

Characterization and mutational analysis of the helicase and NTPase activities of hepatitis C virus full-length NS3 protein

Andrew D. Wardell,¹ William Errington,¹ Giuseppe Ciaramella,² James Merson²
and Michael J. McGarvey¹

¹ Department of Medicine, Imperial College School of Medicine, St Mary's Campus (QEQM Wing), South Wharf Road, London W2 1NY, UK

² Pfizer Ltd, Ramsgate Road, Sandwich, Kent CT13 9NJ, UK

The non-structural protein 3 (NS3) of hepatitis C virus (HCV) possesses three activities which are likely to be essential for virus replication; a serine protease located in the N terminus and helicase and NTPase activities located in the C terminus. Sequence analysis of the helicase/NTPase domain has identified motifs indicative of the DEAD-box family of helicases. Here we present the characterization of the helicase and NTPase activities of full-length NS3, expressed as a His-tagged fusion protein in *E. coli*, and make comparisons with published data of NS3 helicase domain alone. The helicase and NTPase activities of full-length NS3 have been demonstrated and we have characterized the effects of amino acid substitutions on conserved motifs of NS3 helicase. Helicase and NTPase activities were dependent on Mg²⁺ and ATP and inhibited by monovalent cations. NS3 was able to hydrolyse all four NTPs and dNTPs to drive DNA duplex unwinding but with differing abilities. NTPase activity was stimulated by all polynucleotides tested, with poly(U) having the greatest effect. Mutational analysis of conserved motifs of NS3 helicase showed all conserved residues to be required for optimal activity. These results are in accord with a recently proposed model for NS3 helicase activity.

Introduction

Hepatitis C virus (HCV) was first isolated in 1989 and identified as the major causative agent of post-transfusional non-A non-B hepatitis (Choo *et al.*, 1989). Sequence comparison identified HCV as a new member of the family *Flaviviridae*, which also includes flavi- and pestiviruses (Choo *et al.*, 1991). HCV is an enveloped virus with a positive-sense RNA genome of approximately 9.4 kb. The genome encodes a single polypeptide of 3010–3033 amino acids which is cleaved co- or post-translation by host and viral proteases to yield mature viral proteins (Grakoui *et al.*, 1993; Selby *et al.*, 1993). These proteins are in the order NH₂-C-E1-E2-p7-NS2-NS3-NS4A-NS4B-NS5A-NS5B-COOH, where C, E1 and E2 are the structural proteins (core and glycoproteins 1 and 2) and the remainder are non-structural (NS) proteins involved in polyprotein processing and virus replication.

HCV NS3 protein is a multifunctional protein involved in polyprotein processing and virus replication. A serine protease

domain is located in its N terminus and a helicase/NTPase domain is located in its C terminus. The presence of these activities, initially inferred from the identification of amino acid motifs and alignments with related proteins, has subsequently been demonstrated (Suzich *et al.*, 1993; Tomei *et al.*, 1993; Tai *et al.*, 1996). The serine protease domain is required for processing of the viral polyprotein, releasing NS4A, NS4B, NS5A and NS5B, and has been shown to be a chymotrypsin-like protease (Tomei *et al.*, 1993; Hahm *et al.*, 1995; Kim *et al.*, 1996). Conserved motifs in the NS3 helicase domain identify it as a member of the DEAD-box family of RNA helicases. This group of helicases consists of three subfamilies of proteins that contain either a DEAD, DEAH or DExH amino acid motif (Gorbalenya *et al.*, 1989; Koonin, 1991). NS3, which possesses a DECH motif, is therefore a member of the DExH subfamily. Conserved regions within RNA helicases of this type include motif I (GxGKS) and motif II (DExH) required for ATP binding and hydrolysis, and motif VI (QRxGRxGR) involved in ATP hydrolysis and RNA unwinding (Gross & Shuman, 1995, 1996; Kim *et al.*, 1997). Helicase and NTPase activities of various cellular and viral DEAD-box proteins have been demonstrated: for example, eIF4A, vaccinia virus NPH-II, and

Author for correspondence: Michael McGarvey.

Fax +44 171 725 9369. e-mail m.mcgarvey@ic.ac.uk

NS3 of flavi- and pestiviruses (Pause & Sonenberg, 1992; Wengler, 1993; Gross & Shuman, 1996; Lieber *et al.*, 1996). DEAD-box proteins also play a role in mRNA stability and splicing (Iost & Dreyfus, 1994; Wagner *et al.*, 1998) and this suggests further roles for NS3 in virus replication and in the control of host cell functions.

The C-terminal region of NS3 containing only the helicase/NTPase domain has been expressed and characterized (Suzich *et al.*, 1993; Jin & Peterson, 1995; Tai *et al.*, 1996; Gwack *et al.*, 1997). The helicase and NTPase activities are dependent on Mg^{2+} and inhibited by monovalent cations; the NTPase activity is enhanced by the presence of polynucleotides. NS3 is able to unwind RNA, DNA and RNA/DNA duplexes in a 3' to 5' direction dependent on a 3' single-stranded region (Tai *et al.*, 1996). Mutational analyses of the helicase domain have shown that residues of the conserved motifs of DEAD-box helicases are essential for NTPase and helicase activities and also that NTPase activity is essential but not sufficient for helicase activity (Heilek & Peterson, 1997; Kim *et al.*, 1997).

A recent study of activities of an HCV NS3–NS4A complex isolated from transfected COS cells showed the protease activity to be enhanced by poly(U) (Morgenstern *et al.*, 1997), indicating an interdependency between the protease and helicase domains of NS3. In addition, NS3 has been shown to interact with and affect the activities of host cell proteins such as protein kinase A (Borowski *et al.*, 1996, 1997) and the tumour suppressor p53 (Ishido *et al.*, 1997; Muramatsu *et al.*, 1997) which are dependent on C- and N-terminal domains of NS3 respectively and affect the subcellular localization of NS3. This highlights the importance of investigating the activities of full-length NS3 to fully elucidate its role in virus replication and host cell functions.

In this study we present an analysis of the helicase and NTPase activities of full-length NS3 protein expressed in *E. coli*. We have characterized the optimal conditions for these activities and introduced point mutations to replace amino acids of the conserved DEAD-box protein motifs I, II and VI. The effects of these mutations have been demonstrated and compared to those of corresponding mutations in the NS3 helicase domain alone and in other DEAD-box proteins. We also relate our results to the recently published structure of the HCV NS3 helicase domain (Kim *et al.*, 1998) with respect to the model of duplex unwinding activity proposed.

Methods

Materials. NS3-7 antibody used in this study was raised against the NS3 peptide FIPVENLETTMRS (amino acids 1195–1207) in rabbit. Oligonucleotides were from OSWEL and polynucleotides were from Pharmacia or Sigma. Plasmid pBRTMHCV(1–3011) was from C. Rice (Grakoui *et al.*, 1993) and β -galactosidase control plasmid was from Novagen. Plasmid pHEX-NS5A/B encodes amino acids 2328–2516 of HCV NS5 spanning the NS5A/B cleavage site fused to a six-histidine tag. The parental vector, pHEX, was supplied by G. Reid (MRC,

Retrovirus Research Laboratory, University of Glasgow, UK) and was constructed by replacing the GST coding region of pGEX-2T with a six-histidine tag. pHEX-NS5A/B was expressed in *E. coli* to form inclusion bodies. These were isolated in the same way as for NS3 to provide a negative control protein for NTPase and helicase activities.

■ Cloning, expression and isolation of HCV NS3 wild-type and mutant proteins.

The full-length NS3 coding sequence was amplified by PCR from plasmid pBRTMHCV(1–3011) using the sense primer 5' GCGCCATATGGCGCCCATCACGGCG 3' and antisense primer 5' GCGCGGATCCTTACGTGACGACCTCCAG 3' which were designed to introduce a 5' *NdeI* restriction site and 3' *BamHI* restriction site. PCR was for 4 min at 95 °C followed by 20 cycles of 1 min 94 °C, 1 min 55 °C and 1 min 72 °C using *Taq* polymerase (Boehringer Mannheim). The PCR product was ligated into pCRII vector (Invitrogen) to create the construct pCRNS3. The NS3 gene was excised as an *NdeI*–*BamHI* fragment and ligated into *NdeI*- and *BamHI*-digested pET16b vector (Novagen) to produce plasmid pAW3(1027–1657). *E. coli* strain JM109(DE3) harbouring pAW3(1027–1657) was grown at 37 °C in LB media containing ampicillin to an OD_{600} of 0.5 and then cooled to 30 °C. Expression was induced by the addition of 0.5 mM IPTG and growth continued at 25 °C for 3 h. The cells were then harvested by centrifugation at 2000 *g* for 15 min at 4 °C and the pellet was resuspended in 10 ml PBS per 100 ml original culture. Inclusion bodies containing NS3 protein were purified as follows. Aliquots of 10 ml were sonicated using a Mistral Soniprep on ice for four pulses of 15 s at 21 μ m and the insoluble material was harvested by centrifugation at 7000 *g* for 10 min at 4 °C. The insoluble protein was resuspended in PBS and the process repeated four times. Finally, the pellet was resuspended in 5 ml 8 M urea in PBS, centrifuged as before and the supernatant retained. The urea-solubilized NS3 was diluted to 0.2 mg/ml with 8 M urea in PBS and the urea removed by stepwise dialysis against 20 vols buffer A [25 mM Tris–HCl (pH 7.2), 10% (v/v) glycerol, 50 mM NaCl, 1 mM DTT, 0.1% CHAPS] containing 4 M, 2 M, 1 M urea, then three buffer changes without urea. Each dialysis step was for a minimum of 2 h. The sample was clarified by centrifugation at 70 000 *g* for 30 min at 4 °C and dialysed against buffer A containing 50% glycerol for 24 h and stored at –20 °C. NS3 mutants were made by the introduction of point mutations to the plasmid pAW3(1027–1657) using the Chameleon kit (Stratagene). The presence of the desired mutation was confirmed by DNA sequencing using an ABI automated sequencer. NS3 mutant proteins were expressed and purified as above.

■ NTPase assay. NTPase assays were performed using two methods. The first was a colorimetric method performed in a 96-well plate using a malachite green reagent. In a 20 μ l volume, 6 pmol enzyme, 25 mM Tris–HCl (pH 7.5), 5 mM $MgCl_2$, 0.5 mM ATP and 1.5 mM polyuridylic acid (except where indicated) were incubated for 30 min at 25 °C. The reaction was stopped with the addition of 50 mM EDTA and the colour was developed by the addition of 200 μ l AM/MG reagent (0.034% malachite green, 1.05% ammonium molybdate and 0.04% Tween 20 in 1 M HCl). After 5 min 30 μ l 34% (w/v) trisodium citrate was added and the A_{630} read. The amount of NTP hydrolysed was then calculated from an inorganic phosphate standard curve.

For the second assay, 3 pmol enzyme, 25 mM Tris–HCl (pH 7.5), 5 mM $MgCl_2$, 0.1 mM ATP, 1.5 mM polyuridylic acid and 1.25 μ Ci [γ - 32 P] ATP or [γ - 32 P]ATP were incubated for 30 min at 25 °C and the reaction was stopped with 50 mM EDTA. Samples were dotted on to sheets of PEI cellulose and then substrate and products separated by ascending chromatography in 0.4 M KH_2PO_4 (pH 3.4). The chromatogram was air dried and exposed to X-ray film. Regions corresponding to substrate and product were cut out and the bands quantified using a Packard liquid scintillation counter.

■ Helicase activity. The helicase substrate was prepared as follows. A shorter oligonucleotide (release strand) of sequence 5' TATCTGTGCGAGCTCGCTCGATGATCCTCTAGAGTCGAC was ^{32}P end-labelled using T4 polynucleotide kinase. This was annealed to a larger oligonucleotide (template strand) of sequence 5' GAATACAGACTTGACGTGCAGGTCGACTCTAGAGGATCATCGAGCGAGCTCGTATTCCGGTCTCGCTATAGTGAGTCGTATTAATGTCGATAAGCCAG by heating to 90 °C for 5 min and allowing to cool to room temperature overnight. Annealed oligonucleotides were purified from unannealed using nick spin columns (Boehringer Mannheim). The helicase reaction was for 60 min at 37 °C in 15 μl containing 50 mM MOPS (pH 6.5), 5 mM ATP, 3 mM MgCl_2 , 2 mM DTT, 0.25 (w/v) mg/ml BSA, 0.3 pmol substrate and 4.5 pmol enzyme. 10 μl of the reaction was added to 5 μl load buffer [100 mM Tris-HCl (pH 8.0), 40% (w/v) sucrose, 100 mM EDTA, 1% (w/v) SDS, 0.01% (w/v) bromophenol blue] and the products were resolved using a 15% TBE-PAGE gel. The gel was dried and autoradiographed and unwinding was quantified by PhosphorImage analysis using ImageQuant software.

Results

Expression of wild-type NS3

Full-length NS3 (amino acids 1027–1657) was expressed as a histidine-tagged fusion protein in *E. coli* and isolated in a purified form from inclusion bodies. Expression of NS3 was confirmed by SDS-PAGE analysis and Western blot (Fig. 1). The observed size of approximately 70 kDa was consistent with the size estimated by sequence analysis. Soluble protein

was recovered as described above, with a yield of approximately 5 mg per litre of bacterial culture. Smaller immunoreactive species not present in control expressions were observed corresponding to a low level of degradation of NS3 (Fig. 1B). The major degradation product was of approximately 45 kDa and was observed by Western blot in the purified sample (Fig. 1B, lane 5). This product was not detectable by Coomassie staining and therefore constitutes a very small fraction of total protein in the sample. A similar pattern of NS3 degradation has been observed by others when expressed in *E. coli* (Suzich *et al.*, 1993; Kim *et al.*, 1995) and insect cells (Gwack *et al.*, 1995; Jin & Peterson, 1995).

Optimization of conditions for NTPase and helicase activities of NS3

NTPase activity of NS3 was shown to be stimulated by poly(U). This activity was absent in an identical protein sample harvested from *E. coli* containing the control plasmid pHEX-NS5A/B (Fig. 2A). NS3 NTPase activity was inhibited by pre-incubation with sera from an HCV-positive patient with a demonstrated affinity for NS3 (data not shown). Unwinding of a DNA duplex by NS3 was demonstrated (Fig. 2B). Unwinding was not observed in the absence of ATP nor when ATP was replaced with the non-hydrolysable analogue ATP- γS . These activities were characterized with respect to monovalent and

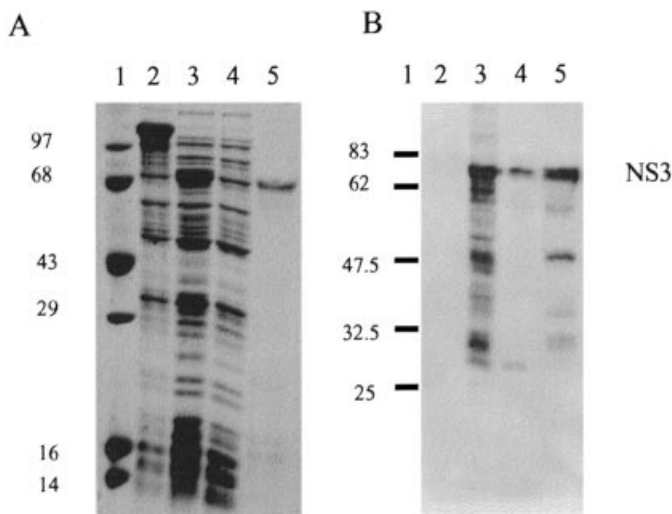


Fig. 1

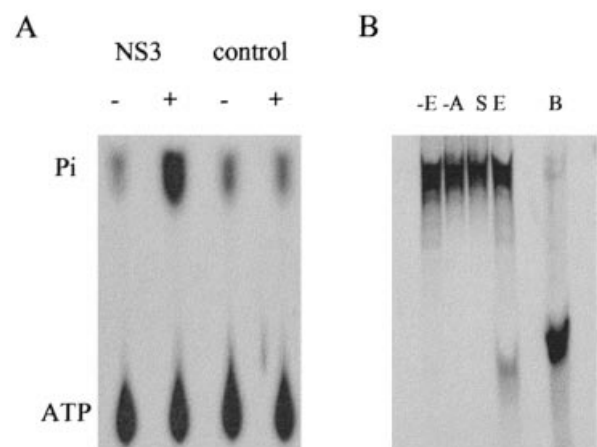


Fig. 2

Fig. 1. SDS-PAGE analysis of full-length NS3 expression and purification performed using a 10% (w/v) acrylamide gel. (A) Coomassie stain. (B) Western blot using NS3-7 (1/3000) NS3 antisera for detection. Lane 1, molecular mass markers with sizes in kDa shown; 2, β -galactosidase-expressing control; 3, total cell extract; 4, soluble extract; 5, isolated and purified inclusion bodies containing NS3.

Fig. 2. (A) Poly(U)-stimulated NTPase activity of NS3. NS3; full-length NS3 protein isolated from inclusion bodies. Control; corresponding preparation from pHEX-NS5A/B expression. Reactions were done in the presence (+) or absence (–) of 1.5 mM poly(U). (B) Unwinding of DNA duplex by NS3. –E, no NS3; –A, no ATP; S, ATP replaced with ATP- γS ; E, NS3 and standard assay conditions; B, boiled substrate.

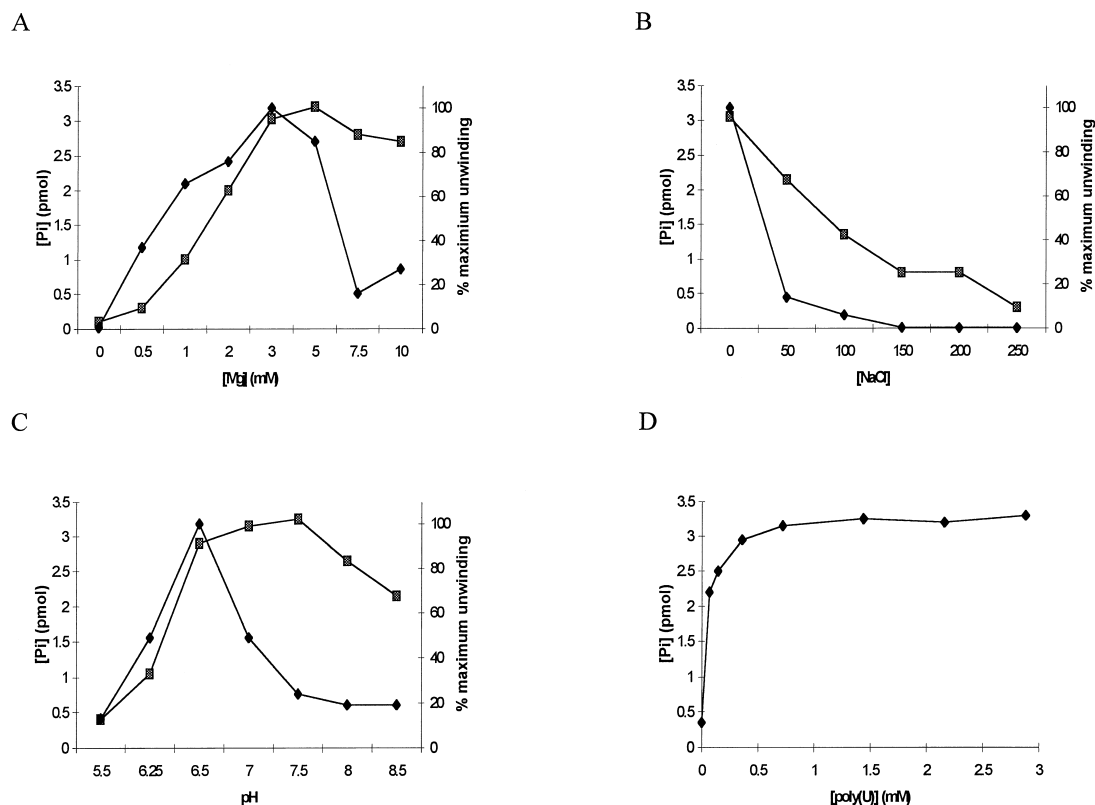


Fig. 3. Effect of polynucleotides, Mg^{2+} , pH and NaCl on NTPase and helicase activities (◆, NTPase; ■, helicase). (A) Dependence on Mg^{2+} at 0.5 mM ATP (NTPase) and 5 mM ATP (helicase). (B) NaCl titration. (C) Effect of pH on helicase and NTPase activity. Buffers used for pH titration were 50 mM MES pH 5.5 and 6.0, 50 mM MOPS-KOH pH 6.5, 7.0 and 7.5, and 50 mM Tris-HCl pH 8.0 and 8.5. All buffers were standardized at assay temperature. (D) Poly(U) stimulation of NTPase activity. Values of polynucleotide used are in μ M polynucleotide. Effects of each variable were determined using the malachite green NTPase assay and helicase assay described above. These results show the average of two experiments. Duplicate data-points varied by less than 20%.

divalent cations, poly(U) and pH (Fig. 3 A–D). NTPase activity was shown to be dependent on the presence of poly(U), with optimal stimulation above 750 μ M. Activity was barely detectable in the absence of the polynucleotide cofactor. Mg^{2+} was required for both activities; under the conditions tested both activities steadily increased to an optimum $MgCl_2$ concentration of 3 mM. Mg^{2+} concentrations greater than 3 mM had little effect on NTPase activity but drastically reduced helicase activity. NTPase activity was significantly less sensitive to pH and monovalent salt than helicase activity. NTPase activity peaked at pH 7.5 and was relatively unaffected over the range pH 6.5–8.0. However, helicase activity was maximal at pH 6.5 but was reduced to 50% maximum by a change of ± 0.5 pH units. Helicase activity was reduced to 10% in the presence of 50 mM NaCl, and zero with 150 mM. NTPase was much less affected; activity was still detected at 200 mM NaCl. KCl showed a similar effect on helicase activity (data not shown).

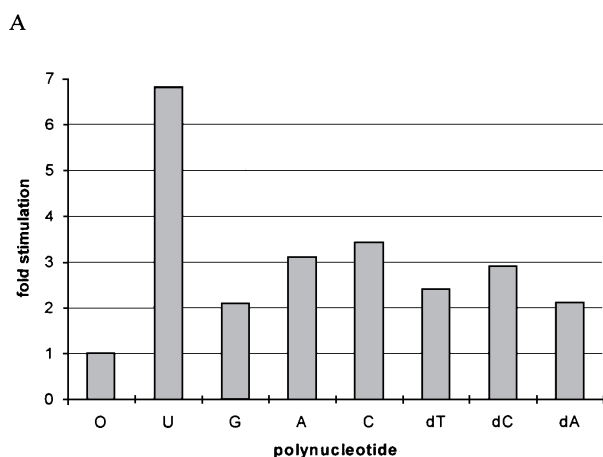
Optimal conditions were determined to be 5 mM $MgCl_2$, 0.5 mM ATP, 750 μ M poly(U), pH 7.5 for NTPase activity and 3 mM $MgCl_2$, 5 mM ATP, pH 6.5 for helicase activity.

Both activities were optimal in the absence of NaCl. These conditions were used for further analysis of the activities.

Polynucleotide stimulation and kinetic analysis of NTPase activity

The stimulatory effects of different polynucleotides on NTPase activity were examined. All polynucleotides tested were able to stimulate ATP hydrolysis (Fig. 4A). Poly(U) demonstrated the greatest stimulation of NTPase activity showing an approximately 7-fold increase over activity in the absence of polynucleotide. This stimulation was twice that of poly(C), the next best cofactor. All other polynucleotides tested in this study stimulated activity between 2 and 3-fold.

The hydrolysis of other NTPs in addition to ATP by HCV NS3 was also studied (Fig. 4B). The kinetic parameters k_{cat} (turnover number) and K_m (Michaelis constant) were determined from Lineweaver-Burk plots of hydrolysis activity for different concentrations of substrate. The k_{cat}/K_m value was calculated for each substrate and is a measure of the overall activity of the enzyme for that substrate. ATP was the best substrate for NS3 showing the highest turnover (32.4 min⁻¹)



B

	K_m (mM)	k_{cat} (min ⁻¹)	k_{cat}/K_m (mM ⁻¹ min ⁻¹)
ATP	0.28	32.4	116
GTP	0.83	23.4	28
CTP	0.36	24	67
UTP	0.59	25.2	43
dATP	0.34	22.2	65
dGTP	1.25	24	19
dCTP	0.43	29.4	68
dTTP	0.18	15.6	87

Fig. 4. (A) The ability of different polynucleotides to stimulate NS3 NTPase activity was assayed using the radioactive method as described in Methods. When used, polynucleotide cofactor was included at 1.5 mM (nucleotides). Stimulation was calculated as a factor of activity in the absence of cofactor, which was taken to be 1.0. (B) K_m and k_{cat} values for NTPase substrates determined from Lineweaver–Burk plots of NTP hydrolysis measured using the malachite green assay over 30 min, with 0.1, 0.25, 0.5, 0.75, 1.0 mM substrate in the presence of 5 μ M poly(U). $MgCl_2$ concentration was maintained at 4.5 mM above NTP level. Data shown represent the average of two experiments. Duplicate data-points varied by less than 20%.

and k_{cat}/K_m value (116 mM⁻¹ min⁻¹). GTP and dGTP were the poorest substrates for NS3.

NTP utilization for helicase activity

The ability of NS3 to utilize different NTPs as energy sources for unwinding of a DNA duplex was examined. NS3 was able to unwind the substrate with all NTPs tested but to different degrees (Fig. 5). Most unwinding was observed with ATP, CTP and dTTP. UTP, dATP and dCTP were equal in their ability to provide an energy source for unwinding (50% maximal activity) whereas GTP and dGTP were the poorest source of energy for unwinding (25% of maximal activity).

Generation of NS3 mutants

The effect of mutations to conserved domains of the DEAD-box family of helicases known to be required for

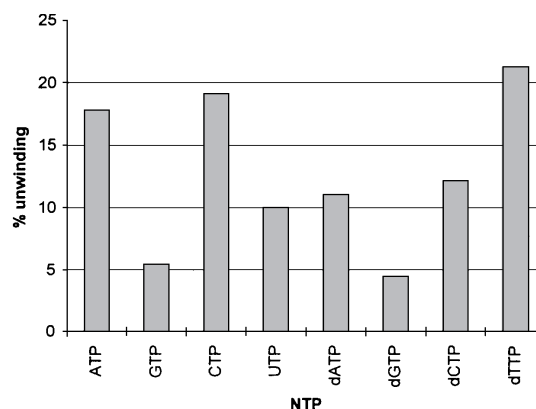


Fig. 5. Ability of NTPs to drive duplex unwinding. ATP, GTP, CTP, UTP, dATP, dGTP, dCTP and dTTP were tested for their ability to act as an energy source for duplex unwinding. Each nucleotide was present at 5 mM and all other conditions were standard (see Methods). Data shown represent the average of two experiments. Duplicate data-points varied by less than 20%.

activity were studied. Amino acid substitutions were made at six positions (K_{1236} , D_{1316} , E_{1317} , C_{1318} , Q_{1486} and R_{1493}) based on previous mutational studies of HCV NS3 and related helicases (Gross & Shuman, 1995, 1996; Kim *et al.*, 1997) and crystallography data (Kim *et al.*, 1998) (Fig. 6A). These residues are proposed to be involved in either the binding and hydrolysis of ATP or the coupling of unwinding to NTPase activity. The mutants were named according to the substituted amino acid and position. Plasmids carrying the desired mutation were expressed in *E. coli* and purified as described above for wild-type NS3 and similar yields of soluble protein recovered (Fig. 6B).

NTPase and helicase activities of NS3 mutants

Each mutant was tested for NTPase and helicase activity (Fig. 6C–E). Substitution of D_{1316} for alanine (motif II) destroyed both NTPase and helicase activities. Mutants $E_{1317}A$ (motif II) and $R_{1493}K$ (motif VI) showed only the basal level of each activity compared to the NS5A/B inclusion body control. Substitution of the lysine residue of motif I ($K_{1236}A$) resulted in the loss of helicase activity but retention of 27% wild-type NTPase activity. Similarly the mutant $Q_{1486}H$ (motif VI) possessed 34% wild-type NTPase activity but helicase activity was detectable only at basal level. Mutation of the variable residue of motif II ($C_{1318}S$) had little effect on NTPase activity but reduced helicase activity to 20% of wild-type.

Discussion

Previously, most work demonstrating the helicase and NTPase activities of NS3 has been carried out with the helicase domain expressed alone (Suzich *et al.*, 1993; Tai *et al.*, 1996) due to the difficulties involved in the isolation of full-length soluble protein. Recently, using a full-length NS3–NS4A complex isolated from transfected COS cells (Morgenstern *et al.*, 1997), it has been demonstrated that NS3 protease activity

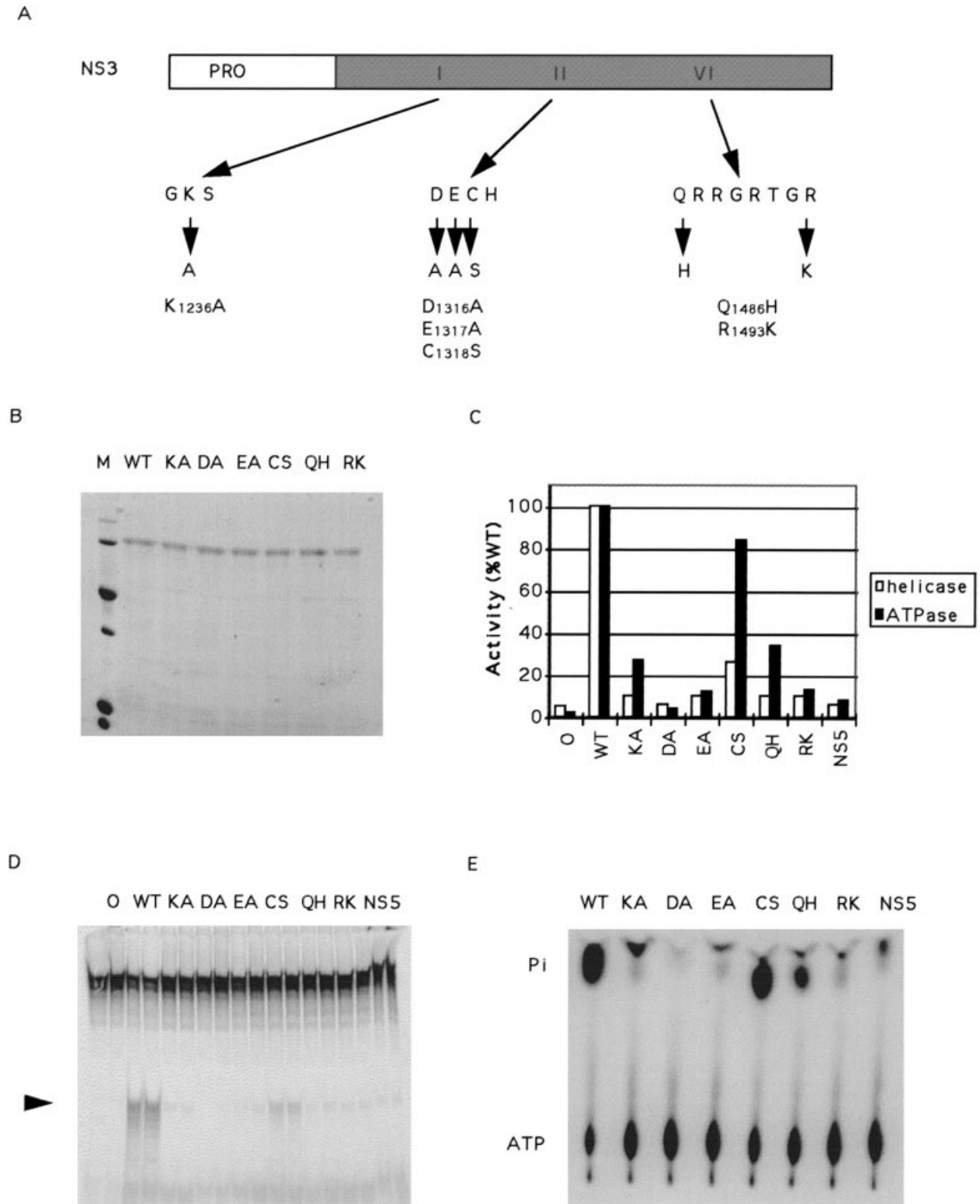


Fig. 6. Mutational analysis of NS3 NTPase and helicase activities. (A) Conserved motifs I, II and VI of DEAD-box helicases in relation to NS3 protein with protease domain shown (PRO). The amino acid residues substituted in this study and nomenclature of the mutants are shown. (B) SDS-PAGE analysis of purified NS3 mutants. Proteins were resolved using 10% (w/v) acrylamide and stained with Coomassie blue. (C) Helicase and NTPase activities of NS3 mutants displayed as a histogram; all

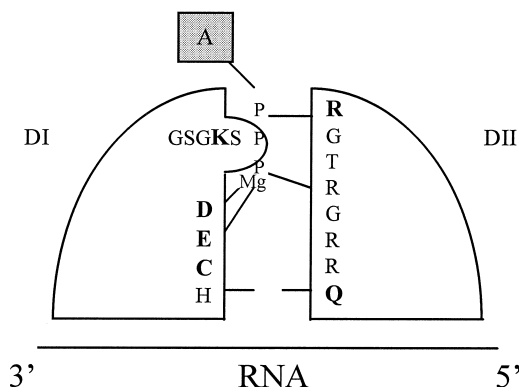


Fig. 7. Schematic representation of the NS3 NTP-binding pocket between domains I (DI) and II (DII) relative to RNA binding as proposed (Kim *et al.*, 1998); adapted from this reference. Conserved amino acid motifs I (G₁₂₃₃SGKS₁₂₃₇), II (D₁₃₁₆ECH₁₃₁₉) and VI (Q₁₄₈₆RRGRTGR₁₄₉₃) are shown. Amino acids substituted in this study are shown in bold. An ATP molecule is shown interacting with specific residues in DI and DII.

is stimulated by poly(U). This suggests that the two domains of NS3 may influence the activities of one another, and consequently provide a possible mechanism of regulation of their activities. In addition, NS3 has been shown to interact with host cell proteins such as protein kinase A and the tumour suppressor p53 and to affect their activities (Borowski *et al.*, 1996, 1997; Ishido *et al.*, 1997; Muramatsu *et al.*, 1997). These interactions, which affect the subcellular localization of NS3, are proposed to be due to the presence of further domains within the protein. It is therefore important to characterize full-length NS3 in order to fully understand its role in virus replication and how it may affect host cell functions.

In this study we have demonstrated the NTPase and helicase activities of full-length HCV NS3 expressed in *E. coli* and studied the effect of mutations on these activities. NS3 expressed in *E. coli* formed inclusion bodies which were purified and then refolded through a denaturation/renaturation procedure. This refolding strategy was necessary due to the very low levels of soluble NS3 observed in the cell extracts. It proved impossible to purify this apparently soluble moiety which is likely to be carry-over insoluble protein. NTPase and helicase activities were shown for NS3 protein isolated in this way. Activity was absent from control extracts of pHEXNS5A/B expressed in *E. coli*; this demonstrates that these were authentic activities of NS3 and not those of an *E. coli*-derived contaminant.

Optimal conditions for helicase and NTPase activity were shown to be similar to those previously reported for the helicase domain expressed alone (Suzich *et al.*, 1993; Tai *et al.*, 1996). However, NTPase activity was more stable at pH

greater than 7.0 than the helicase domain alone, as has also been shown with NS3–NS4A (Morgenstern *et al.*, 1997). In contrast, helicase activity was optimal at pH 6.5 both in our study and for the helicase domain alone (Tai *et al.*, 1996). The pH requirement for helicase activity of the NS3–NS4A protein was not shown (Morgenstern *et al.*, 1997). The difference between the pH optima of NTPase activity in full-length NS3 and the helicase domain may be a result of the protease domain stabilizing the structure of the helicase domain. An interesting difference observed between poly(U)-stimulated NTPase activity and helicase activity was the effect of monovalent cations. Helicase activity was much more sensitive to Na⁺ than was NTPase activity. This suggests that salt does not significantly affect NTP or polynucleotide binding but has an effect on either NS3 translocation, duplex separation or release of ssDNA.

The NTPase activity of NS3 was shown to be stimulated by all the polynucleotides tested. Poly(U) demonstrated the greatest stimulation, an enhancement of approximately 7-fold over activity in the absence of any polynucleotide. The other polynucleotides stimulated activity by 2- to 3.5-fold. In a study of the NS3 helicase domain (Suzich *et al.*, 1993) poly(U) was also shown to have the greatest stimulatory effect. However, poly(G) appeared to inhibit activity. The significance of this is unclear since in our study poly(G) clearly stimulated activity (by 2-fold) which could represent a difference in the biochemical properties of full-length and helicase domain proteins.

The ability of different NTPs to act as a substrate of full-length NS3 and provide an energy source for helicase activity was investigated. All NTPs tested were able to be hydrolysed by NS3 in the order ATP > dTTP > CTP = dATP = dCTP > UTP > GTP > dGTP. Previous studies of the substrate preferences of NTPase activities of helicase domain alone produced a similar profile (Suzich *et al.*, 1993). All NTPs were also able to be used by full-length NS3 to provide a source of energy for DNA duplex unwinding with a similar profile to their ability to be hydrolysed (ATP = dTTP = CTP = dATP = dCTP = UTP = GTP = dGTP). The slight differences between the abilities of the substrates to be hydrolysed and provide an energy source for unwinding may be a result of the different reaction conditions of NTPase and helicase assays, or a result of stabilization of unwound duplex by different NTPs.

HCV NS4A is an NS3 cofactor which binds to its N terminus and is required for optimal NS3 protease activity. It should be considered that NS4A binding may affect helicase and NTPase activities since poly(U) binding by NS3 has been shown to affect protease activity. The full-length NS3 described here may now be employed to investigate the effects of NS4A on helicase and NTPase activities.

activities are expressed as a percentage of wild-type (WT) activity. Data shown represent the average of two experiments and duplicate data-points varied by less than 20%. (D) Helicase activity of NS3 mutants (migration of release strand arrowed). (E) NTPase activity of NS3 mutants (positions of ATP and P_i indicated). WT, wild-type NS3; mutants KA, K₁₂₃₆A; DA, D₁₃₁₆A; EA, E₁₃₁₇A; CS, C₁₃₁₈S; QH, Q₁₄₈₆H; RK, R₁₄₉₃K. Control samples: O; no protein; NS5, NS5 polypeptide inclusion body control.

The effects of mutating the conserved motifs of DEAD-box NTPase/helicases have been studied and related to the proposed mechanism of ATP hydrolysis and helicase activity (Kim *et al.*, 1998). According to this model, the ATP molecule lies in an interdomain cleft where the β - and γ -phosphates interact with the phosphate-binding loop of motif I and with the acidic residues (D₁₃₁₆ and E₁₃₁₇) of motif II via Mg²⁺. The binding of arginine residues of motif VI to the β - and γ -phosphates closes the domain resulting in translocation of NS3 and unwinding of the bound duplex (Fig. 7). It is suggested that H₁₃₁₉ and Q₁₄₈₆ of motifs II and VI interact to assist opening and closing of this domain, thus coupling NTPase activity to unwinding. The mutagenesis data presented here, which demonstrate that D₁₃₁₆ and E₁₃₁₇ of motif II and R₁₄₉₃ of motif VI are required for NTPase activity and that C₁₃₁₈ of motif II is not essential, support this mechanism. The observation that Q₁₄₈₆H retains one-third of wild-type NTPase activity and yet is unable to unwind duplex DNA is consistent with the proposal that Q₁₄₈₆ is involved in the coupling of NTPase to helicase activity but not directly involved in NTP hydrolysis.

Substitution of the invariant lysine of motif I (K₁₂₃₆) for alanine in mutant K₁₂₃₆A resulted in 30% of wild-type NTPase activity. This observation was also made with the NS3 helicase domain alone (Kim *et al.*, 1997). However, this residue is present in all members of the DEAD-box family and is required for the binding of ATP by its side-chains. It has been shown to be essential for NTP hydrolysis in the closely related helicase of vaccinia virus, NPH-II (Gross & Shuman, 1995). Therefore, the dispensable nature of this residue was unexpected. Mutational studies of the NTPase activity of *E. coli* rho protein have shown that an alternate lysine residue is able to compensate for a similar mutation (Dombroski *et al.*, 1988) and it is possible that the proximal lysine (K₁₂₃₉) of HCV NS3 is also able to compensate for this mutation.

Mutant C₁₃₁₈S was demonstrated to have almost full NTPase activity and retained 20% wild-type helicase activity. This was in contrast to previous observations (Kim *et al.*, 1997) where mutational analysis of the helicase domain showed this mutation to abrogate both activities. In this study, substitution of this residue for glycine reduced NTPase activity but had little effect on helicase activity. Structural data have demonstrated that the cysteine residue is buried within the secondary structure (Kim *et al.*, 1998). These data suggest that the cysteine residue is not involved in the catalysis of hydrolysis or unwinding but that the introduction of novel side-chains reduces activity. The amino acid serine possesses a polar side-chain containing a hydroxyl group. It is possible that this side-chain is solvent exposed and interferes with the opening and closing of the two domains as shown (Fig. 7), thus affecting protein translocation and therefore unwinding. The effect on NTPase activity may be a result of side-chains interfering with nucleotide binding or due to uncoupling of the two activities. That this change has a greater effect on helicase activity of helicase domain than full-length NS3 suggests the presence of

structural differences that stabilize the active conformation in full-length NS3, thereby partially overcoming the effect of this mutation.

Here we have characterized the helicase and NTPase activities of the full-length HCV NS3 protein and have shown that optimal conditions and enzyme kinetics are essentially the same as for helicase domain alone. There were exceptions to this. For example, NTPase activity was more stable across a pH range in full-length NS3 than helicase domain. Differences were also observed where C₁₃₁₈, the variable residue of the DExH motif, was substituted for serine. This substitution was deleterious to both activities in the helicase domain alone but had little effect on NTPase and reduced helicase activity to only 20% of wild-type in full-length NS3. These results suggest subtle differences between activities of full-length and helicase domain NS3 which may be important for studying the biological role of NS3 and for the development of inhibitory compounds.

We would like to thank Lesley Fishburn for technical assistance during this work. This work was supported by grants from the Medical Research Council and Pfizer.

References

- Borowski, P., Heiland, M., Oehlmann, K., Becker, B., Kornetzky, L., Feucht, H. & Laufe, L. (1996). Non-structural protein 3 of hepatitis C virus inhibits phosphorylation mediated by cAMP-dependant protein kinase. *European Journal of Biochemistry* **237**, 611–618.
- Borowski, P., Oehlmann, K., Heiland, M. & Laufs, R. (1997). Nonstructural protein 3 of hepatitis C virus blocks the distribution of the free catalytic subunit of cyclic AMP-dependent protein kinase. *Journal of Virology* **71**, 2838–2843.
- Choo, Q. L., Kuo, G., Weiner, A. J., Overby, L. R., Bradley, D. W. & Houghton, M. (1989). Isolation of a cDNA clone derived from a blood-borne non-A, non-B viral hepatitis genome. *Science* **244**, 359–361.
- Choo, Q. L., Richman, K. H., Han, J. H., Berger, K., Lee, C., Dong, C., Gallegos, C., Coit, D., Medina-Selby, A., Barr, P. J., Weiner, A. J., Bradley, D. W., Kuo, G. & Houghton, M. (1991). Genetic organisation and diversity of the hepatitis C virus. *Proceedings of the National Academy of Sciences, USA* **88**, 2451–2455.
- Dombroski, A. J., Brennan, C. A., Spear, P. & Platt, T. (1988). Site-directed alterations in the ATP-binding domain of rho protein affect its activities as a termination factor. *Journal of Biological Chemistry* **263**, 18802–18809.
- Gorbalenya, A. E., Koonin, E. V., Donchenko, A. P. & Blinov, V. M. (1989). Two related superfamilies of putative helicases involved in replication, recombination, repair and expression of DNA and RNA genomes. *Nucleic Acids Research* **17**, 4713–4728.
- Grakoui, A., Wychowski, C., Lin, C., Feinstone, S. M. & Rice, C. M. (1993). Expression and identification of hepatitis C virus polyprotein cleavage products. *Journal of Virology* **67**, 1385–1395.
- Gross, C. H. & Shuman, S. (1995). Mutational analysis of vaccinia virus nucleoside triphosphate phosphohydrolase II, a DExH box RNA helicase. *Journal of Virology* **69**, 4727–4736.
- Gross, C. H. & Shuman, S. (1996). 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. *Journal of Virology* **70**, 1706–1713.

- Gwack, Y., Kim, D. W., Han, J. H. & Choe, J. (1995). NTPase activity of hepatitis C virus NS3 protein expressed in insect cells. *Molecules and Cells* **5**, 171–175.
- Gwack, Y., Kim, D. W., Han, J. H. & Choe, J. (1997). DNA helicase activity of the hepatitis C virus nonstructural protein 3. *European Journal of Biochemistry* **250**, 47–54.
- Hahm, B., Han, D. S., Back, S. H., Song, O. K., Cho, M.-J., Kim, C. J., Shimotohno, K. & Jang, S. K. (1995). NS3–4A of hepatitis C virus is a chymotrypsin-like protease. *Journal of Virology* **69**, 2534–2539.
- Heilek, G. M. & Peterson, M. G. (1997). A point mutation abolishes the helicase but not the nucleoside triphosphatase activity of hepatitis C virus NS3 protein. *Journal of Virology* **71**, 6264–6266.
- Iost, I. & Dreyfus, M. (1994). mRNAs can be stabilized by DEAD-box proteins. *Nature* **372**, 193–196.
- Ishido, S., Muramatsu, S., Fujita, T., Iwanaga, Y., Tong, W. Y., Katayama, Y., Itoh, M. & Hotta, H. (1997). Wild-type, but not mutant-type, p53 enhances nuclear accumulation of the NS3 protein of hepatitis C virus. *Biochemical and Biophysical Research Communications* **230**, 431–436.
- Jin, L. & Peterson, D. L. (1995). Expression, isolation and characterisation of the hepatitis C virus ATPase/RNA helicase. *Archives of Biochemistry and Biophysics* **323**, 47–53.
- Kim, D. W., Gwack, Y., Han, J. H. & Choe, J. (1995). C-terminal domain of the hepatitis C virus NS3 protein contains an RNA helicase activity. *Biochemical and Biophysical Research Communications* **215**, 160–166.
- Kim, J. L., Morgenstern, K. A., Lin, C., Fox, T., Dwyer, M. D., Landro, J. A., Chambers, S. P., Markland, W., Lepre, C. A., O'Malley, E. T., Harbeson, S. L., Rice, C. M., Murcko, M. A., Caron, P. R. & Thomson, J. A. (1996). Crystal structure of the hepatitis C virus NS3 protease domain complexed with a synthetic NS4A cofactor peptide. *Cell* **87**, 343–355.
- Kim, D. W., Kim, J., Gwack, Y., Han, J. H. & Choe, J. (1997). Mutational analysis of the hepatitis C virus RNA helicase. *Journal of Virology* **71**, 9400–9409.
- Kim, J. L., Morgenstern, K. A., Griffith, J. P., Dwyer, M. D., Thomson, J. A., Murcko, M. A., Lin, C. & Caron, P. R. (1998). Hepatitis C virus NS3 RNA helicase domain with a bound oligonucleotide: the crystal structure provides insights into the mode of unwinding. *Structure* **6**, 89–100.
- Koonin, E. V. (1991). Similarities in RNA helicases. *Nature* **352**, 290.
- Lieber, A., He, C. Y., Polyak, S. J., Gretch, D. R., Barr, D. & Kay, M. A. (1996). Elimination of hepatitis C virus RNA in infected human hepatocytes by adenovirus-mediated expression of ribozymes. *Journal of Virology* **70**, 8782–8791.
- Morgenstern, K. A., Landro, J. A., Hsiao, K., Lin, C., Gu, Y., Su, M. S. S. & Thomson, J. A. (1997). Polynucleotide modulation of the protease, nucleoside triphosphate and helicase activities of a hepatitis C virus NS3-NS4A complex isolated from transfected COS cells. *Journal of Virology* **71**, 3767–3775.
- Muramatsu, S., Ishido, S., Fujita, T., Itoh, M. & Hotta, H. (1997). Nuclear localization of the NS3 protein of hepatitis C virus and factors affecting localization. *Journal of Virology* **71**, 4954–4961.
- Pause, A. & Sonenberg, N. (1992). Mutational analysis of a DEAD box RNA helicase: the mammalian translation initiation factor eIF-4A. *EMBO Journal* **11**, 2643–2654.
- Selby, M. J., Choo, Q. L., Berger, K., Kuo, G., Glazer, E., Eckart, M., Lee, C., Chien, D., Kuo, C. & Houghton, M. (1993). Expression, identification and subcellular localization of the proteins encoded by the hepatitis C viral genome. *Journal of General Virology* **74**, 1103–1113.
- Suzich, J. A., Tamura, J. K., Palmer Hill, F., Warrenner, P., Grakoui, A., Rice, C. M., Feinstone, S. M. & Collett, M. S. (1993). Hepatitis C virus NS3 protein polynucleotide-stimulated nucleoside triphosphatase and comparison with the related pestivirus and flavivirus enzymes. *Journal of Virology* **67**, 6152–6158.
- Tai, C. L., Chi, W. K., Chen, D. S. & Hwang, L. H. (1996). The helicase activity associated with hepatitis C virus nonstructural protein 3 (NS3). *Journal of Virology* **70**, 8477–8484.
- Tomei, L., Failla, C., Santolini, E., De Francesco, R. & La Monica, N. (1993). NS3 is a serine protease required for processing of hepatitis C virus polyprotein. *Journal of Virology* **67**, 4017–4026.
- Wagner, J. D. O., Jankowsky, E., Company, M., Pyle, A. M. & Abelson, J. N. (1998). The DEAH-box protein PRP22 is an ATPase that mediates ATP-dependent mRNA release from the spliceosome and unwinds RNA duplexes. *EMBO Journal* **17**, 2926–2937.
- Wengler, G. (1993). The NS3 nonstructural protein of flaviviruses contains an RNA triphosphatase activity. *Virology* **197**, 265–273.

Received 28 July 1998; Accepted 30 October 1998