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The QRxGRxGRxxxG Motif of the Vaccinia Virus DExH Box RNA Helicase NPH-II Is Required for ATP Hydrolysis and RNA Unwinding but Not for RNA Binding

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Vaccinia virus NPH-II is an essential nucleic acid-dependent nucleoside triphosphatase that catalyzes unidirectional unwinding of duplex RNA containing a 3′ tail. NPH-II is the prototypal RNA helicase of the DExH box protein family, which is defined by several shared sequence motifs. The contribution of the conserved QRKGRVGRVNPG region to enzyme activity was assessed by alanine-scanning mutagenesis. Ten mutated versions of NPH-II were expressed in vaccinia virus-infected BSC40 cells and purified by nickel affinity chromatography and glycerol gradient sedimentation. The mutated proteins were characterized with respect to RNA helicase, nucleic acid-dependent ATPase, and RNA binding functions. Individual alanine substitutions at invariant residues Q-491, G-494, R-495, G-497, R-498, and G-502 caused severe defects in RNA unwinding that correlated with reduced rates of ATP hydrolysis. None of these mutations affected the binding of NPH-II to single-strand RNA or to the tailed duplex RNA used as a helicase substrate. Mutation of the strictly conserved position R-492 inhibited ATPase and helicase activities and also caused a modest decrement in RNA binding. Alanine mutations at the nonconserved position N-500 and the weakly conserved residue P-501 had no apparent effect on any activity associated with NPH-II, whereas a mutation at the weakly conserved position K-493 reduced helicase to one-third and ATPase to two-thirds of the activity of wild-type enzyme without affecting RNA binding. We conclude that the QRxGRxGRxxxG motif of this DExH helicase is required for ATP hydrolysis and RNA unwinding but not for RNA binding. Because mutations in the HRxGRxxR motif of the prototypal DEAD box RNA helicase eIF-4A abolish or severely inhibit RNA binding, we surmise that the contribution of conserved helicase motifs to overall protein function is context dependent.

Vaccinia virus encodes an essential RNA helicase that catalyzes unidirectional unwinding of 3′-tailed duplex RNAs (25, 26). The vaccinia virus enzyme, known previously as nucleoside triphosphate phosphohydrolase-II, or NPH-II (17, 18), is a member of the DExH family of RNA-dependent nucleoside triphosphatases (NTPases) (10, 25). The DExH family is defined by six conserved motifs arrayed in a collinear fashion (6, 23) (for four of these motifs, see Fig. 1). The size of the DExH family is expanding rapidly as the signature elements are encountered in newly cloned genes and during genome-sequencing efforts. Typically, the products of these genes are designated as putative helicases in the absence of supporting biochemical data. Although DNA-dependent or RNA-dependent NTPase activity has been demonstrated for many DExH proteins, relatively few have actually been shown to unwind duplex DNA or RNA.

Vaccinia virus NPH-II, which was the first RNA-dependent ATPase to be characterized (17, 18), can be regarded as the prototype of the DExH-type RNA helicases. DExH family members can be classified on the basis of their degree of similarity to NPH-II in the immediate vicinity of the four conserved motifs shown in Fig. 1. The NPH-II-like subgroup includes human helicase A, an RNA-specific helicase with 3′-to-5′ directionality that is biochemically very similar to the vaccinia virus enzyme (13, 14). Related proteins include the yeast splicing factors PRP2 and PRP16, which are RNA-dependent NTPases (9, 24). DExH proteins that lack one or more residues that are strictly conserved in the NPH-II-like subfamily are grouped separately. The relevant residues include the Gly, Gln, and Lys-Gln positions flanking the GxGKT box, the Glu and Asp moieties just downstream of the DExH box, and various constituents of the QRxGRxGRxxxG motif (see Fig. 1). The non-NPH-II-like group includes three vaccinia virus-encoded DNA-dependent ATPases (2, 3, 18), the bacterial RecG and RecQ DNA helicases (15, 28), the yeast RAD3 DNA helicase (27), the potyvirus C1 RNA helicase (12), and the bovine viral diarrhea virus NS3 RNA helicase (29). The DExH family members are more closely related to each other within these four protein segments than they are to the eponymous DEAD box RNA helicase eIF-4A (see Fig. 1).

Membership in the DExH or DEAD box family does not guarantee an associated helicase activity. Furthermore, even among the family members that are helicases, motif conservation has no clear predictive value regarding the key properties of the helicase, e.g., directionality of unwinding, specificity for DNA versus RNA, etc. Insights into the function of the conserved motifs have come primarily from mutational analyses of representative family members. Among the RNA helicases, eIF-4A, the prototypal DEAD box protein, has been mutagenized extensively (19–22). Sonenberg et al. have shown clearly that mutations within the GKT box, the DEAD box, and the HRxGRxxR motif can impair RNA unwinding by eIF-4A by inhibiting ATP binding, ATP hydrolysis, and/or RNA binding (19, 20).

We are pursuing a structure-function analysis of vaccinia virus NPH-II, the prototypal DExH box RNA helicase. We demonstrated previously the importance of the conserved GKT and DExH motifs in NTPase and helicase activities (7). Alanine substitutions for Lys in the GKT element or for Asp and Glu in the DExH box severely impaired ATP hydrolysis and RNA unwinding without affecting the binding of the mu-

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tated proteins to single-strand (ss) RNA. Changing the DExH box His residue to Ala resulted in a protein that was constitutively active for ATPase in the absence of a nucleic acid effector; this mutant did not unwind duplex RNA, even though RNA binding was unaffected (7). In the present study, we have targeted alanine substitution mutations to 10 residues within the QRKGRVGRVNPG motif, anticipating, on the basis of studies of eIF-4A, that this arginine-rich region might be involved in RNA binding. We demonstrate a role for the conserved amino acid side chains in ATP hydrolysis and RNA unwinding; however, unlike the case of eIF-4A (19), mutations in this region of NPH-II did not significantly impair RNA binding. Our findings illustrate that the contribution of individual motifs to overall protein function is context dependent.

MATERIALS AND METHODS

Alanine mutagenesis of His-NPH-II. Phagemid pTM-His_{10}-NPH-II containing a His-tagged NPH-II gene under the control of a T7 promoter and a picornavirus translational enhancer was described previously (7). For the present study, a unique *Sal*I site in the polylinker 3′ of the NPH-II gene was eliminated by restriction with *Sal*I and filling in with Klenow DNA polymerase; this maneuver facilitated the exchange of *Acc*I-*Xho*I fragments containing mutations of the QR KGRVGRVNPG region. Uracil-substituted single-strand pTM-His_{10}-NPH-II DNA was used as a template for oligonucleotide-directed mutagenesis (11). Mutagenic DNA primers were designed to create alanine substitutions at residues Q-491, R-492, K-493, G-494, R-495, G-497, R-498, N-500, P-501, and G-502 in the QRKGRVGRVNPG motif. The presence of the desired mutations was screened initially by the gain or loss of a restriction site and confirmed by DNA sequencing. An 877-bp *Acc*I-*Xho*I fragment containing the mutated region was exchanged for the corresponding wild-type fragment in the parent vector to generate a series of pTM-His-NPH-II(Ala) plasmids. The entire NPH-II 877-bp *Acc*I-*Xho*I fragment was sequenced in each case to ensure that no unwanted mutations were present in the expression vectors.

Expression and purification of His_{10}-NPH-II. Expression of wild-type His_{10}-NPH-II was achieved by coinfection of BSC40 cell monolayers with vaccinia viruses vTF3 and vHis_{10}-NPH at a multiplicity of infection of 10 for each virus (5, 7). Infected cells were incubated at 31°C for 24 h. Soluble cell lysates were prepared as described previously (7). The His_{10}-NPH-II protein was adsorbed to Ni-nitrilotriacetic acid agarose. After extensive washing of the resin with buffer containing 50 mM imidazole, His_{10}-NPH-II was recovered by elution with 500 mM imidazole.

Expression of each Ala-substituted His_{10}-NPH-II protein was achieved by transient transfection of vTF3-infected BSC40 cells with pTM-His-NPH-II(Ala) plasmids by using the electroporation procedure described previously (7). A control transfection was performed with the pTM1 vector (4). Cells were harvested 24 h after transfection and lysates were prepared as described previously (7). The His_{10}-NPH-II-Ala proteins were purified by adsorption to Ni-agarose and elution with 500 mM imidazole as was done for the wild-type protein.

Wild-type His_{10}-NPH-II and each His_{10}-NPH-II-Ala mutant protein were purified further by sedimentation of 0.3-ml aliquots of each Ni-agarose fraction through a 4.7-ml 15-to-30% glycerol gradient containing 0.5 M NaCl in buffer A (50 mM Tris HCl [pH 8.0], 2 mM dithiothreitol, 1 mM EDTA, 10% glycerol, 0.1% Triton X-100). The gradient was centrifuged for 20 h at 50,000 rpm in a Beckman SW50 rotor. Fractions (0.17 ml) were collected from the bottom of the tube.

The concentrations of NPH-II in the peak glycerol gradient fractions were determined by quantitative Western blotting (immunoblotting). The NPH-II standard used in this assay was expressed by using a recombinant baculovirus containing the NPH-II gene driven by the polyhedrin promoter and purified to homogeneity from virus-infected Sf9 cells (8). The concentration of the recombinant-baculovirus NPH-II was first determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) analysis of a twofold dilution series of the protein in parallel with known amounts of bovine serum albumin (BSA). The gel was stained with Coomassie blue, and the extent of dye binding to NPH-II and BSA was quantitated by densitometric scanning of the lanes. The concentration of NPH-II was calculated by extrapolation to the BSA standard curve. The NPH-II standard and serial dilutions of wild-type and mutant NPH-II proteins purified from BSC40 cells were then applied to a nitrocellulose membrane with a slot blot apparatus. The membrane was probed with anti-NPH-II rabbit serum, and the immunoreactive protein was visualized by the chemiluminescence method (ECL kit; Amersham). The autoradiographic signal was quantitated by densitometric scanning of the slot blot; for all proteins, the signal varied linearly with the volume of enzyme sample applied to the blot (not shown). The concentration of each NPH-II preparation was calculated by extrapolation to the recombinant-baculovirus NPH-II standard. All subsequent functional assays of wild-type and mutated enzymes were performed with equivalent amounts of immunoreactive NPH-II protein.

RNA helicase substrate. Radiolabeled standard double-strand RNA helicase substrate was prepared as described previously (7, 13). In brief, the component RNA strands were transcribed in vitro from linear plasmid DNA templates by using SP6 RNA polymerase. The 38-mer strand, 5′-GAAUACACG<u>GAAUUCG AGCUCGCCCGGGGAUCCUCUAG</u>, was radiolabeled to high specific activity with [α-^{32}P]GTP. The partially complementary 98-mer strand, 5′-GAAUACAA GCUUGGGCUGCAGGUCGACU<u>CUAGAGGAUCCCCGGGCGAG CUCGAAUUC</u>GGGUCUCCCUAUAGUGAGUCGUAUUAAUUUCGAU AAGCCAG, was labeled with [α-^{32}P]GTP at a 300-fold-lower specific activity. (The 29-bp duplex region is underlined.) The transcription reaction products were subjected to polyacrylamide-urea gel electrophoresis; radiolabeled transcripts were localized by autoradiography and recovered from an excised gel slice. Strand annealing was performed at a 3:1 molar ratio of 98-mer to 38-mer. The tailed RNA duplexes were then purified by native gel electrophoresis (7, 13).

Helicase assay. Reaction mixtures (20 μl) contained 40 mM Tris HCl (pH 8.0), 2 mM dithiothreitol, 1 mM $MgCl_2$, 1 mM ATP, and 25 fmol of [α-^{32}P]GMP-labeled standard double-strand RNA substrate. After incubation for 15 min at 37°C, the reactions were halted by addition of 5 μl of a solution containing 0.1 M Tris HCl (pH 7.4), 5 mM EDTA, 0.5% SDS, 50% glycerol, 0.1% xylene cyanol, and 0.1% bromophenol blue. Aliquots (20 μl) were electrophoresed at 15 mA of constant current through an 8% polyacrylamide gel containing 0.5× Tris-borate-EDTA. Labeled RNAs were visualized by autoradiographic exposure and by scanning the gel with a Fuji BAS1000 phosphorimager. The extent of unwinding (calculated as the amount of displaced RNA/total RNA) was quantitated with the phosphorimager.

Assay of RNA binding. Reaction mixtures (20 μl) contained 40 mM Tris HCl (pH 8.0), 2 mM dithiothreitol, 1 mM $MgCl_2$, and 25 fmol of [α-^{32}P]GMP-labeled 98-mer ssRNA (corresponding to the 98-mer strand of the helicase substrate and labeled to high specific activity). After incubation for 15 min at 37°C, samples were adjusted to 8% glycerol, and 20-μl aliquots were electrophoresed at 15 mA through a native 8% polyacrylamide gel containing 0.25× Tris-borate-EDTA. The extent of RNA-protein complex formation (calculated as the amount of bound RNA/total RNA) was quantitated with a phosphorimager.

RESULTS

Mutagenesis strategy. The QRKGRVGRVNPG region of vaccinia virus NPH-II was targeted for mutational analysis. This 12-amino-acid segment includes 7 residues that are strictly conserved among the 18 different proteins that we classify as NPH-II-like DExH family members (Fig. 1). The conserved positions are Gln-491, Arg-492, Gly-494, Arg-495, Gly-497, Arg-498, and Gly-502. Mutations were introduced into the NPH-II gene such that each of these residues was replaced by alanine. Alanine substitutions were also introduced at three other positions, Lys-493, Asn-500, and Pro-501, that are not well conserved among the DExH proteins. Because alanine substitution eliminates the side chain beyond the β-carbon, usually without perturbing global protein structure, this mutational approach provides an indication of the essentiality of the side chain for enzyme activity.

Expression and purification of wild-type and mutated NPH-II proteins. Wild-type NPH-II was expressed in cultured mammalian cells coinfected with a vaccinia virus (vTF-3) that expresses T7 RNA polymerase (4, 5) and a second recombinant virus that carries an inserted copy of the NPH-II gene under the control of a T7 promoter (7). Mutated NPH-II proteins were expressed by transfection of vTF-3-infected cells with T7-based plasmids carrying the NPH-II-Ala genes (7). We avoided using recombinant viruses bearing mutated NPH-II genes because of the potential for gene conversion of the mutant allele by the endogenous wild-type NPH-II gene. Each protein was expressed as a His-tagged derivative. The N-terminal histidine-rich leader peptide facilitated affinity purification based on adsorption of the tagged protein to an immobilized nickel resin. Affinity purification is critical for structure-function analysis, in which mutated versions of the helicase expressed under T7 control must be resolved from native NPH-II encoded by the endogenous viral gene and driven by vaccinia virus promoters. Note that we have already shown that the biochemical properties of the recombinant His-tagged

	GxTGxGKTSQxxK ST Q	DExHE D	TAT S	SK Q	QRxGRxGRxxxG ***** ** ***
NPH2*	VVLTGGTGVGKTSQVPKLLL	LFSYGTLIIDEVHEHDQIGDIIIAVAR	MFLMTATLE	ISKSMRDQRKGRVGRVNPGTYVYFY	
FPV	IVVTGSTGIGKTSQLPKVIM	LSNYNIIIVDEIHEHDRIADIIISVLR	LVLMSATLE	ISKSMMTQRKGRVGRVSKGIYVYFY	
HuHelA*	VIIRGATGCGKTTQVPQFIL	IRGISHVIVDEIHERDINTSFLLVVLR	IVFMSATID	ASKTNLEQRKGRAGRSTAGFCFHLC	
BoHel2*	VIIRGATGCGKTTQVPQFIL	IRGISHVIVDEIHERDINTDFLLVVLR	IVLMSATID	ASKTNLEQRKGRAGRVRPGFCFHLC	
PRP2	LIIMGETGSGKTTQLPQYLV	LSKYSCIMIDEAHERTLATDILIGLLK	LLISSATMN	CSRASVDQRAGRAGRVGPGKCFRIF	
PRP16	VVIIGETGSGKTTQLAQYLY	LDKYSCVIIDEAHERSLNTDILLGFFK	LIITSATMN	ISKANADQRSGRAGRTAPGTAYRLY	
PRP22	LVIVGETGSGKTTQITQYLD	MSKYSVIMLDEAHERTVATDVLFALLK	VIVTSATLN	ISQAQANQRKGRAGRTGPGKCYRLY	
MLE	VIIRGNTGCGKTTQIAQYIL	LRGVSHIIVDEIHERDVNSDFLLVILR	VILMSATID	ASKTNLEQRKGRAGRVRPGFCFTLC	
HRH1	LIVIGETGSGKTTQITQYLA	LTQYAIIMLDEAHERTIHTDVLFGLLK	LIVTSATLD	ISQAQAKQRAGRAGRTGPGKCYRLY	
hrpA	VIVAGETGSGKTTQLPKICM	LMQYDTIIIDEAHEHSLNIDFLLGYLK	IIITSATID	ISQASANQRKGRCGRVSEGICIRLY	
PRH1	IVVVGETGSGKSTQIPQFLN	LSQYHTLILDEAHERTLMTDMLLGFVK	VIIMSATLN	ISQSAAMQRSGRAGREAAGQCYRIY	
L9931.2	VLITGETGSGKSTQVVQFIL	MLENTIVVIDEVHERSIDTDLIVTLMK	IVLMSATVN	ISKAEVKQRRGRAGRVREGLSYKLF	
9553.04	VIICGETGSGKTTQVPQFLY	LTKYSSIIIDEAHERNINTDILIGMLS	LIIMSATLR	VSKASANQRSGRAGRTGPGHCYRLY	
YKL078w	TVLIGETGSGKSTQIPQFVL	LREYSVIVIDEAHERTVLTDLILGFLK	IIVMSATLQ	ISQASAMQRSGRAGRESEGKSFRLY	
K03H1.2	VIIVGETGSGKTTQLAQYLL	LDQYSAIIMDEAHERSLNTDVLFGLLR	LIVTSATMD	VSQASANQRTGRAGRTGPGQCYRLY	
F56D2	ITLVGETGSGKTTQIPQWAV	LDKYKVLILDEAHERTLATDILMGLIK	VVIMSATLD	ISKASAMQRAGRAGRTKPGKCFRLY	
6E1.10	TVVCGETGSGKTTQIPQFLY	LKKYSVILIDEAHERSMYSDVLIGMLS	LVIMSATLR	ISQASGDQRAGRAGRISAGHAYRLY	
EEED8.5	LVVVGETGSGKTTQMTQYAI	LSGYSLIMLDEAHERTIHTDVLFGLLK	LIITSATLD	ISQAAAKQRSGRAGRTGPGKCYRLY	
DmKurz	VIVAGETGSGKTTQLPQFLY	LSKYSVMILDEAHERSVYTDILVGLLS	LIIMSATGG	TSKASADQRAGRAGRISAGHCYRLY	
ASFV	LIIKSRTGSGKSTALPVHVF	ASRYAFMIIDEAHERALGIDLMLMYIK	VILTSATID	AAQSRIEQRKGRVGRVFPGHFYPLY	
BVDV*	KQITLATGAGKTTELPKAVI	MVEYSYIFLDEYHCATPEQLAIIGKIH	VVAMTATPA	VTVGEQAQRRGRVGRVKPGRYYRSQ	
PPV*	ILIRGAVGSGKSTGLPFHLS	LDNYKCIIFDECHVHDASAMAFRCLLS	ILKVSATPP	ISYGERIQRLGRVGRNKPGMALRIG	
UvrB*	QTLLGVTGSGKTFTIANVIA	LPADGLLVVDESHVTIPQIGGMYRGDR	TIYVSATPG	RSERSLIQTIGRAARNVNGKAILYG	
RecG*	RLVQGDVGSGKTLVAALAAL	FNGLALVIIDEQHRFGVHQRLALWEKG	QLIMTATPI	LGLAQLHQLRGRVGRGAVASHCVLL	
RecQ*	CLVVMPTGGGKSLCYQIPAL	HWNPVLLAVDEAHCISQWGHDFRPEYA	FMALTATAD	RNIESYYQETGRAGRDGLPAEAMLF	
SGS1	VFVLMPTGGGKSLCYQLPAV	DGKLARIVVDEAHCVSNWGHDFRPDYK	MIALTATAS	RTLEGYYQETGRAGRDGNYSYCITY	
RAD3*	SILEMPSGTGKTVSLLSLTI	VSKDSIVIFDEAHNIDNVCIESLSLDL	VIITSGTIS	DAMRHAAQCLGRVLRGKDDYGVMVL	
vvA18*	ITLHLACGFGKTITTCYLMA	NKHYDLFILDESHTYNLMNNTAVTRFL	CYFLTATPR	INNMQIEQLLGRVCRETELLDRTVY	
vvNPH1	LLLFHETGVGKTMTTVYILK	SKSRICVIIDECHNFISKSLIKEDGKI	MICLSATPI	WNEASLRQIVGRAIRLNSHVLTPPE	
vvD6	VLLFHIMGSGKTIIALLFAL	RYNNSIFIVDEAHNIFGNNTGELMTVI	FLLLSGSPI	DTFSQYNQILGRSIRKFSYADISEP	
EIF4A*	VIAQAQSGTGKTATFAISIL	PKYIKMFVLDEADEMLSRGFKDQIYDI	VVLLSATMP	TNRENYIHRIGRGGRFGRKGVAINM	

FIG. 1. Conserved sequence elements define the DExH protein family. Eighteen DExH proteins were classified as NPH-II-like on the basis of sequence conservation in the vicinity of four motifs that are shared among family members. The amino acid sequences of each protein are aligned immediately below the sequence of NPH-II. Inclusion in this subgroup is based on amino acid identity at the positions specified above the NPH-II sequence. Selected DExH proteins that diverge from the NPH-II-like consensus sequence at one or more positions are aligned as a separate subgroup. (The DExH box family includes many other proteins that are not shown in this alignment.) The homologous motifs of the DEAD box protein eIF-4A are shown below the DExH proteins. Amino acid residues in the QRxGRxGRxxxG motif of NPH-II that were targeted for alanine substitution are indicated by asterisks above the NPH-II sequence. The aligned polypeptides and their GenBank accession numbers are as follows: vaccinia virus NPH-II (M35027); fowlpox virus (FPV) homolog of NPH-II (G48563); human helicase A (HuHelA) (L13848); bovine helicase 2 (BoHel2) (X82829); *Saccharomyces cerevisiae* splicing factors PRP2 (X55936), PRP16 (M31524), and PRP22 (X58681); *Drosophila* MLE protein (M7412); human HRH1 (D50487); *Escherichia coli* HrpA (D42105); *Schizosaccharomyces pombe* PRH1 (D13249), *S. cerevisiae* reading frames L9931.2 (U20162), 9553.04 (Z48622), and YKL078w (Z28078); *Caenorhabdites elegans* reading frames K03H1.2 (Z29560), F56D2.6 (U13644), 6E1.10 (L16559, L18807), and EEED8.5 (U23484); *Drosophila* kurz protein (DmKurz) (S22609); African swine fever virus (ASFV) helicase-like protein (U18466); bovine viral diarrhea virus NS3 protein (BVDV) (L35851); plum pox potyvirus CI protein (PPV) (M92280); *E. coli* UvrB (X03678), RecG (M64367), and RecQ (M30198); yeast SGS1, a RecQ homolog (L07870); yeast RAD3 (K03293); vaccinia virus (vv) proteins A18, NPH-I, and D6 (M35027); mouse eIF-4A (X03040). Proteins with asterisks are those for which helicase activity has been demonstrated.

NPH-II are identical in every respect to the native enzyme purified from vaccinia virions (7).

The wild-type and mutant NPH-II proteins were purified by adsorption to Ni-agarose and elution with 0.5 M imidazole. The proteins were then purified further by glycerol gradient sedimentation. As a control, extracts of mock-transfected cells were taken through an identical purification procedure. The wild-type and all 10 His-NPH-II-Ala proteins sedimented as discrete peaks during glycerol gradient centrifugation, as indicated by Western blot analysis with anti-NPH-II serum and by RNA binding and ATPase assays of the gradient fractions (8). The peak gradient fractions from each of the enzyme preparations were used for biochemical studies of NPH-II activity. It was not possible to determine the protein concentration of the glycerol gradient preparations by using standard solution dye-binding assays. Hence, the concentration of His-NPH-II protein was gauged by quantitative Western blotting (described in Materials and Methods). Equivalent amounts of each protein preparation were then resolved by SDS-PAGE and probed by Western blotting; a single immunoreactive 70-kDa species corresponding to His-NPH-II was detected in each case (Fig. 2A). No immunoreactive protein was seen in the glycerol gradient fraction of the mock-transfected control preparation (Fig. 2A).

To assess overall purity, aliquots of each preparation containing an equivalent amount of immunoreactive NPH-II were resolved by SDS-PAGE and stained with Coomassie brilliant blue. A single 70-kDa polypeptide was detected that corresponded to His-NPH-II; similar amounts of this stained species were present in each lane (Fig. 2B). No polypeptides were seen in the mock-transfected control preparation (Fig. 2B).

Helicase activity of mutated His-NPH-II proteins. Helicase activity was tested by using the standard substrate, which consists of a 98-nucleotide RNA strand hybridized to a 38-nucleotide radiolabeled RNA strand to produce a tailed molecule with a 29-bp duplex region (7, 13). The amount of RNA unwinding by wild-type NPH-II was proportional to the level of input protein (Fig. 3). The titration profiles of the proteins with either an N-to-A mutation at position 500 (N500A) or P501A were identical to that of the wild type. In contrast, the other eight mutant proteins all displayed reduced specific activity in RNA unwinding, seen as a shift to the right in the titration curves and a more pronounced sigmoidicity at lower enzyme levels (Fig. 3). Of these, the K493A mutation elicited the mildest effect (reduction to about one-third of wild-type activity), whereas the remaining mutations reduced helicase activity by an order of magnitude or more. The Q491A mutation had

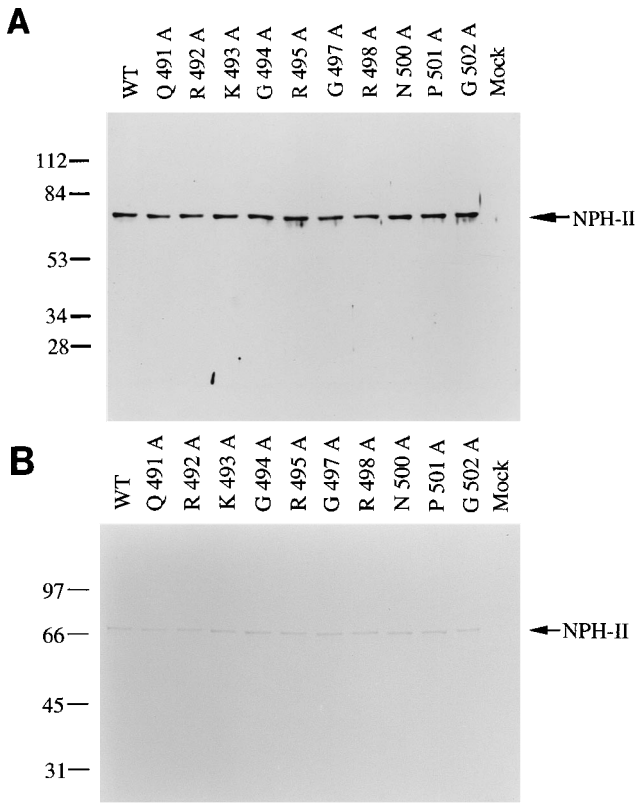

FIG. 2. Assessment of the purity of recombinant NPH-II proteins. (A) Western blot analysis. Aliquots of the glycerol gradient preparations of wild-type and mutant NPH-II (containing 12 ng of immunoreactive NPH-II) were electrophoresed through an 8% polyacrylamide gel containing 0.1% SDS. Proteins were transferred electrophoretically to a nitrocellulose membrane that was blocked in TBST buffer (10 mM Tris-HCl [pH 8.0], 150 mM NaCl, 0.05% Tween 20) containing 5% BSA and 1% dried milk. Membranes were incubated for 2 h at room temperature with rabbit anti-NPH-II serum diluted 1:6,000 in TBST. After removal of serum and washing with TBS-Tween (0.4%), bound antibodies were localized by incubation with anti-rabbit immunoglobulin conjugated with horseradish peroxidase. Immunoreactive polypeptides were visualized with an enhanced chemiluminescence system (ECL; Amersham) according to the instructions of the manufacturer. (B) Polypeptide composition. Aliquots of the glycerol gradient NPH-II preparations (containing 45 ng of immunoreactive NPH-II) were electrophoresed through an 8% polyacrylamide gel containing 0.1% SDS. Polypeptides were visualized by staining with Coomassie blue dye. The positions and sizes (in kilodaltons) of marker proteins are indicated on the left of each panel. WT, wild type.

the strongest effect on RNA unwinding, reducing specific activity to less than 1% of that of the wild-type enzyme (Fig. 3). The helicase activity of the NPH-II-Ala proteins was completely dependent on added ATP (data not shown). RNA unwinding by the mock-transfected preparation was undetectable (data not shown).

RNA binding by His-NPH-II mutants. The ability to bind single-strand nucleic acid in the absence of NTPs is characteristic of NPH-II (7, 25, 26). The complex of NPH-II bound to ssRNA is quite stable and can be easily resolved from free RNA by native gel electrophoresis. The radiolabeled RNA ligand used in standard binding assays corresponds to the 98-mer strand of the helicase substrate. The amount of the shifted protein-RNA complex formed by wild-type NPH-II and by each of the Ala mutants varied linearly with input protein; every one of the mutated proteins retained the ability to bind RNA (Fig. 4). The electrophoretic mobility of the RNA-protein complex formed by each of the mutant His-NPH-II proteins was similar to that of the wild-type enzyme analyzed in parallel (data not shown). No such RNA-protein complex was formed by the mock-transfected control preparation (data not shown). With the exception of R492A, all the NPH-II-Ala proteins displayed an affinity for ssRNA that was essentially identical to that of the wild-type enzyme (Fig. 4). The R492A protein showed about a two- to threefold decrement in ssRNA binding.

To relate the RNA binding properties of the mutant proteins more directly to the observed effects on helicase activity, we examined the binding of the NPH-II mutants to the helicase substrate itself. Prior studies showed that wild-type NPH-II forms a stable complex with the tailed duplex RNA in the absence of nucleotide that is resolved from free RNA substrate by native gel electrophoresis (26). The extent of complex formation was again proportional to the amount of input protein for the wild-type NPH-II and for all 10 mutated proteins (Fig. 5). As was the case for binding to the 98-mer ssRNA, every mutated protein except R492A bound to the helicase substrate with an affinity similar to that of wild-type NPH-II; the R492A titration curve was shifted to the right, and we estimate by extrapolation to the other binding profiles that R492A caused about a two- to threefold decrease in binding affinity to the helicase substrate. It is worth pointing out that the apparent requirement for higher amounts of NPH-II to form an equivalent amount of RNA-protein complex on the tailed helicase substrate compared with the 98-mer ssRNA is a consequence of the method of preparation of the tailed RNA duplex rather

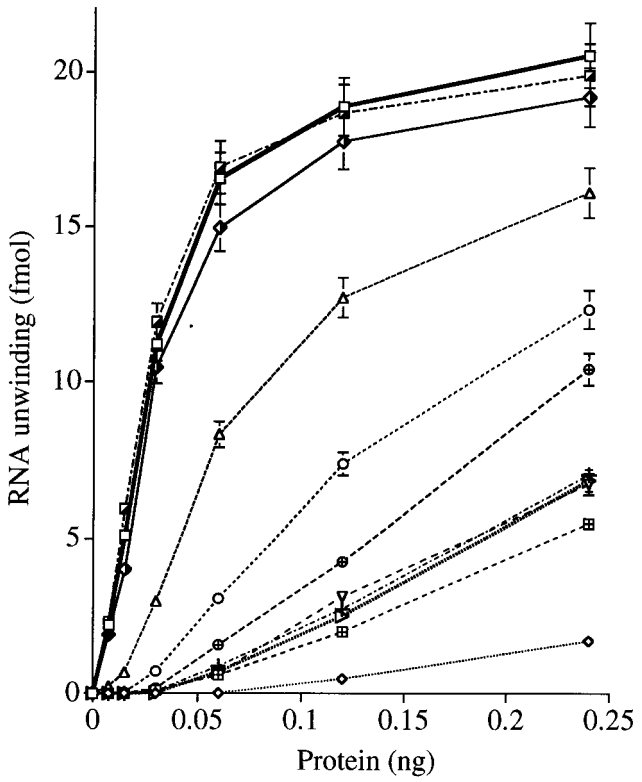

FIG. 3. RNA unwinding by wild-type and mutant His-NPH-II proteins. Helicase assays were performed as described in Materials and Methods. The extent of RNA unwinding by wild-type (WT) and mutated NPH-II proteins is plotted as a function of the amount of input enzyme. Each datum point represents the average of two independent determinations; standard error bars are shown. Symbols: □, WT; ◇, Q491A; ○, R492A; △, K493A; ⊞, G494A; +, R495A; ⊕, G497A; ▽, R498A; ◪, N500A; , P501A; ▷, G502A.

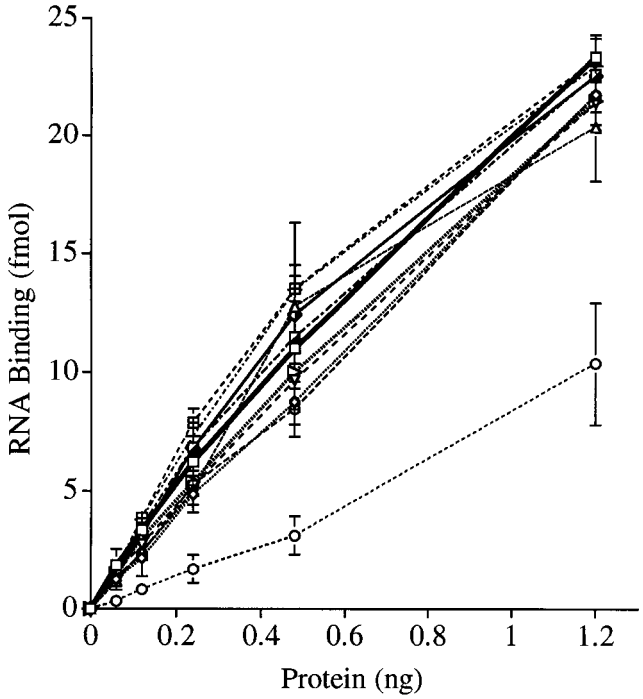

FIG. 4. Binding of wild-type (WT) and mutated His-NPH-II proteins to single-strand RNA. Binding of NPH-II to a radiolabeled 98-mer ssRNA was measured by using a gel shift assay as described in Materials and Methods. The extent of protein-RNA complex formation is plotted as a function of input enzyme. Each datum point represents the average of two independent determinations; standard error bars are shown. Symbols are defined as for Fig. 3.

than an inherently lower affinity of NPH-II for the helicase substrate than for the ssRNA. The amount of input helicase substrate is determined on the basis of the molar amount of the radiolabeled 38-mer strand; the gel purification step of substrate preparation resolves the hybrid from the free 38-mer but does not completely remove the excess cold 98-mer present during the annealing step. Hence, the helicase substrate, which contains more molecules of RNA ligand than accounted for by the amount of hybridized 38-mer, necessarily requires more protein to bind a given fraction of the input labeled RNA.

These experiments suggested that the reduced helicase activities of the Q491A, K493A, G494A, R495A, G497A, R498A, and G502A proteins could not be attributed to mutational effects on interaction with the helicase substrate. Although the R492A mutation did diminish RNA binding by two- to threefold, this effect appeared insufficient to explain the eightfold effect of the R492A mutation on RNA unwinding (Fig. 3). That the N500A and P501A mutations did not affect RNA binding was in keeping with their lack of impact on helicase activity.

Mutational effects on nucleic acid-dependent ATPase. The extent of ATP hydrolysis by NPH-II in the presence of an ssDNA cofactor was proportional to the amount of input enzyme (Fig. 6A). The specific ATPase activities of the N500A and P501A mutants were indistinguishable from that of wild-type NPH-II, whereas the specific activity of the K493A protein was about 60 to 70% of that of the wild type (Fig. 6A). In contrast, the activities of the Q491A, R492A, G494A, R495A, R498A, and G502A mutants were reduced by about an order of magnitude (Fig. 6A). ATP hydrolysis in the presence of an RNA cofactor was also proportional to input enzyme (Fig. 6B). Mutational effects on RNA-dependent ATPase mirrored those observed for the DNA-dependent ATPase, i.e., the N500A, P501A, and K493A mutations had little or no effect on activity, whereas the remaining mutations reduced activity by about an order of magnitude (Fig. 6B). In light of previous findings that a mutation of the His residue in the DExH box rendered the ATPase activity of NPH-II nucleic acid independent (7), we assayed the mutated proteins for ATP hydrolysis in the presence and absence of polynucleotide cofactors. All 10 Ala-substituted proteins showed a 10- to 30-fold stimulation of ATP hydrolysis by nucleic acid (not shown).

Mutational effects on ATPase activity could be caused either by an increase in K_m for ATP or by a decrease in V_{max}. To discriminate between these possibilities, we analyzed the kinetic parameters for ATP hydrolysis for the wild-type and seven of the mutant proteins. DNA-dependent ATP hydrolysis was determined as a function of ATP concentration in the range of 0.2 to 2 mM. K_m and V_{max} were calculated from double-reciprocal plots of the data (not shown). The kinetic parameters are presented in Table 1. The wild-type K_m value of 1.2 mM ATP agrees with values reported previously (7, 17). The Q491A, R492A, and G494A mutants did not greatly affect the affinity for ATP; however, these mutations reduced V_{max} to between 5 and 13% of that of the wild type (Table 1). The R495A, G497A, R498A, and G502A mutations did reduce affinity for ATP while also depressing V_{max} by a factor of 3 to 10 (Table 1). Summary effects on ATPase activity were judged by the ratio of V_{max} to K_m; mutational reductions in V_{max}/K_m varied from 9-fold in the case of G497A to 40-fold for R498A. (Kinetic parameters were not determined for the K493A, N500A, and P501A proteins, each of which had ATPase activity comparable to that of wild-type NPH-II.)

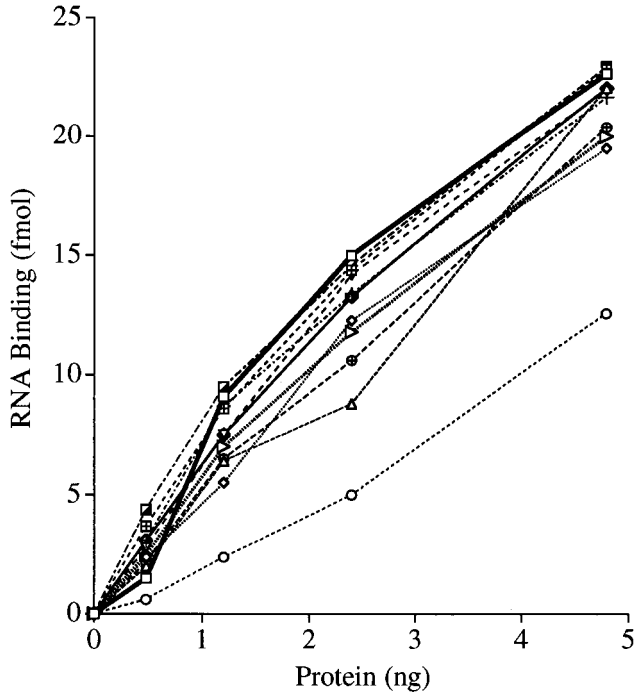

FIG. 5. Binding of wild-type (WT) and mutated His-NPH-II proteins to the RNA helicase substrate. Binding of NPH-II to the tailed double-stranded RNA substrate was measured by using a gel shift assay. The extent of protein-RNA complex formation is plotted as a function of input enzyme. Symbols are defined as for Fig. 3.

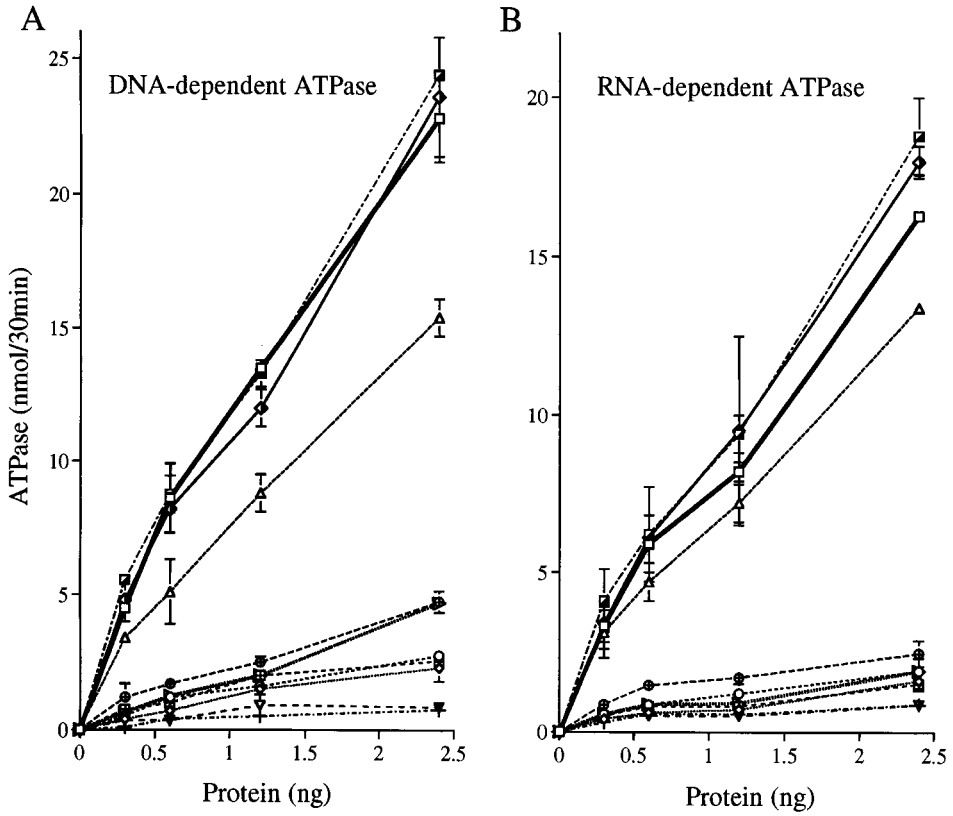

FIG. 6. ATP hydrolysis by wild-type (WT) and mutated His-NPH-II proteins. ATPase hydrolysis by NPH-II in the presence of a DNA cofactor (M13mp18 DNA [A]) or an RNA cofactor [poly(C) (B)] was determined as described elsewhere (7). ATPase is expressed as nanomoles of $^{32}P_i$ released from [γ-^{32}P]ATP during a 30-min incubation at 37°C and is plotted as a function of input enzyme. Each datum point represents the average of two independent determinations; standard error bars are shown. Symbols are defined as for Fig. 3.

DISCUSSION

We have presented a mutational analysis of the QRKGR VGRVNPG motif in vaccinia virus NPH-II, a DExH box RNA helicase. The biochemical activity of each mutant protein was determined by titration and compared with that of wild-type NPH-II. Our results demonstrate that individual residues within the targeted motif are important for NTP hydrolysis and duplex RNA unwinding but apparently not for RNA binding. Ala substitution effects followed a simple rule: mutations at residues not strictly conserved among the NPH-II-like DExH subgroup had little or no effect on any biochemical function of NPH-II, whereas mutations at conserved residues had a significant impact on NTPase and helicase activities.

TABLE 1. Kinetic parameters of ATP hydrolysis by His-NPH-II mutants[a]

NPH-II	K_m (mM)	V_{max} (% of WT)
WT	1.2 ± 0.3	100
Q491A	0.7 ± 0.1	5
R492A	1.9 ± 0.3	10
G494A	1.9 ± 0.8	13
R495A	3.1 ± 0.6	11
G497A	3.2 ± 0.7	29
R498A	5.8 ± 0.3	10
G502A	3.3 ± 0.5	15

[a] DNA-dependent ATPase was measured as a function of ATP concentration (at 0.2, 0.4, 0.6, 1.0, and 2.0 mM ATP). Reaction mixtures (50 μl) contained a saturating amount (0.33 μg) of M13mp18 ssDNA cofactor. Kinetic parameters were calculated from double-reciprocal plots of each titration series. The K_m values shown are averages of three independent ATP titration experiments with standard deviations. V_{max} is expressed relative to the wild-type enzyme (100%). WT, wild type.

The most benign mutations were at Lys-493, Asn-500, and Pro-501. None of the other NPH-II-like proteins aligned in Fig. 1 contains an Asn residue at the position equivalent to Asn-500; indeed, three NPH-II-like proteins normally contain an Ala at this position (Fig. 1). A Pro residue analogous to Pro-501 is found in 10 of 18 of the NPH-II-like proteins, yet 3 of 18 have Ala at this position (Fig. 1). A homolog of Lys-493 is present in 6 of 18 NPH-II-like proteins, compared with 5 of 18 that have Ala at this position. In this light, it is not surprising that Ala replacements at the Lys-493, Asn-500, and Pro-501 residues of NPH-II did not have dramatic effects on protein function. Of course, it is possible that other amino acid substitutions at these positions would not be so well tolerated.

The residues at which Ala substitution had the greatest impact—Gln-491, Arg-492, Gly-494, Arg-495, Gly-496, Arg-497, and Gly-502—are strictly conserved in 18 of 18 NPH-II-like proteins. It is also noteworthy that none of the non-NPH-II-like DExH proteins shown in Fig. 1 contains Ala at a position analogous to Gln-491, Arg-492, Gly-492, Arg-495, Gly-497, or Arg-498, and only one of these, RecG, contains Ala in lieu of Gly-502. Each of the Ala substitutions at the conserved residues of NPH-II-Ala caused a defect in ATP hydrolysis and RNA unwinding. There was no discernible mutational effect on RNA binding, except in the case of R492A. Even for R492A, the two- to threefold reduction in RNA binding affinity

did not account for the quantitatively greater impact of this Ala substitution on ATPase and helicase activities. We infer that the QRxGRxGRxxxG motif is a component of the NTPase active site of NPH-II; this is supported by kinetic analysis, which implicates all seven of these conserved residues in catalysis (reflected by lowered V_{max}) and four of them—R495, G497, R498, and G502—in ATP binding (inferred from a >2-fold increase in K_m). It is likely that further dissection of structure-function relationships by more intensive mutagenesis of these seven residues would be informative; such analysis is beyond the scope of the present study.

We are confident that our results accurately reflect the relative specific activities of the mutated enzymes because (i) activities were normalized to equivalent amounts of immunoreactive NPH-II protein, (ii) each preparation contained a single immunoreactive polypeptide, i.e., proteolytic fragments of NPH-II did not complicate quantitation, and (iii) each preparation contained essentially equivalent amounts of a single Coomassie blue-stained polypeptide corresponding to NPH-II. We note, however, that the determination of NPH-II concentration by Western blotting probably understates the absolute weight-to-volume concentration of purified NPH-II. This became clear from the RNA binding experiments, because the relationship between the moles of ssRNA molecules bound and the moles of input enzyme (calculated from the concentration of immunoreactive protein) indicated that one RNA molecule was bound per 0.5 molecule of NPH-II. This is anomalous given that NPH-II forms a single shifted complex on the 98-mer ssRNA used in RNA binding assays (7, 25). Although we have not determined the stoichiometry of the complex, we consider it likely that one or perhaps two protein molecules are bound per RNA molecule. Note that the NPH-II standard used in the Western assays was quantitated by Coomassie blue dye binding relative to BSA. BSA is known to bind twofold more Coomassie blue dye per unit of weight than a bovine gamma globulin standard (Bio-Rad protein assay; Bio-Rad Laboratories). We suggest therefore that the underestimation of NPH-II concentration is a consequence of lower dye binding per unit of weight of protein by NPH-II than by BSA. We have, in prior studies, used RNA binding as the normalization factor for determining specific activity and turnover number. We have referred to an RNA binding unit as the amount of NPH-II sufficient to shift 1 fmol of RNA (7). If the stoichiometry of the shifted complex is 1:1, then the Western assay understates the absolute concentration by a factor of two. Note that this point does not in any way affect our conclusions about the magnitude of mutational effects on NPH-II function.

It is informative to compare our mutagenesis results with those of Pause et al., who targeted six mutations to four residues within the HRxGRxxR motif of the DEAD box protein eIF-4A (19). They found that changing the His residue to Gln abolished RNA unwinding and reduced ATPase activity to 30% of that of the wild-type eIF-4A. Mutating the adjacent Arg to Gln abolished helicase and ATPase activities. Changing either of the other Arg residues to Gln eliminated helicase activity and reduced ATPase activity to 15 to 18% of the activity of the wild-type enzyme. Replacing the same two Arg moieties with Lys preserved ATPase but still caused complete loss of RNA unwinding. Most remarkably, every one of these mutations eliminated or strongly inhibited the binding of eIF-4A to RNA, as determined by UV cross-linking of purified recombinant eIF-4A to a bromo-UMP-substituted RNA probe (19).

The conclusion of Pause et al. (19) that residues in motif VI of eIF-4A are involved in ATP hydrolysis is consistent with our conclusions regarding NPH-II. However, they attribute their mutational effects to an underlying defect in RNA binding, whereas we draw opposite conclusions. The disparity is likely to reflect fundamental differences in the properties of the two helicases as well as the nature and limitations of the different assays used to measure RNA binding. Foremost among these differences is that eIF-4A binds to RNA with very low affinity. No stable complex can be detected by a native gel mobility shift assay, while nitrocellulose filter-binding assays indicate a K_d of $>10^{-6}$ M (19). The dependence of RNA cross-linking on eIF-4A concentration indicates that this assay also measures a low-affinity interaction with a dissociation constant in the micromolar range (19). Moreover, the cross-linking of eIF-4A to RNA is dependent on the inclusion of hydrolyzable ATP in the reaction (19). In contrast, NPH-II forms a very stable complex with RNA in the absence of nucleotide that can be isolated by the mobility shift technique; indeed, the NPH-II RNA complex, once formed, does not dissociate readily, even when challenged with competitor RNA (25). The binding data of Fig. 4 indicate a K_d for NPH-II of $<10^{-9}$ M (this is the case even if we assume that the NPH-II concentration is underestimated by severalfold); this binding affinity is 3 orders of magnitude greater than that of eIF-4A. Our analysis shows that the high-affinity binding site of NPH-II is not disrupted by mutations of the conserved residues in motif VI. If the NPH-II mutations described in this study affected a putative low-affinity RNA binding interaction that was relevant to ATP hydrolysis and RNA unwinding, it is inevitable that such effects would be obscured in our assays by the otherwise unperturbed high-affinity binding site. An important direction for future studies will be to localize the high-affinity site within the NPH-II polypeptide. The amino acid sequence of NPH-II provides no obvious clues to the location of this site.

UV cross-linking is an indirect method to assay RNA binding. Cross-linking requires not only that the RNA be bound to protein but also that the photoactive RNA moiety be situated close to a reactive substituent of the protein. Thus, mutations that reduce photoadduct formation may do so by reducing RNA binding or by altering the reactive protein substituent without actually interfering with the binding event. Interpretations of mutational effects on RNA cross-linking are most clear when an independent assay for RNA binding is available and (ideally) when the site of photoadduct formation by the wild-type protein is known. As noted by Pause et al. (19), the finding that Arg-to-Lys mutations in motif VI of eIF-4A eliminated RNA cross-linking, but did not have a commensurate effect on RNA-dependent ATPase, raises questions about what is being measured by the cross-linking assay. For this reason, we have not performed cross-linking studies with the NPH-II mutants but have relied instead on the gel shift assay.

Other properties distinguish NPH-II from eIF-4A. For example, eIF-4A requires a second protein cofactor, eIF-4B, to act as a helicase (21). Even with eIF-4B, eIF-4A is a relatively inefficient helicase, requiring a significant molar excess of protein over RNA to elicit unwinding. eIF-4B, like eIF-4A, binds to RNA with an affinity in the micromolar range (1, 16). This, plus the prospect that eIF-4B might act to promote reannealing of the RNA strands displaced by the helicase (1), may account for the unfavorable stoichiometry of unwinding.

None of these issues bears on studies of unwinding by NPH-II because the vaccinia virus helicase acts alone to unwind a large molar excess of tailed RNA duplexes. The preparation of wild-type NPH-II used in the present study unwound 30 fmol of helicase substrate per RNA binding unit during a 15-min reaction. Several Ala substitution mutations in the QRxGRxGRxxxG motif reduced specific helicase activity to stoichiometric or substoichiometric levels. The single most det-

rimental effect on RNA unwinding was caused by mutation of Gln-491, which is present in every DExH protein and is one of the defining residues as far as distinctions between DEAD and DExH families are concerned. In general, the mutational effects on unwinding could be attributed to defects in ATP hydrolysis. However, some mutations caused an apparently greater effect on unwinding than others in proportion to their effects on ATPase; this suggests that some of these residues may be involved in coupling energy utilization to strand displacement.

In conclusion, we have shown that the QRxGRxGRxxxG motif is involved in NTP hydrolysis and RNA unwinding and that individual residues in this motif are not critical to high-affinity binding of NPH-II to RNA. Our results underscore again that the contribution of individual residues to overall protein function, as assessed by mutational analysis and biochemical assays in vitro, can vary significantly from one helicase to another. There is considerable incentive to extend structure-function analyses to several representative members of each helicase family in order to gain general insight into common mechanistic themes while illuminating the basis for properties like directionality and cofactor specificity that vary so widely within each family.

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