of proteins participating in both transcription and DNA repair, including factor TFIIF/ERCC3 (7, 16, 23, 49). In the case of the TFIIF factor, the core complex of five polypeptides is modified with different proteins for activity in transcription or DNA repair (56). Although the evidence for a vaccinia virus DNA repair system is limited, vaccinia virus can repair its DNA during infection of cells deficient in DNA repair (27), and vaccinia virus-encoded proteins have been identified that could function in a vaccinia virus DNA NER pathway, including a uracil *N*-glycosylase (55), DNA ligase (26), and DNases that could serve as an apyrimidinic-apurinic endonuclease (58). During NER, a short oligonucleotide containing the DNA damage is excised (12 to 13 nt in prokaryotes, 27 to 29 nt in eukaryotes), the oligonucleotide is removed by a helicase, and the resulting gap is filled in and ligated to complete the repair process (46). The observation that the A18R helicase activity was unable to unwind DNA duplexes greater than 20 to 22 nt in length implicates A18R involvement in an analogous vaccinia virus NER pathway. It is also possible that the limited A18R helicase processivity observed in vitro may be a minimal

estimate of its activity, because it could be stimulated during infection to unwind longer DNA duplexes by additional viral or cellular proteins that were lost during the purification procedure. In this regard, the addition of SSBs to the helicase reaction neither stimulated nor inhibited A18R helicase activity. Clearly, identifying proteins that interact with the A18R enzyme during infection will provide insights into vaccinia virus RNA and DNA metabolism.

The data presented here permit us to address a previous hypothesis that the A18R protein, acting as an RNA-RNA helicase, inhibits activation of the cellular antiviral 2-5A pathway by unwinding the dsRNA duplexes formed as a normal consequence of vaccinia virus infection (28). We believe this is unlikely, because the His-A18R ATPase activity was not stimulated by ss- or dsRNA and the His-A18R helicase failed to unwind the short RNA-RNA duplexes used in our *in vitro* assay. Therefore, without invoking the presence of additional viral or cellular proteins responsible for mediating a change in A18R protein specificity *in vivo*, it is unlikely the A18R protein functions directly in abrogating the effects of the 2-5A pathway.

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