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Enzymatic Characterization of Hepatitis C Virus NS3/4A Complexes Expressed in Mammalian Cells by Using the Herpes Simplex Virus Amplicon System

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The hepatitis C virus (HCV) NS3 protein possesses three enzymatic activities: an N-terminal serine protease activity, a C-terminal RNA-stimulated NTPase activity, and an RNA helicase activity. To characterize them, the full-length $NS3_{631}$/4A and three C-terminal truncated proteases ($NS3_{201}$/4A, $NS3_{181}$/4A, and $NS3_{155}$/4A) were expressed in mammalian cells with HSV amplicon-defective viruses. Our results revealed that all of the NS3/4A proteins produced in mammalian cells (except $NS3_{155}$/4A) are active in processing both *cis* and *trans* cleavage sites. Temperature optimization studies revealed that the protease is more active at temperatures ranging from 4 to 25°C and is completely inactive at 42°C. The RNA-stimulated ATPase activity was characterized with a partially purified $NS3_{631}$/4A fraction and has a higher optimal temperature at 37 to 42°C. The effects of detergents on both NS3 protease and RNA-stimulated ATPase were similar. Nonionic detergents such as Triton X-100, Nonidet P-40 and Tween 20 did not affect the activities, while anionic detergents such as sodium dodecyl sulfate and deoxycholic acid were inhibitory. Zwitterionic detergent such as 3-[(3-cholamidopropyl)-dimethyl-ammonio]-1-propanesulfonate (CHAPS) inhibited protease activity at a concentration of 0.5% (8 mM), which had no effect on ATPase activity. Finally, RNA-unwinding activity was demonstrated in the $NS3_{631}$/4A fraction but not in the similarly purified $NS3_{181}$/4A and $NS3_{201}$/4A fractions. $NS3_{631}$/4A unwinds RNA duplexes with 3′ but not 5′ single-stranded overhangs, suggesting that the NS3 RNA helicase functions in a 3′-to-5′ direction.

Hepatitis C virus (HCV) is the major etiological agent of posttransfusion as well as community-acquired non-A non-B hepatitis (1, 7, 26). Chronic and persistent infection by HCV often leads to liver cirrhosis and hepatocellular carcinoma (4, 37).

HCV is believed to be a small enveloped virus containing a positive-stranded linear RNA genome of approximately 9.5 kb in size (23). It has been classified as a separate genus in the family of *Flaviviridae*, on the basis of its genome organization and hydropathy profile, which are similar to those of flaviviruses and pestiviruses (8, 35, 42).

The HCV genome encodes a single large polyprotein of approximately 3,010 amino acids (8, 42). Proteolytic processing by both host signal peptidases and viral proteases results in at least 10 viral proteins in the following order: NH_2-C-E1-E2-p7-NS2-NS3-NS4A-NS4B-NS5A-NS5B-COOH (16, 17, 32). C, E1, and E2 are believed to be viral structural proteins which are encoded by the 5′ one-fourth of the long open reading frame (ORF), and the role of p7 has not been established. The remaining viral proteins (NS2, NS3, NS4A, NS4B, NS5A, and NS5B) are believed to be nonstructural proteins, components of the viral replication machinery (22, 36).

Two virally encoded proteases in the HCV polyprotein have been identified: an NS2/3 metalloprotease and an NS3 serine protease (15, 18). The NS2/3 protease, a novel Zn^{2+}-dependent protease, is responsible for cleavage at the NS2/NS3 junction (15, 18). The NS3 protease is required for cleavages at the NS3/4A, NS4A/4B, NS4B/5A, and NS5A/5B junction sites (2, 14, 45). The catalytic domain of this protease has been mapped to the N-terminal 180-amino-acid region of NS3, containing a characteristic catalytic triad (His-Asp-Ser). The HCV NS3 protease is likely to be an essential enzyme for viral growth, because its analog from flaviviruses has been shown to be essential for viral growth (6). Thus, NS3 protease has become the focus of intensive studies to develop anti-HCV drugs.

Recent work from several laboratories has revealed that NS4A is a cofactor for the NS3 protease because it is absolutely required by the NS3 protease for cleavage at the 4B/5A junction (10, 38, 44). A similar requirement for a cofactor has been shown for flaviviruses (5, 12) and suggested for pestiviruses (49), indicating a common strategy utilized by members in the family *Flaviviridae*. The domains for physical interaction between NS3 and NS4A have been mapped to the N-terminal 22 amino acids of NS3 (11, 38, 44) and to the central region of NS4A (10, 34, 44). It is believed that NS4A stabilizes the active conformation of the NS3 protease domain and recruits NS3 to the membranes, where, presumably, proteolytic processing takes place (19, 33, 44).

In addition to the N-terminal protease domain, NS3 is thought to possess an RNA helicase domain in the C-terminal two-thirds of the NS3 protein on the basis of the presence of a nucleoside triphosphate (NTP)-binding motif and a DEAH box for RNA helicases (13). The RNA-stimulated NTPase activity (41) and the RNA helicase activity (25) of HCV NS3 have been demonstrated and require the C-terminal 465 amino acids of NS3. The RNA-stimulated NTPase activity (43, 47, 48)

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and the RNA helicase activity (46) for the flavivirus and pestivirus NS3 analogs have also been characterized.

The herpes simplex virus (HSV) amplicon has been developed as a novel eukaryotic expression vector, which contains an HSV *ori* for DNA replication and a cleavage/packaging *pac* signal (28, 40). In the presence of HSV helper virus, amplicon DNA is amplified into a head-to-tail concatemer, which is then packaged into the defective HSV particles up to one genome size (~150 kb) (40). One of the applications of the defective amplicon virus system is to efficiently transfer high copy numbers of foreign genes into a broad range of mammalian cells for high-level expression and gene therapy (24, 28). For this reason, we have chosen to use the amplicon system to produce high levels of the NS3 protease in mammalian cells.

In this report, we describe the characterization of three NS3-associated enzymatic activities expressed in mammalian cells: serine protease, RNA-stimulated NTPase, and RNA helicase. Our main objectives were (i) to produce large amounts of NS3 proteases using the mammalian cell expression system; (ii) to characterize the protease activities of the full-length $NS3_{631}$/4A protein as well as three truncated protease domains, $NS3_{201}$/4A, $NS3_{181}$/4A, and $NS3_{155}$/4A; and (iii) to characterize the $NS3_{631}$/4A-associated NTPase/RNA helicase activities.

MATERIALS AND METHODS

Cells, virus and antibodies. Rabbit skin cells (RSC) and human epidermoid 2 cells (HEp-2) were obtained from Bernard Roizman, University of Chicago. African green monkey kidney cells (Vero) were obtained from the American Type Culture Collection (CCL 81). Vero cells, RSC, and HEp-2 cells were grown in medium 199 (JRH Biosciences) supplemented with 5% fetal bovine serum (Bio Whittaker). The type 1 (HSV-1) *tsk* mutant, which has a temperature-sensitive mutation in the ICP4 gene, was obtained from H. S. Marsden at the MRC Virology Unit, Institute of Virology, Glasgow, United Kingdom. The mutant viruses were propagated at 33°C in HEp-2 cells with medium 199 containing 1% fetal bovine serum. Anti-NS3 (α-NS3) antiserum was raised in rabbits against a bacterially expressed $NS3_{183}$ protein, which was gel purified. Anti-NS4A (α-NS4A) antiserum was raised in rabbits against a 20-amino-acid peptide derived from the C terminus of NS4A. Anti-hemagglutinin (α-HA) is a monoclonal antibody against a hemagglutinin epitope from influenza virus purchased from Boehringer Mannheim Corp.

Construction of the amplicon vector and NS3/4A expression plasmids. The expression cassette of plasmid pCMVβ (Clontech), containing the major immediate-early promoter of cytomegalovirus as well as the simian virus 40 late region polyadenylation signal, was excised by *Sph*I and inserted at the *Bam*HI site of the amplicon vector, pF1′-Pα (27). A polylinker containing a series of unique restriction enzyme sites (*Stu*I-*Not*I-*Bgl*II-*Nsi*I-*Spe*I-*Kpn*I-*Acc*III) was inserted downstream of the cytomegalovirus promoter between the *Stu*I and the *Acc*III sites for convenience in introducing genes of interest. The map of the resulting amplicon plasmid, pF1′-CMV, is shown in Fig. 1. The T7-HCV expression plasmid, pBRTM/HCV(1-3011), which contains the entire ORF of the HCV-1a (H strain) genome, was obtained from Charles M. Rice, Washington University at St. Louis. The 5′ end of the NS3 gene was modified by inserting a linker containing a unique *Spe*I site and an ATG start codon. The 3′ end of NS4A was modified by removing most of the NS4B and all of the NS5 region downstream of the *Cel*II site. A stop codon (TAG) and a unique *Kpn*I site were inserted after the *Cel*II site. The resulting fragment contains the full-length $NS3_{631}$/4A and the N-terminal 15 amino acids from NS4B and thus possesses both *cis* and *trans* cleavage sites for the NS3 protease (see Fig. 1). The C-terminal truncated NS3/4A protease domains were created by PCR amplification of the corresponding fragments at the 5′ region of the NS3 ORF and then by linking the fragments to the *Nsi*I site near the 3′ end of the NS3 ORF. The resulting constructs, $NS3_{201}$/4A, $NS3_{181}$/4A, and $NS3_{155}$/4A, lack the C-terminal RNA helicase domain except for 11 amino acids (Thr-Cys-Met-Ser-Ala-Asp-Leu-Glu-Val-Val-Thr) at the very C terminus of NS3 (see Fig. 1). An influenza virus HA epitope containing 9 amino acids (Tyr-Pro-Tyr-Asp-Val-Pro-Asp-Tyr-Ala) was inserted in frame in front of the stop codon in all of the constructs, after the N-terminal 15 amino acids of NS4B. The HA epitope can thus be used to monitor the cleavage at the NS4A/4B site. All of the NS3/4A constructs were excised by *Spe*I and *Kpn*I and inserted into the amplicon vector pF1′-CMV.

Generation of the defective amplicon viruses. To generate the defective viruses, pF1′-CMV expression plasmids were transfected into RSC by using the calcium phosphate precipitation method and then by superinfection at 33°C by the helper virus, a temperature-sensitive HSV-1 mutant, *tsk* (24). The resulting viral stocks were then serially propagated in HEp-2 cells for at least three generations. Titers of the helper virus were determined (~10^6 to 10^7 PFU/ml) by plaque assaying at 33°C. The relative amounts of the amplicon DNA were determined by ^{32}phosphate labeling and restriction enzyme analysis of the amplicon DNA in comparison with the helper virus DNA. The titers of the amplicon-defective viruses were then estimated by the helper virus titers as well as the ratios of the amplicon DNA to the helper virus DNA (24).

Expression of the HCV NS3/4A proteins in mammalian cells. Vero cells were infected by the amplicon viruses at a multiplicity of infection of 3. Infected cells were incubated either at 37°C (nonpermissive temperature for the helper virus *tsk*) or at 33°C (permissive temperature). The cells were harvested 24 h postinfection and were lysed in a lysis buffer containing 50 mM *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid (HEPES; pH 7.3), 1 mM EDTA, 2 mM dithiothreitol (DTT), 50 mM NaCl, 1% Nonidet P-40, 10% glycerol, 300 nM antipain, and 200 nM leupeptin. Aliquots of the lysates which are equivalent to 5×10^4 cells were loaded onto a 10 to 20% sodium dodecyl sulfate (SDS)-polyacrylamide gradient gel. Expression and proteolytic processing of the NS3/4A products were detected by Western blotting (immunoblotting) analysis with NS3, NS4A, or HA-specific antibodies. All of the lysates can be stored at −80°C for months without loss of NS3 activities.

Partial purification of the $NS3_{631}$/4A complex. Four roller bottles of Vero cells were infected with the amplicon viruses at 37°C for 24 h. Cells were harvested and lysed in 10 ml of the lysis buffer. The cell lysates were precipitated with 25% saturated ammonium sulfate solution at 4°C. The precipitate was resolubilized in buffer A (25 mM HEPES [pH 7.3], 25 mM NaCl, 1 mM EDTA, 2 mM DTT, 0.1% Nonidet P-40, 10% glycerol) and loaded onto a Mono Q column. The NS3/4A complexes were eluted off the Mono Q column at 0.6 M NaCl. The resulting Mono Q fraction was then passed through a Mono S column and one-step eluted at 1 M NaCl. The resulting Mono S fraction was finally loaded on a poly(U)-Sepharose affinity column (Pharmacia). The $NS3_{631}$/4A complex was eluted off the column at 1.0 M NaCl. The resulting poly(U) fraction contains partially purified $NS3_{631}$/4A complex (~10 to 20% pure). Aliquots from each fraction during the purification were analyzed on an SDS-10 to 20% polyacrylamide gradient gel by Coomassie brilliant blue staining and Western blotting (see Fig. 4).

Immunoblotting analysis. Immunoblotting or Western blotting analysis was carried out as described previously (20). Briefly, proteins were separated on a 10 to 20% polyacrylamide gradient gel (Integrated Separation Systems) and were transferred onto an Immobilon P membrane (Millipore Corp.) with a semidry electroblotter (Integrated Separation Systems). Incubations of the membrane with primary antibodies or alkaline phosphatase-conjugated secondary antibodies took place in phosphate-buffered saline solution (PBS; pH 7.3) containing 1% evaporated milk and 0.3% Tween 20. The membrane was then washed in PBS containing 0.3% Tween 20 and was developed in the presence of chromogenic substrates, 1.7 mg of 5-bromo-4-chloro-3-indolyl phosphate (BCIP) per ml, and 3.3 mg of nitroblue tetrazolium.

In vitro *trans* cleavage assaying. A bacterial T7 expression plasmid, pTS102, containing a truncated ΔNS5A/B recombinant substrate (amino acid positions 2312 to 2621), was obtained from Elizabeth B. Smith (Schering-Plough Research Institute). The substrate was synthesized and ^{35}S labeled with in vitro transcription and translation systems (Promega). Labeled substrate contained the NS5A/5B cleavage site and had an apparent molecular weight of 45,000 by SDS-polyacrylamide gel electrophoresis (PAGE) analysis. Incubation of labeled substrate with the NS3/4A proteases resulted in the release of two products: the C-terminal portion of NS5A (ΔNS5A [18 kDa]) and the N-terminal portion of NS5B (ΔNS5B [27 kDa]) (see Fig. 3B). The in vitro *trans* cleavage assay was carried out at 25°C for 30 min in a buffer containing 50 mM Tris (pH 8.0), 0.5 mM EDTA, and 6 mM DTT, which was similar to conditions described by Bouffard et al. (3).

ATPase assaying. The ATPase assay has been described previously (30, 48). Briefly, ATPase assaying measures the hydrolysis of ATP to ADP, in which the enzymes were incubated with 10 μCi of [α-^{32}P]ATP at 37°C for 30 min in a 10-μl reaction mixture containing 50 mM HEPES (pH 7.3), 1 mM DTT, 100 μg of bovine serum albumin (BSA) per ml, 10 mM NaCl, 3 mM $MgCl_2$, and 5 mM ATP. The reaction was stopped by the addition of EDTA to a final concentration of 45 mM. A 0.5-μl volume of the reaction mixture was spotted on a polyethyleneimine-cellulose plate and was analyzed by thin-layer chromatography (43). RNA-stimulated ATPase assaying was carried out in the presence of 1 mM poly(U) homopolymer (Pharmacia).

Preparation of RNA duplexes and RNA helicase assaying. The substrates for RNA helicase assaying were prepared as described previously (30). Three types of RNA duplexes, standard, 3′-tailed, and 5′-tailed, were made and are shown in Fig. 6. The RNA helicase assay measures the displacement of the ^{32}P-labeled primers from the RNA duplexes. The reaction was carried out as described elsewhere (30). Briefly, partially purified $NS3_{631}$/4A was incubated with 50 fmol of ^{32}P-labeled RNA duplexes at 37°C for 30 min in a 20-μl reaction mixture containing 20 mM HEPES (pH 7.3), 2.5 mM $MgCl_2$, 5 mM ATP, 2 mM DTT, 100 μg of BSA per ml, and 1 U of RNase inhibitor (Prime Inhibitor; 5′→3′ Inc.). The reaction was stopped by the addition of 5 μl of RNA loading buffer (100 mM Tris-HCl [pH 7.5], 20 mM EDTA, 0.5% SDS, 0.1% Nonidet P-40, 0.1% bromophenol blue, 0.1% xylene cyanol, 50% glycerol). The entire mixture was then loaded onto a 15% polyacrylamide gel (30:1) and electrophoresed in 0.1% SDS and 0.5× Tris-borate-EDTA (TBE) buffer at 10 mA for 16 h. The gel was dried

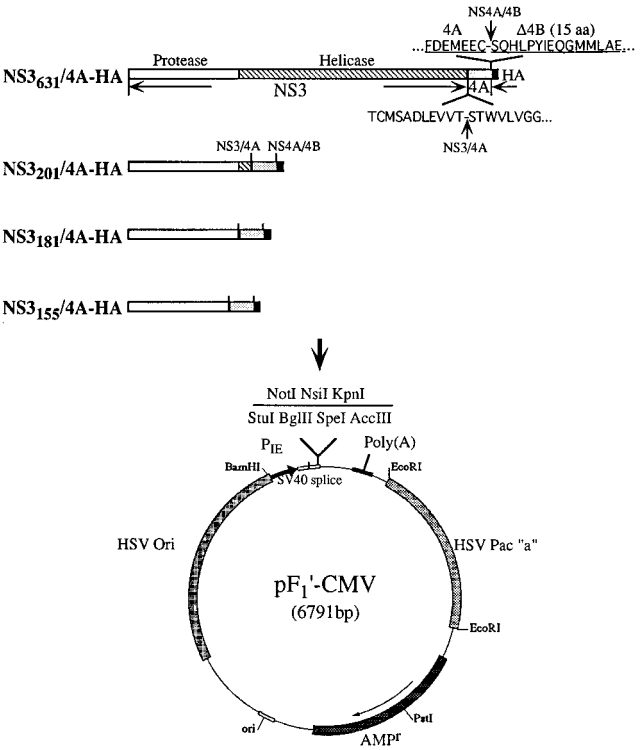

FIG. 1. Construction of the amplicon expression plasmids. The N-terminal protease domain (open bar) and the C-terminal RNA helicase domain (hatched bar) of NS3 are indicated. NS4A is indicated by a shaded bar. Amino acid sequences at both the *cis* (NS3/4A) and *trans* (NS4A/4B) cleavage sites are shown. Arrows, scissile bonds where the cleavages occur. The same *cis*- and *trans*-cleavable sites are present in all of the constructs. The amplicon vector pF1′-CMV contains both HSV *ori* (DNA replication origin) and HSV *pac* (packaging signal). P_{IE}, the cytomegalovirus (CMV) immediate-early promoter; SV40, simian virus 40; aa, amino acids.

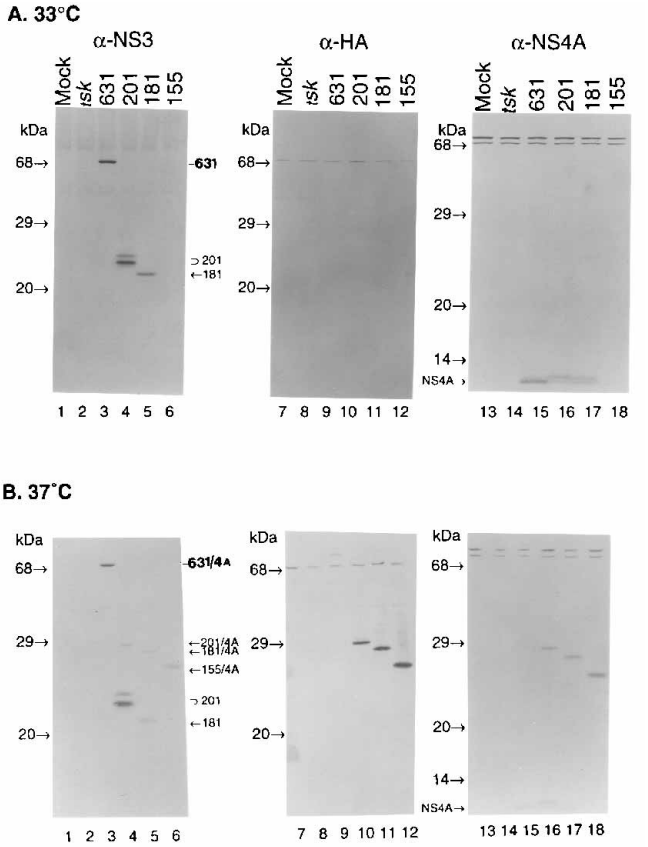

FIG. 2. Expression of NS3/4A-HA constructs in mammalian cells with the HSV amplicon system. (A and B) Expression at 33 and 37°C, respectively. Western blotting analyses were carried out with three different antibodies: α-NS3 (lanes 1 to 6), α-HA (lanes 7 to 12), and α-NS4A (lanes 13 to 18). 631, 201, and 181, $NS3_{631}$, $NS3_{201}$, and $NS3_{181}$, respectively; 631/4A, 201/4A, 181/4A, and 155/4A, the uncleaved precursors $NS3_{631}$/4A-HA, $NS3_{201}$/4A-HA, $NS3_{181}$/4A-HA, and $NS3_{155}$/4A-HA, respectively.

and exposed for autoradiography. The amount of helicase activity was determined by the appearance of the smaller RNA primer bands, which run faster than the RNA duplexes on the gel.

RESULTS

Construction of amplicon expression plasmids and generation of defective amplicon viruses. To achieve high-level expression of NS3 protease in mammalian cells, genes encoding the full-length $NS3_{631}$/4A as well as a series of the C-terminal truncated protease domains, $NS3_{201}$/4A, $NS3_{181}$/4A, and $NS3_{155}$/4A, were constructed and cloned into the amplicon vector (Fig. 1). All of the NS3 genes were fused in frame with the NS4A ORF because NS4A is required as a cofactor for NS3 protease activity (10, 38, 44). All of the constructs retained both the NS3/4A *cis* cleavage site and the NS4A/4B *trans* cleavage site as indicated (Fig. 1). An influenza virus HA epitope was fused to the C termini of all of the NS3/4A proteases and was used to monitor *trans* cleavage at the NS4A/B site.

Expression of proteolytically active NS3/4A complexes in mammalian cells. Amplicons containing different NS3/4A constructs were used to infect Vero cells at a multiplicity of infection of approximately 3. Infected cells were incubated at either 33 or at 37°C for 24 h. The products were analyzed by Western blots with three different antibodies, α-NS3, α-NS4A, or α-HA. The results demonstrated that the $NS3_{631}$/4A-HA, $NS3_{201}$/4A-HA, $NS3_{181}$/4A-HA, and $NS3_{155}$/4A-HA constructs were expressed at different levels (Fig. 2, left blots). The $NS3_{155}$/4A-HA protein was not detected at 33°C (Fig. 2A, lane 6) and was produced only at a low level at 37°C (Fig. 2B, lane 6). The expression level of $NS3_{181}$/4A-HA (Fig. 2A and B, lane 5) was somewhat lower than that of $NS3_{631}$/4A-HA or $NS3_{201}$/4A-HA (Fig. 2A and B, lanes 3 and 4). The $NS3_{201}$/4A-HA construct produced two $NS3_{201}$ products which might be due to N-linked glycosylation, since there is a putative N-linked glycosylation site at the C-terminal end of $NS3_{201}$. However, tunicamycin, a potent inhibitor of N-linked glycosylation, failed to inhibit the production of the larger $NS3_{201}$ product. Thus, it is not clear why more than one $NS3_{201}$ product was produced. All of the NS3/4A-HA products except for $NS3_{155}$/4A-HA are active in processing the *cis* cleavage site at the NS3/4A junction, releasing the NS4A protein shown at the bottom of the Western blot (Fig. 2A, right blot). A product which is slightly larger than the NS4A protein was also released from the $NS3_{201}$/4A-HA and $NS3_{181}$/4A-HA constructs. This product is not NS4A-HA because it cannot be detected by the α-HA monoclonal antibody (Fig. 2A, center blot). It is possible that this product results from inaccurate cleavage at the NS3/4A site because of spatial and conformational constraints created in the corresponding constructs. The observation that no products could be detected on the blot with α-HA monoclonal antibodies (Fig. 2A, lanes 9 to 11) suggested that the HA epitope was quickly removed because of *trans* cleavage at the NS4A/4B site. Interestingly, no

or very little precursors can be detected in the lysates from cells incubated at 33°C (Fig. 2A), suggesting that processing at both *cis* and *trans* cleavage sites must occur very rapidly. In contrast, at 37°C, uncleaved $NS3_{201}$/4A-HA, $NS3_{181}$/4A-HA, and $NS3_{155}$/4A-HA products which were readily detected by the α-HA antibody accumulated (Fig. 2B, lanes 10 to 12). A light band corresponding to the $NS3_{631}$/4A-HA precursor was detected at 37°C (Fig. 2B, lane 9) but not at 33°C (Fig. 2A, lane 9). The uncleaved precursors were also detected by the α-NS4A antibodies (Fig. 2B, lanes 16 to 18), and considerably less NS4A product was released at 37°C from *cis* cleavage. These results demonstrated that the NS3/4A-HA proteases expressed at 37°C were less active than those at 33°C, suggesting that the protease domain may not fold properly at higher temperatures.

Partial purification of the $NS3_{631}$/4A complex. It has been shown that the NS3 protease domain forms complexes with NS4A (11, 34, 38). The results shown in Fig. 2 suggested that the autoproteolytic processing of the NS3/4A-HA products occurred rapidly, removing the HA epitope and the N-terminal 15 amino acids of NS4B. Thus, the product from each gene construct was designated NS3/4A complex, such as $NS3_{631}$/4A complex, $NS3_{201}$/4A complex, and $NS3_{181}$/4A complex. To further characterize the protease activity as well as the NS3-associated NTPase/RNA helicase activities, we attempted to purify the $NS3_{631}$/4A complex expressed in mammalian cells. The purification includes 25% ammonium sulfate precipitation and then passage through Mono Q, Mono S, and poly(U) affinity columns (Materials and Methods). This protocol was utilized to purify both the $NS3_{631}$/4A and the $NS3_{201}$/4A complexes expressed in mammalian cells. The purification was monitored by Western blotting analysis with α-NS3 antibodies (Fig. 3A) as well as by a protease assay with a substrate containing the NS5A/5B site (Fig. 3B). Both the $NS3_{631}$/4A and $NS3_{201}$/4A complexes exhibited the same chromatographic behavior up until the final poly(U) affinity column passage, which was designed to purify the $NS3_{631}$/4A complex on the basis of the RNA-binding activity of the C-terminal RNA helicase domain (46). The $NS3_{201}$ protein was absent in the final poly(U) fraction (Fig. 3A, lane 12) because it lacks the RNA helicase domain and does not bind to the poly(U) column. RNA-stimulated ATPase activity was also analyzed at each purification step in the absence or presence of 1 mM poly(U) homopolymers (Fig. 3C). RNA-stimulated ATPase activity was observed in all of the $NS3_{631}$/4A fractions (lanes 2 to 11). In contrast, only background ATPase activity was detected in the starting lysate and the 25% ammonium sulfate precipitate of the $NS3_{201}$/4A fractions. This suggested that the endogenous ATPase activities were efficiently removed from the partially purified $NS3_{631}$/4A fraction. Proteins in each fraction during the purification were also analyzed on a 10 to 20% polyacrylamide gel stained with Coomassie brilliant blue. A 70-kDa protein band is present in the final poly(U) fraction derived from the $NS3_{631}$/4A lysate (Fig. 4A, lane 6) but not from the $NS3_{201}$/4A lysate (Fig. 4A, lane 7). Western blotting analysis confirmed that the 70-kDa protein is the full-length $NS3_{631}$ protease (Fig. 4B, lane 2). Although the NS4A protein is difficult to detect on the gel stained with Coomassie brilliant blue because of its small size (~6 kDa) (Fig. 4A, lane 6), it copurified with the $NS3_{631}$ protease as detected by α-NS4A antibodies (Fig. 4B, lane 5). The purity of the $NS3_{631}$ protein was estimated at approximately 10 to 20% compared with the amounts of other contaminating proteins, which were present in the final poly(U) fractions of both $NS3_{631}$/4A and $NS3_{201}$/4A (Fig. 4A, lane 6 and 7). The same protocol had been used to purify the $NS3_{631}$/4A complex produced in insect cells with the baculovirus system, which yielded a purity of approximately 90% (Fig. 4C, lane 1). The NS4A again copurified with the full-length NS3 protein (Fig. 4C, lane 2). The levels of expression as well as the enzymatic activities of the $NS3_{631}$/4A complexes from both expression systems (HSV amplicon versus baculovirus) are comparable (21). However, for large-scale production of the proteins, we prefer the baculovirus system.

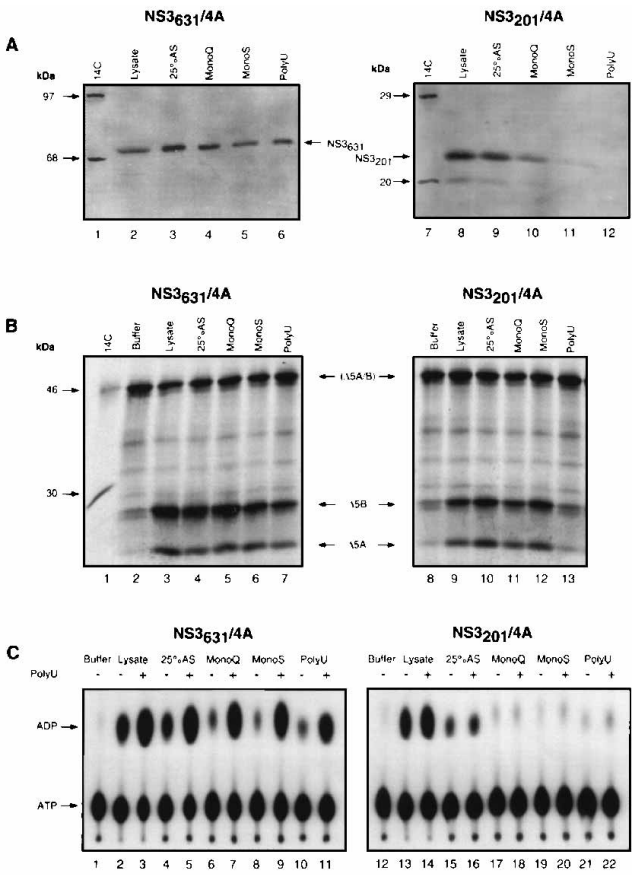

FIG. 3. Partial purification of the $NS3_{631}$/4A complex in parallel with the $NS3_{201}$/4A complex. (A) Monitoring of the $NS3_{631}$/4A complex (lanes 2 to 6) and the $NS3_{201}$/4A complex (lanes 8 to 12) during purification by Western blotting analysis with α-NS3 antibodies. (B) Monitoring of the purification by in vitro *trans* cleavage protease assaying with an in vitro-translated substrate containing the NS5A/5B site. Δ5A/B, the uncleaved substrate labeled with [^{35}S]methionine; Δ5A and Δ5B, cleavage products. Substrate alone (buffer) without NS3 proteins is the negative control, as indicated in lanes 2 and 8. Lanes 3 to 7, samples from the $NS3_{631}$/4A purification; lanes 9 to 13, samples from the $NS3_{201}$/4A purification. (C) Comparative analysis of NS3-associated ATPase activities during the purification of $NS3_{631}$/4A and $NS3_{201}$/4A. ATPase assaying was performed in the absence (−) or in the presence (+) of 1 mM poly(U) nucleotides, respectively. The amounts of samples used in the above analyses were normalized between $NS3_{631}$/4A and $NS3_{201}$/4A fractions by protein concentration.

Differential optimal temperatures for NS3 protease and NS3-associated RNA-stimulated ATPase. To further characterize NS3 protease activity and the NS3-associated ATPase activity, in vitro assays for both enzymes were established. To confirm the observations that NS3 proteases were less active when expressed at 37°C than at 33°C (Fig. 2), partially purified $NS3_{631}$/4A and $NS3_{201}$/4A were used in an in vitro assay to characterize the optimal temperatures for the protease activity. The results revealed that while the proteases were most active at lower temperatures ranging from 4 to 25°C, they were obviously less active at 37°C than at 30°C and were completely inactive at 42°C (Fig. 5A). The decrease in protease activity at

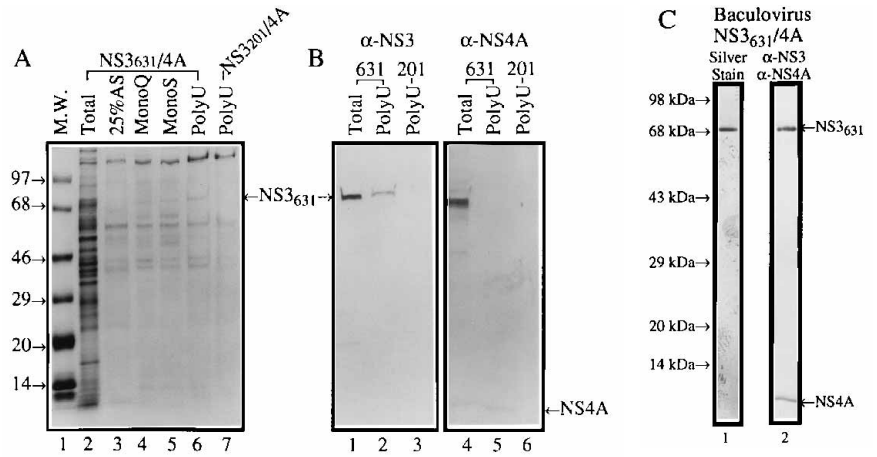

FIG. 4. Purity of the $NS3_{631}$/4A complex. (A) Protein analysis on an SDS-10 to 20% polyacrylamide gel stained with Coomassie brilliant blue. MW, molecular weight. (B) Western blotting analysis of the total lysate (lanes 1 and 4) and the poly(U) fractions (lanes 2, 3, 5, and 6) with α-NS3 antibodies (lanes 1 to 3) and α-NS4A antibodies (lanes 4 to 6). (C) SDS-PAGE analysis of the $NS3_{631}$/4A complex produced in a baculovirus expression system. Lanes: 1, silver stain; 2, Western blotting with both α-NS3 and α-NS4A antibodies.

higher temperatures is probably not due to any host-related protease inactivation, because no degradation was detected by Western blotting analysis before and after incubation at higher temperatures (data not shown). To characterize the optimal temperature for the NS3-associated RNA-stimulated ATPase activity, the ATPase activities of the poly(U) fractions from both $NS3_{631}$/4A and $NS3_{201}$/4A were compared. In contrast to the protease, the NS3-associated RNA-stimulated ATPase has a higher optimal temperature of 37 to 42°C (Fig. 5B). The optimal temperatures for both enzyme activities were also verified with the 90% pure $NS3_{631}$/4A derived from the baculovirus system (data not shown). One explanation for the differential optimal temperatures is that the NS3-associated RNA-stimulated ATPase domain may be more thermostable than the NS3 protease domain. To address the thermostability of both domains, heat inactivation of the $NS3_{631}$/4A complex was

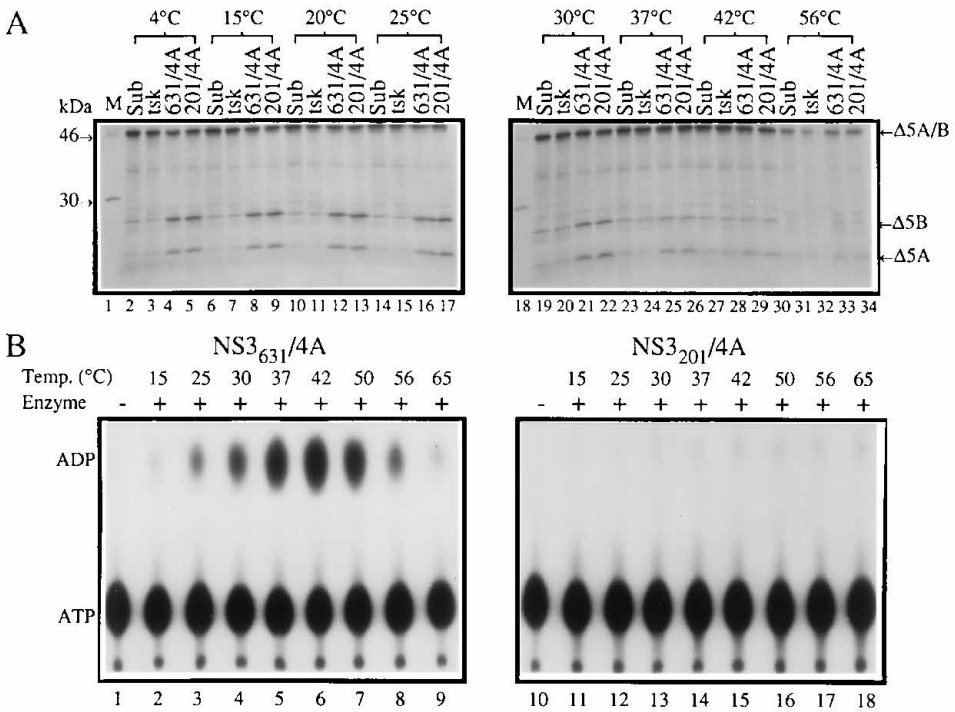

FIG. 5. Optimal temperatures for NS3 protease and RNA-stimulated ATPase. (A) Temperature optimization for protease activity with the in vitro *trans* cleavage assay. Sub, substrate alone; tsk, *tsk* helper virus-infected cell lysate; 631/4A and 201/4A, the corresponding Mono S fractions during the purification; Δ5A/B, the uncleaved substrate labeled with [^{35}S]methionine; Δ5A and Δ5B, the cleavage products. (B) Temperature (temp) optimization for RNA-stimulated ATPase activity in the presence (+) of 1 mM poly(U). The poly(U) fractions of $NS3_{631}$/4A (lanes 2 to 9) and $NS3_{201}$/4A (lanes 11 to 18) were used in the ATPase assay analyzed by thin-layer chromatography.

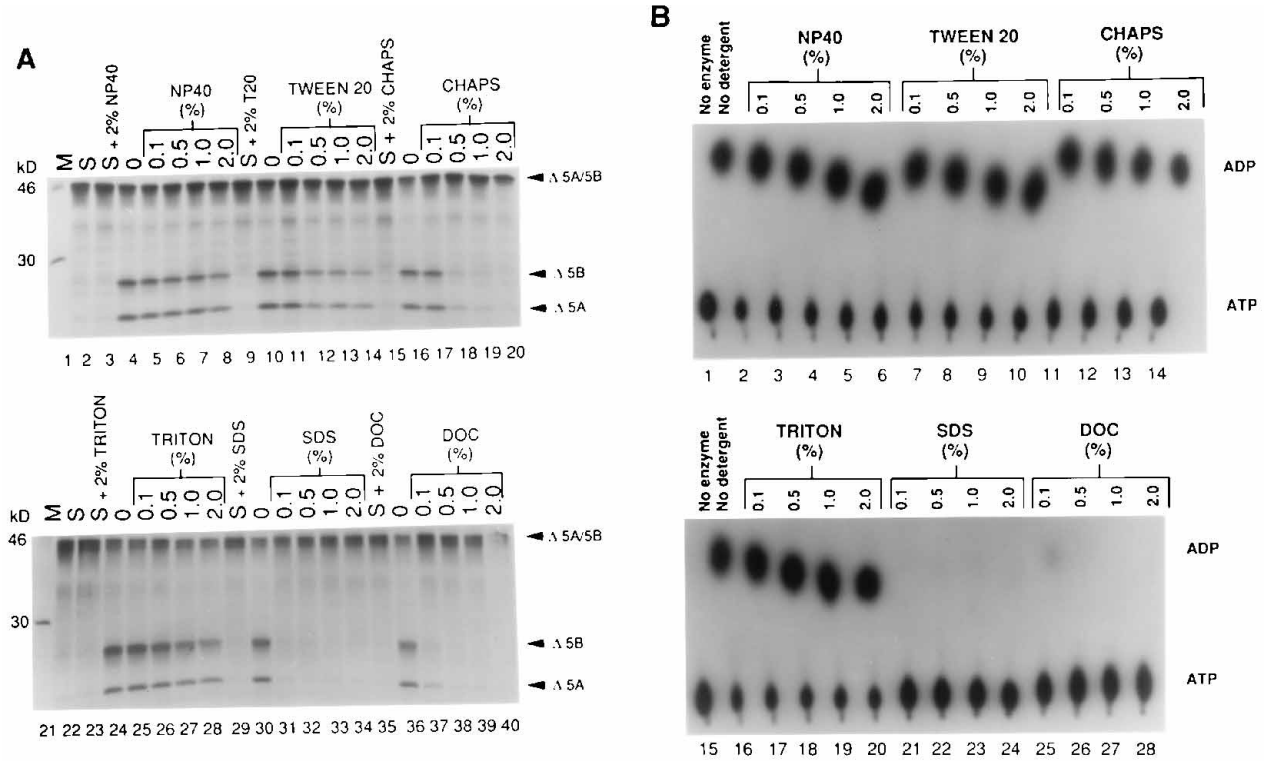

FIG. 6. The effect of detergents on NS3 protease and RNA-stimulated ATPase. (A) Effects of detergent on protease activity. S, substrate (Δ5A/B) alone without the $NS3_{631}$/4A complex; T20, Tween 20; Δ5A/B, uncleaved substrate labeled with [^{35}S]methionine; Δ5A and Δ5B, the cleavage products. (B) Effects of detergent on RNA-stimulated ATPase activity. The concentration of each detergent can be converted to molar concentrations as follows: 1% Triton X-100, 16 mM; 1% Nonidet P-40, 17 mM; 1% Tween 20, 8 mM; 1% SDS, 35 mM; 1% deoxycholate (DOC), 24 mM; 1% CHAPS, 16 mM.

carried out by preincubating the proteins at different temperatures. The results indicated that both enzyme activities were inactivated at 50°C after preincubation (21). Furthermore, the thermoinstability of the protease domain is probably not due to the dissociation of NS4A, since adding excess amounts of NS4A peptide (2 μM) failed to prevent the NS3 protease from being inactivated.

Detergent effects on the activities of NS3 protease and NS3-associated RNA-stimulated ATPase. It has been proposed that a potential role of NS4A is to recruit NS3 to membranes where the HCV virus assembly is believed to occur (19, 33, 44). Thus, it is interesting to know how detergents affect the NS3 functions. The effect of detergents on NS3 protease activity has been reported with somewhat contradictory results (3, 9, 33). We investigated the effects of detergent on both protease activity and RNA-stimulated ATPase activity. The results in Fig. 6 showed that while nonionic detergents such as Triton X-100, Nonidet P-40, and Tween 20 did not affect both enzymatic activities, anionic detergents such as SDS and deoxycholic acid were inhibitory. Interestingly, D'Souza et al. (9) reported stimulation of NS3 protease activity with a zwitterionic detergent, 3-[(3-cholamidopropyl)-dimethyl-ammonio]-1-propanesulfonate (CHAPS), at concentrations of up to 2.5% (40 mM); in our hands, 0.5% (8.0 mM) CHAPS is inhibitory (Fig. 6A, lane 18). However, RNA-stimulated ATPase activity was not affected (Fig. 6B, lane 12). Our results regarding the effects of detergent on protease activity are similar to those reported by Bouffard et al. (3), with the exception that, in their hands, 1% SDS was not inhibitory, whereas we observed complete inhibition with 0.1% SDS (Fig. 6A, lane 31). Although we failed to observe any significant stimulating effect on protease activity with the addition of detergents using the in vitro cleavage assay, in a more quantitative high-performance liquid chromatography assay, stimulation with detergent such as 0.1% Nonidet P-40 was detected (21).

The $NS3_{631}$/4A complex produced in mammalian cells has RNA helicase activity with a 3′-to-5′ directionality. An in vitro RNA-unwinding (strand displacement) assay for RNA helicase activity was developed under conditions similar to those described previously (30). Three types of RNA helicase substrates were prepared to determine RNA helicase activity and its directionality (31, 46). The poly(U) fractions were used to characterize the $NS3_{631}$/4A-associated RNA helicase with both $NS3_{181}$/4A and $NS3_{201}$/4A fractions as controls. As shown in Fig. 7A, the $NS3_{631}$/4A complex unwound the standard duplex RNA substrate (lanes 5 and 7), while the similarly purified $NS3_{181}$/4A and $NS3_{201}$/4A fractions failed to unwind the substrate (lanes 4 and 6). As a positive control, RNA helicase A (31) also unwound the standard RNA duplexes (Fig. 7A, lane 3). In a test of directionality, the $NS3_{631}$/4A-associated RNA helicase unwound the 3′-tailed RNA duplexes (Fig. 7B, lanes 4 and 5) but not the 5′-tailed RNA duplexes (Fig. 7C, lane 4 and 5), suggesting that the NS3 RNA helicase is a 3′-to-5′ RNA helicase. The control RNA helicase A has the same directionality as the NS3 RNA helicase (Fig. 7B, lane 3; Fig. 7C, lane 3). Further characterization to optimize the NS3 RNA helicase activity and to compare activities from different RNA helicase constructs has been carried out and will be presented elsewhere. RNA helicases from other positive-stranded RNA viruses such as the NS3 protein of bovine viral diarrhea virus

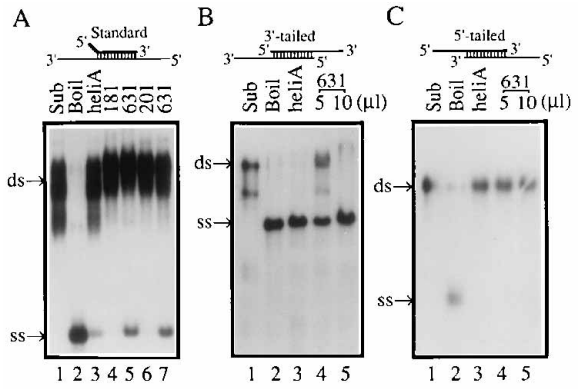

FIG. 7. NS3 RNA helicase activity. (A) RNA-unwinding activity with the standard substrate. Sub, RNA duplex; Boil, denatured single-stranded RNA molecules made by heating the RNA duplex at 95°C for 30 min; heli A, RNA helicase A; 181, 201, and 631, the partially purified poly(U) fractions of $NS3_{181}$/4A, $NS3_{201}$/4A, and $NS3_{631}$/4A complexes, respectively. (B) RNA-unwinding activity with the 3′-tailed substrate. (C) RNA-unwinding assay with the 5′-tailed substrate.

(46) and the cylindrical inclusion (CI) protein of plum pox viruses (29) have shown properties similar to those of the HCV NS3 RNA helicase.

DISCUSSION

In this report, we have demonstrated three enzymatic activities that are associated with the HCV NS3 protein. NS3 protease activity and NS3 RNA-stimulated NTPase activity have been characterized previously (2, 14, 41, 45). Our results have further extended the effort to characterize and optimize the effects of temperature on protease activity in comparison with NTPase activity. We have utilized the HSV amplicon system to express NS3/4A proteins in mammalian cells. More importantly, for the first time, conventional purification of the native $NS3_{631}$/4A complex without any fusion tag is presented, and we have demonstrated RNA helicase activity, the third activity of the NS3 protein.

An interesting finding in our report is the comparison of the optimal temperatures and the detergent effects on both protease and RNA-stimulated ATPase activities (Fig. 5 and 6). While the protease is most active at 4 to 25°C, the ATPase is more active at 37 to 42°C. Although a similar observation for the protease activity has been reported previously (3), it has not been discussed systematically. The difference in the optimal temperatures may suggest that the protease domain is less thermostable than the ATPase domain (Fig. 5). However, heat inactivation experiments have indicated that the thermostabilities are similar between the protease domain and the NTPase domain. Furthermore, inactivation of the protease may not be due to the dissociation of NS4A (21). Detergents seem to affect both activities similarly, although a slight difference in the effect of the zwitterionic detergent CHAPS was observed (Fig. 6B). These results suggest that both the protease domain and the RNA-stimulated NTPase domain have similar requirements for enzyme stability. In a recent report by Lin et al. (32), the protease was shown to be more active at 30 or 37°C, and the protease was inhibited by nonionic detergents at concentrations that did not affect the protease activity in our assays. Several reasons may explain the contradictory observations: (i) different systems were used to produce the enzymes for the in vitro cleavage assays, (ii) different substrates were used containing different cleavage sites, and (iii) different gene constructs were used. It is also quite possible that different HCV isolates or cDNA clones encode proteases with different temperature sensitivities. In our hands, the difference seems not to result from the temperatures at which various NS3 enzymes have been produced. We have generated our proteases at different temperatures (33 versus 37°C), which show similar requirements for lower optimal temperatures. Furthermore, after incubation at 37°C for 30 min, the protease is equally active compared with the protease incubated at 25°C (data not shown). The slower autoproteolytic processing of the NS3 protease when expressed at 37°C (Fig. 2) also confirms that the NS3 protease is less active at higher temperatures. This result seems to contradict the fact that HCV replicates in human liver, where the temperature is approximately 37°C. It is possible that there is a specific cellular or viral factor which stabilizes enzyme activity at 37°C. Alternatively, HCV may have developed a suboptimal enzyme activity to regulate its growth in humans, and this might reflect the observation that HCV replicates poorly in cultured cells (39, 50).

One role proposed for NS4A is to recruit NS3 to membranes for its activity (19, 33, 44) or to stabilize the active conformation of the protease domain. Indeed, it has been shown that NS4A forms complexes with the N-terminal domain of NS3 (11, 38, 44). We have demonstrated that NS3 and NS4A form stable complexes and are copurified during the entire purification procedures. The complexes are very stable and cannot be disrupted in the presence of 1% Nonidet P-40 detergent and 2 M salt (NaCl). Furthermore, the complexes are eluted off a gel filtration column (FPLC Superose 12 column; Pharmacia) right after the void volume (molecular weight, ≈2,000,000), suggesting that the NS3/4A complexes form aggregates, possibly with detergents in the form of micelles (21).

The RNA-stimulated ATPase activity in the poly(U) fraction of $NS3_{631}$/4A showed requirements for optimal activity similar to those previously reported (41), such as temperature, $MgCl_2$, and stimulation by polynucleotides with a preference for poly(U) (data not shown). This activity is also inhibited by KCl. A slight difference was observed, i.e., that $NS3_{631}$/4A has a slightly broader range of pH values for optimal ATPase activity (data not shown), in contrast to the stringent optimal pH (6.5) reported previously (41). The difference could be due to the fact that a truncated C-terminal domain was used in the previous characterization. The RNA helicase activity demonstrated in this report also showed properties similar to those reported for other positive-strand RNA viruses (29, 46). For example, it unwinds RNA duplexes only with the 3′-unpaired region and KCl is inhibitory. Further characterization of the HCV NS3 RNA helicase is currently in progress to determine: (i) if it binds to single-stranded RNA or DNA in the absence of ATP; (ii) if it can displace DNA/RNA hybrid duplexes or DNA/DNA duplexes; and (iii) what its requirements in the 3′ unpaired region for optimal RNA-unwinding activity are.

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