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Vaccinia Virus RNA Helicase: Nucleic Acid Specificity in Duplex Unwinding

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Vaccinia virus RNA helicase (NPH-II) catalyzes nucleoside triphosphate-dependent unwinding of duplex RNAs containing a single-stranded 3′ RNA tail. In this study, we examine the structural features of the nucleic acid substrate that are important for helicase activity. Strand displacement was affected by the length of the 3′ tail. Whereas NPH-II efficiently unwound double-stranded RNA substrates with 19- or 11-nucleotide (nt) 3′ tails, shortening the 3′ tail to 4 nt reduced unwinding by an order of magnitude. Processivity of the helicase was inferred from its ability to unwind a tailed RNA substrate containing a 96-bp duplex region. NPH-II exhibited profound asymmetry in displacing hybrid duplexes composed of DNA and RNA strands. A 34-bp RNA-DNA hybrid with a 19-nt 3′ RNA tail was unwound catalytically, whereas a 34-bp DNA-RNA hybrid containing a 19-nt 3′ DNA tail was 2 orders of magnitude less effective as a helicase substrate. NPH-II was incapable of displacing a 34-bp double-stranded DNA substrate of identical sequence. 3′-Tailed DNA molecules with 24- or 19-bp duplex regions were also inert as helicase substrates. On the basis of current models for RNA-DNA hybrid structures, we suggest the following explanation for these findings. (i) Unwinding of duplex nucleic acids by NPH-II is optimal when the polynucleotide strand of the duplex along which the enzyme translocates has adopted an A-form secondary structure, and (ii) a B-form secondary structure impedes protein translocation through DNA duplexes.

Vaccinia virus encodes an essential RNA helicase that catalyzes unidirectional unwinding of 3′-tailed duplex RNAs (12, 13). The vaccinia virus helicase, known originally as nucleoside triphosphate (NTP) phosphohydrolase-II, or NPH-II (8, 9), is a member of the DExH family of RNA-dependent NTPases (5, 11, 12). NPH-II affords an excellent model system for biochemical analysis of a DExH-type RNA helicase. The enzyme acts catalytically, unwinding a molar excess of 3′-tailed RNA duplexes in the presence of any NTP and a divalent cation cofactor (3, 12, 13). NPH-II forms a stable complex with RNA single strands in the absence of nucleotide. It has been suggested that NTP hydrolysis by NPH-II bound to an RNA tail drives translocation of the protein in a 3′ to 5′ direction along the RNA strand and that the enzyme, once engaged, can readily translocate through duplex RNA regions. Remarkably, NPH-II was found to be unable to unwind a 3′-tailed duplex DNA, even though single-stranded DNA (ssDNA) is a perfectly effective cofactor for NTP hydrolysis and the enzyme readily bound to the double-stranded DNA (dsDNA) helicase substrate (13). To explain this finding, it was suggested that energy utilization by DNA-bound enzyme would lead either to dissociation without extensive protein movement or to arrest of translocation by the DNA duplex.

Several issues arising from this hypothesis are addressed in the present study, including (i) the requirement for the 3′ tail, (ii) processivity of unwinding, and (iii) the specificity of the helicase for DNA versus RNA strands. We performed these experiments with recombinant NPH-II that was either expressed in vaccinia virus-infected BSC40 cells under the control of a T7 RNA polymerase promoter as described previously (3) or expressed in baculovirus-infected Sf9 cells under the control of a polyhedrin promoter (Fig. 1). An N-terminal His tag permitted purification of the protein from cell extracts by Ni-agarose chromatography, as described previously (3). NPH-II from Sf9 cells was nearly homogeneous after adsorption to Ni-agarose and elution by 0.5 M imidazole (Fig. 1). Levels of NPH-II are specified as RNA binding units; one unit of NPH-II binds 1 fmol of 98-mer ssRNA (3). Results obtained with baculovirus-expressed NPH-II regarding nucleic acid strand specificity were confirmed by using the vaccinia virus-expressed protein (3a).

Effect of 3′ single-stranded tail length on helicase activity. RNA unwinding by NPH-II from vaccinia virus cores requires a 3′ single-stranded tail (13). The effect of 3′ tail length on helicase activity was examined by comparing the efficacy of three dsRNA substrates that differed in the length of the 3′ tail. The substrates consisted of a 34-bp duplex region with two 5′ tails of 19 and 11 nucleotides (nt) and either a 4, 11, or 19-nt 3′ tail (Fig. 2). In the experiment shown in Fig. 2, 25 fmol of the indicated substrate was incubated with 1.4, 2.8, or 14 fmol of recombinant NPH-II (as RNA binding units). The extent of RNA unwinding clearly decreased as the 3′ tail was shortened, with the sharpest decrement occurring at the transition from 11 to 4 nt. In the case of the 3′ 19-nt substrate, NPH-II displaced at least 16 molecules of RNA per RNA binding unit in a 15-min reaction. This is an underestimate, as the reaction was saturated at the lowest level of enzyme tested (Fig. 2). The 3′ 11-nt substrate was unwound to a lesser extent, with eight molecules of RNA unwound per RNA binding unit at the lowest level of protein tested. The 4-nt 3′-tailed substrate was unwound only at higher levels of input protein, such that NPH-II displaced 0.5 RNA molecules per RNA binding unit in 15 min (Fig. 2). Longer time points were not examined.

The apparent loss of helicase activity as the 3′ tail was shortened most likely reflects a length requirement for the binding of NPH-II to ssRNA. We suspect that the two 5′ single-stranded tails (19 and 11 nt long) in each substrate tested are competitors for protein binding to the 3′ tail. The existence of *cis*-competitor strands may account for the observation that unwinding of the 3′ 11-mer tailed substrate was neither quantitative nor strictly linear with respect to input

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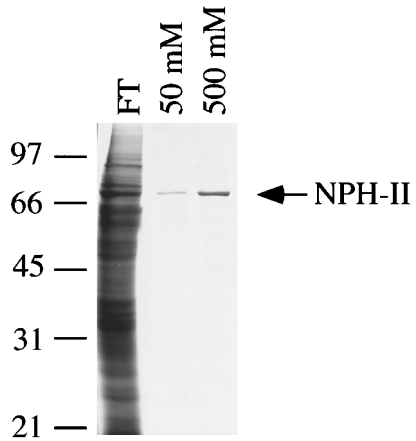

FIG. 1. Baculovirus His-NPH-II expression and purification. The His-10-NPH-II gene (3) was cloned into the baculovirus transfer vector pBacPAK-9 (Clonetech) so as to place the NPH-II gene under the control of the baculovirus polyhedrin promoter. A recombinant baculovirus (vCG-1) expressing the His-10-NPH-II protein was constructed and selected according to protocols supplied by Clonetech. Thirty Sf9 cell monolayers (150-cm^2 dishes) grown at 27°C were infected with vCG-1 at a multiplicity of 10. Cells were harvested at 72 h postinfection. Subsequent purification steps were performed at 4°C. A soluble whole-cell extract (3) was mixed with 1.5 ml of Ni-NTA-agarose resin (Qiagen) that had been equilibrated with buffer C (20 mM Tris HCl [pH 8.0], 300 mM NaCl, 10% glycerol, 0.1% Triton X-100). The resin was recovered by centrifugation, washed four times with buffer C (13 ml per wash), and then eluted sequentially with 10, 50, and 500 mM imidazole in buffer C. The polypeptide composition of the material that did not bind to Ni-agarose (FT) and the 50 and 500 mM imidazole eluate fractions was analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. A Coomassie blue-stained gel is shown. The majority of NPH-II protein was recovered as a single homogeneous polypeptide in the 500 mM imidazole eluate (arrow). Immunoblotting with anti-NPH-II antiserum confirmed that this polypeptide was indeed NPH-II (3a). Prior to use in enzyme assays, the 0.5 M imidazole fraction was equilibrated against buffer A (50 mM Tris HCl [pH 8.0], 2 mM dithiothreitol, 1 mM EDTA, 10% glycerol, 0.1% Triton X-100) by repeated centrifugal concentration steps by using a Centricon-30 apparatus (Amicon).

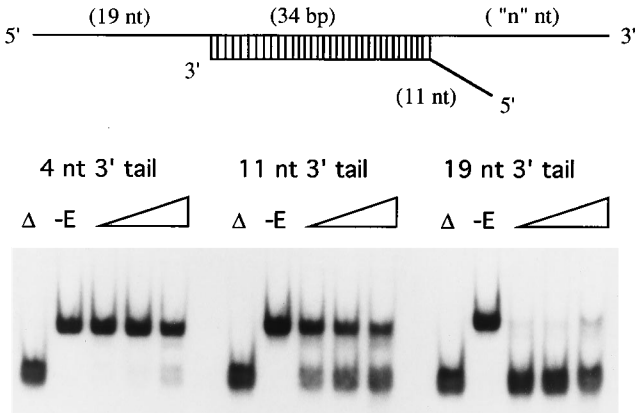

FIG. 2. Effect of 3′ single-stranded tail length on helicase activity. The helicase substrates consisted of a 34-bp duplex region with two 5′ tails of 19 and 11 nt and varied only by the lengths of their 3′ tails ("n" nt). The [α-^{32}P]GMP-labeled 45-mer bottom strand (5′-GGGCGAAUUGGAUCGAAUUCCUGCAGCCCGGGGGAUCCACUAGUU-3′) was transcribed by T7 RNA polymerase at high specific radioactivity from a modified pBluescript template. The longer top strand containing the 3′ tail was labeled at 300-fold lower specific activity. The top strand with the 19-nt 3′ tail (5′-GGGAACAAAAGCUGGCUAGAACUAGUGGAUCCCCCGGGCUGCAGGAAUUCGAUAUCAAGCUUAUCGAUACCG-3′) was synthesized by T3 RNA polymerase from a modified pBluescript template. The duplex region is underlined. The top strands used to construct the 11- and 4-nt 3′-tailed dsRNAs were transcribed from the same plasmid that had been linearized closer to the transcription start site. Annealing of the RNA strands and gel purification of the tailed duplexes were performed as described previously (3, 6). Helicase reaction mixtures (20 μl) contained 40 mM Tris HCl (pH 8.0), 2 mM dithiothreitol, 1 mM $MgCl_2$, 1 mM ATP, 25 fmol of each substrate, and either 1.4, 2.8, or 14 fmol (as RNA binding units) of NPH-II (glycerol gradient fraction from vaccinia virus-infected BSC40 cells [3]). Enzyme was omitted from control reactions (−E). After incubation for 15 min at 37°C, reactions were halted by the addition of 5 μl of 0.1 M Tris HCl (pH 7.4), 5 mM EDTA, 0.5% sodium dodecyl sulfate, 50% glycerol, 0.1% xylene cyanol, and 0.1% bromophenol blue. The samples were electrophoresed through an 8% polyacrylamide gel containing 0.5× TBE (3). Labeled RNAs were visualized by autoradiographic exposure of the dried gel. A sample containing RNA substrate that was denatured by heating at 95°C for 5 min followed by cooling on ice is shown in lane Δ.

enzyme. The present results showing that helicase activity is acutely dependent on 3′ tail length are consistent with earlier studies demonstrating that affinity for polynucleotides, as measured by activation of the ATPase, was influenced strongly by chain length (13).

Unwinding of a 96-bp RNA duplex. The vaccinia virus helicase readily unwound duplex regions of 34 bp (Fig. 2). To determine whether the enzyme was capable of unwinding a longer dsRNA, we constructed a 96-bp RNA substrate with a 3′ 113-nt tail (Fig. 3). NPH-II unwound this RNA quantitatively; at least 15 RNA molecules were unwound per RNA binding unit during the 15-min reaction. Thus, we inferred that the helicase could unwind processively.

Nucleic Acid Substrate Specificity. NPH-II purified from virions was unable to displace a 3′-tailed dsDNA substrate (13). To understand the basis for discrimination between DNA and RNA strands in the unwinding reaction, we tested helicase activity on RNA-DNA hybrid molecules (Fig. 4). Substrates were constructed from DNA oligonucleotides prepared by automated synthesis and RNAs transcribed in vitro by bacteriophage RNA polymerase. The hybrids, formed by annealing a 72-mer top strand to a 45-mer bottom strand, contained a 34-bp duplex region and single-stranded tails, as shown in Fig. 4. The 45-mer bottom strand was radiolabeled in each case. Unwinding reaction mixtures contained 25 fmol of duplex nucleic acid substrate and various amounts of NPH-II. An RNA-DNA hybrid with a 3′ RNA tail was unwound readily. The extent of displacement varied linearly with input enzyme (Fig. 4). We estimated that 30 fmol of RNA-DNA hybrid was unwound per fmol of NPH-II. A DNA-RNA hybrid with a 3′ DNA tail was also unwound, albeit much less efficiently than the RNA-DNA hybrid (Fig. 4). We estimated that about 0.2 fmol of DNA-RNA hybrid was unwound per unit of NPH-II; hence, the specific helicase activity with the DNA-RNA hybrid was 2 orders of magnitude lower than that with the RNA-DNA hybrid substrate. A 3′-tailed dsDNA substrate was inert, even at stoichiometric levels of input NPH-II (Fig. 4). Note that the sequences of the component strands were the same in the RNA-DNA, DNA-RNA, and DNA-DNA substrates.

NPH-II bound stably to the RNA-DNA helicase substrate in the absence of an NTP cofactor. Three discrete complexes were detected and resolved from free substrate by native gel electrophoresis (Fig. 5). Their evolution as a function of input protein was consistent with sequential recruitment of one, two, or three NPH-II molecules to the tailed duplex, presumably via binding to the three single-stranded tails. Complexes of similar mobility were formed when NPH-II was incubated with the DNA-RNA hybrid with the 3′ DNA tail, which was unwound with low efficiency in the presence of NTP, and with the DNA-DNA duplex that was not unwound at all (Fig. 5). A plot of the extent of ligand binding as a function of input protein indicated that NPH-II bound to each duplex with similar affinity (Fig. 4). Hence, the observed nucleic acid strand specificity of the helicase activity reflected discrimination at the level of duplex unwinding, not substrate binding.

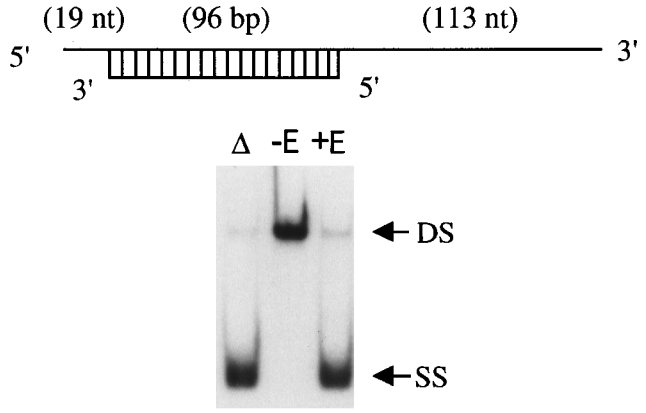

FIG. 3. Unwinding of a 96-bp RNA duplex. The helicase substrate contained a 96-bp duplex region and a 113-nt 3′ tail, as illustrated. The 228-nt RNA top strand was transcribed by T3 RNA polymerase from a modified pSKII(−) template linearized with *Pvu*II. The complementary 96-mer RNA bottom strand was transcribed by T7 RNA polymerase (and radiolabeled at high specific activity) from a pSKII(−) plasmid linearized with *Eag*I. (The sizes of the constituent RNAs were verified by denaturing gel electrophoresis.) The helicase reaction mixture contained 25 fmol of the substrate shown and 1.4 fmol (as RNA binding units) of NPH-II purified from BSC40 cells (+E). Enzyme was omitted from a control reaction (−E). A sample containing RNA substrate that was denatured by heating at 95°C for 5 min followed by cooling on ice is shown in lane Δ. The positions of the dsRNA substrate (DS) and released ssRNA (SS) are indicated (arrows).

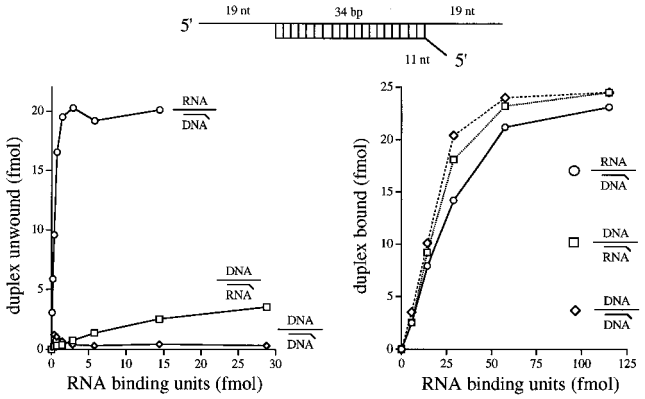

FIG. 4. Nucleic acid substrate specificity. The helicase substrates consisted of a 34-bp duplex region with 19- and 11-nt 5′ tails and a 19-nt 3′ tail, as illustrated. The sequences of the component strands were as described in the legend to Fig. 2. RNAs were transcribed in vitro by bacteriophage RNA polymerase. DNA strands of identical sequence (with T in place of U) were prepared by automated phosphoramidite chemistry. The 45-mer DNA strand was 5′ end labeled with [γ-^{32}P]ATP, using T4 polynucleotide kinase. (Left) Helicase reaction mixtures contained 40 mM Tris HCl (pH 8.0), 2 mM dithiothreitol (DTT), 2 mM $MgCl_2$, 2 mM ATP, 25 fmol of nucleic acid substrate (either RNA-DNA hybrid, DNA-RNA hybrid, or DNA-DNA duplex), and NPH-II purified from baculovirus-infected Sf9 cells. The reactions were halted after 15 min at 37°C, and the reaction products were resolved by gel electrophoresis. The extent of strand unwinding was quantitated by scanning the gel, using a Fuji BAS1000 PhosphorImager. The amount (femtomoles) of displaced 45-mer strand was plotted as a function of input enzyme (RNA binding units). (Right) Assays of NPH-II binding to the helicase substrates were performed in reaction mixtures (20 μl) containing 40 mM Tris HCl (pH 8.0), 2 mM DTT, 2 mM $MgCl_2$, 25 fmol of nucleic acid ligand (either RNA-DNA hybrid, DNA-RNA hybrid, or DNA-DNA duplex), and 5.8, 14, 29, 58, or 115 fmol of NPH-II. After incubation for 15 min at 37°C, the samples were adjusted to 8% glycerol and then electrophoresed through a native 8% polyacrylamide gel containing 0.25× TBE (13). Protein-RNA complexes were visualized by autoradiographic exposure of the dried gel (see Fig. 5). The extent of RNA-protein complex formation (bound RNA/total RNA) was quantitated by using a PhosphorImager and was plotted as a function of input NPH-II (RNA binding units).

Although the length of the duplex segment does not appear to be a limiting factor in detecting unwinding by NPH-II on duplex structures containing 3′ RNA tails, we considered that this parameter might constrain unwinding of DNA-DNA duplexes (14). Hence we tested helicase activity with a 24-bp DNA duplex containing a 29-nt 3′ tail; an RNA-DNA hybrid of identical sequence was tested in parallel as a positive control. Again, the RNA-DNA hybrid was unwound catalytically (22 fmol displaced per unit of NPH-II in the linear response range), whereas the DNA-DNA duplex was not unwound at all, even when NPH-II was added in molar excess over substrate (data not shown). NPH-II was also unable to unwind a DNA-DNA duplex when the duplex segment was shortened to 19 bp (data not shown). We surmise that purified NPH-II is unable to unwind duplex DNA.

The RNA-DNA hybrid structures that were so readily unwound by NPH-II contained a 5′ single-stranded tail on the bottom strand of the duplex. An otherwise identical hybrid substrate lacking the 5′ DNA tail was unwound just as well by NPH-II (data not shown). Thus, it was not necessary to have a preexisting fork structure at the presumptive point of entry of the helicase into the duplex segment. The potential role of the 5′ RNA tail on the top strand was tested by shifting the duplex segment to the very 5′ end of the 3′-tailed RNA strand. This structure was unwound catalytically, indicating that a 5′ RNA tail is not required for strand displacement (data not shown).

Conclusions and implications. The present study of the substrate requirements for duplex unwinding, together with earlier work, provides a working model for helicase action. A 3′ single-stranded tail is essential for the unwinding reaction. We speculate that the function of the tail is to recruit the enzyme to the helicase substrate such that it is poised on the proper side of the duplex segment to be unwound. Initial binding of NPH-II to the nucleic acid is neither dependent on, nor stimulated by, a nucleotide cofactor. The observed effects of 3′ tail length on helicase activity most likely reflect the chain length requirements for binding of enzyme to single-stranded nucleic acid.

The energy of NTP hydrolysis is hypothesized to drive translocation of the protein 3′ to 5′ along the strand to which it is initially bound. Note that unidirectional translocation is merely inferred from the polarity of unwinding (13). The helicase assay only registers events in which protein translocation is unimpeded by duplex structure. Thus, a molecule to which the enzyme binds may score as a poor helicase substrate either

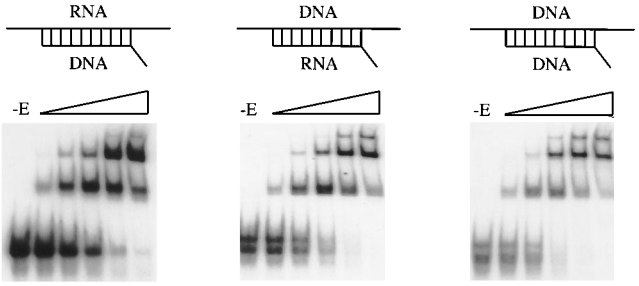

FIG. 5. Binding of NPH-II to tailed duplex nucleic acids. Complexes formed between NPH-II and radiolabeled RNA-DNA hybrid (left panel), DNA-RNA hybrid (middle panel), or DNA-DNA duplex substrates (right panel) were resolved by native gel electrophoresis and visualized by autoradiography. Binding reactions contained 25 fmol of nucleic acid ligand and either 5.8, 14, 29, 58, or 115 fmol of NPH-II (from left to right in each titration series). Enzyme was omitted from control reactions (−E).

because NPH-II does not translocate adequately along the 3′ tail or because the enzyme does not continue moving into or through the duplex region. We have shown that the vaccinia virus helicase can unwind through at least 96 bp of RNA duplex. The enzyme could also displace a dsRNA substrate containing a 189-bp duplex, albeit at very low efficiency (3a). We suspect that the apparent decrement in unwinding with the 189-bp duplex is caused by reannealing of the displaced RNA strands behind the translocating enzyme molecule; propagation of an RNA bubble under these circumstances would not score in the unwinding assay.

The vaccinia virus helicase is unable to unwind a 3′-tailed dsDNA molecule, even though the protein binds to the substrate and can be activated to hydrolyze ATP by ssDNA (3, 8, 13). The earlier suggestion that ATP hydrolysis by DNA-bound enzyme might lead to protein dissociation without translocation has been vitiated by the present finding that a 3′-tailed DNA-RNA hybrid can be unwound, albeit much less efficiently than a dsRNA molecule. In such a substrate, the enzyme is recruited to the 3′ DNA tail and, according to our hypothesis, translocates along the DNA strand through a 34-bp DNA-RNA duplex in order to score in the assay. Because this cannot be achieved when the same 3′-tailed strand is hybridized to DNA, we conclude that NPH-II is impeded from moving into or through the DNA duplex. It is not simply the case that NPH-II cannot displace a DNA strand. Indeed a DNA strand can be displaced very well when it is base paired with a 3′-tailed RNA. These experiments suggest that translocation of NPH-II may be differentially affected by the helical form of the nucleic acid duplex.

Structural studies of DNA-RNA hybrids by X-ray fiber diffraction and nuclear magnetic resonance methods indicate that these molecules do not assume a B-form conformation typical of duplex DNA (1, 10). Although DNA-RNA hybrids have an A-form character, their structure does differ from that of pure A-form dsRNA. Remarkably, the RNA and DNA chains adopt quite different helical conformations within the DNA-RNA hybrid, with the RNA strand retaining the A-form helical conformation (as found in dsRNA), whereas the DNA strand adopts a conformation that is neither strictly A nor B, but is instead intermediate in character between these two forms (10). This structure, which has been termed a heteromerous duplex (1), engenders a plausible model for the strand specificity of NPH-II as a 3′ to 5′ helicase. We suggest that functional asymmetry, whereby an RNA-DNA hybrid is unwound much better than a DNA-RNA hybrid, is a consequence of the structural asymmetry of these molecules, i.e., by positing that NPH-II will translocate most effectively through a duplex region when the strand along which it is moving is in an A-form helical conformation. This is the case when the 3′-tailed strand is RNA and the duplex region is either dsRNA or an RNA-DNA hybrid. However, when the 3′-tailed strand is DNA and the duplex is a DNA-RNA hybrid, then the strand along which the helicase moves will adopt an intermediate conformation, which we propose is less favorable for helicase movement. We further suggest that NPH-II will not translocate through a duplex when the strand along which it is moving is a B-form helix, as will be the case in a 3′-tailed DNA-DNA molecule.

The nucleic acid specificity for duplex unwinding by NPH-II is very similar to that of two other DExH box proteins: human RNA helicase A (6, 7) and pestivirus NS3 protein (15). Helicase A and NS3 both act unidirectionally (3′ to 5′) to displace RNA-RNA and RNA-DNA duplexes with a 3′ RNA tail, and both are inactive on duplex DNAs. Helicase A and NS3 were found not to unwind a DNA-RNA hybrid with a 3′ DNA tail; however, unwinding of this molecule at low efficiency (such as we observed for NPH-II) might not have been appreciated by performing the substrate specificity assays at a single level of input enzyme (6, 15). Nuclear helicase II, which was purified from calf thymus on the basis of its DNA unwinding activity, also has RNA helicase activity (16). The cDNA sequence obtained via reverse genetics from peptide sequence reveals that helicase II is the bovine equivalent of human helicase A (92% identity; 96% similarity) (17). Because the RNA and DNA unwinding activities of the nuclear helicase II preparation from calf thymus displayed differential sensitivities to salt (16), a definitive conclusion that both reactions are catalyzed by the same polypeptide must await characterization of the recombinant helicase II protein. We note that the inability of recombinant vaccinia virus NPH-II to unwind dsDNA is in full agreement with the properties of native NPH-II purified from vaccinia virions (13).

The new finding that NPH-II unwinds RNA-DNA hybrid molecules has implications for the functions that NPH-II might perform during early mRNA synthesis within the vaccinia virus core. Specifically, NPH-II might facilitate transcription elongation by preventing R-loop formation behind the elongating RNA polymerase. R-loop formation is favored by the negative superhelicity generated during transcription of divergently oriented genes (2), as is the case with early transcription by vaccinia virions. Note that disruption of R-loops by NPH-II could occur by either of two pathways, involving (i) NPH-II movement in a 3′ to 5′ polarity along the nascent RNA strand or (ii) NPH-II movement in a 3′ to 5′ direction along the template strand of the DNA. In the former case, the helicase is moving away from the elongating polymerase, whereas in the latter model the helicase moves in the same direction as the transcription elongation complex. The observation that purified NPH-II unwinds hybrids more effectively when moving along the RNA strand might favor the first pathway. However, the rate of elongation of vaccinia virus RNA polymerase (20 to 50 nt/s [4]) may be slow enough to allow disruption of R-loops by NPH-II moving along the DNA template strand. The lack of DNA helicase activity argues that NPH-II is not likely to unwind the DNA template during transcription. Of course, our data do not exclude the existence of a separate viral protein that might confer on NPH-II the ability to displace duplex DNA. An earlier suggestion that NPH-II might facilitate the extrusion of newly completed mRNA chains out of the virion core by acting as an energy-coupled RNA transporter (12, 13) is not impacted by the present findings.

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