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Expression and Purification of a Hepatitis C Virus NS3/4A Complex, and Characterization of Its Helicase Activity with the Scintillation Proximity Assay System

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The C-terminal two-thirds of nonstructural protein 3 (NS3) of hepatitis C virus (HCV) possesses RNA helicase activity. This enzyme is considered to be involved in viral replication, and is expected to be one of the target molecules of anti-HCV drugs. Previously, we established a high-throughput screening system for HCV helicase inhibitors using the Scintillation Proximity Assay (SPA) system [Kyono, K. *et al.* (1998) *Anal. Biochem.* 257, 120–126]. Here, we show improvement of the preparation method for the HCV NS3/4A complex. Alteration of the expression region led to an increase in protein expression. The partially purified full-length NS3 protein showed higher NS3 protease activity without the cofactor NS4A peptide than the truncated protease domain with the cofactor peptide, suggesting that this protein formed a complex with NS4A. NS3 further purified to homogeneity, as judged on silver staining, remained in a complex with NS4A. Characterization of the helicase activity of this full NS3/4A complex using the SPA helicase assay system revealed that this enzyme preferred Mn^{2+}, and that the optimal pH was 6.0–6.5. The NS3/4A complex could act on a DNA template but could not unwind the M13DNA/DNA substrate.

Key words: HCV, NS3/4A complex, expression, helicase activity, SPA system.

Abbreviations: AcNPV, *Autographa californica* nuclear polyhedrosis virus; BVDV, bovine viral diarrhea virus; CBB, Coomassie brilliant blue; MES, 2-(*N*-morpholino)ethanesulfonic acid; MOPS, morpholinepropanesulfonic acid.

Hepatitis C virus (HCV) is the major etiologic agent for non-A, non-B viral hepatitis (*1*, *2*). Chronic and persistent infection by HCV often leads to liver cirrhosis and hepatocellular carcinomas (*3*, *4*). HCV is a positive-strand RNA virus and a member of family *Flaviviridae* (*5–7*). The viral genome comprises about 9,500 nucleotides and contains a single open reading frame, which encodes a polyprotein of 3,010 to 3,033 amino acids (*5*, *7*, *8*). This polyprotein is processed into structural (C, E1, and E2) and nonstructural (NS2, NS3, NS4A, NS4B, NS5A, and NS5B) proteins by both host signal peptidases and viral proteases (*9–12*). While the structural proteins arise through the action of host proteinases, the nonstructural proteins are produced by two viral proteinases. The NS2-NS3 junction is cleaved by a zinc-dependent proteinase composed of NS2 and the N-terminal one-third of the NS3 protein (*11*, *13*).

One third of the N-terminal of the NS3 protein (20 kDa) is a serine endopeptidase (hepacivirin, EC 3.4.21.98), which cleaves the NS3-NS4A, NS4A-NS4B, NS4B-NS5A, and NS5A-NS5B junctions (*11*, *14–18*), and has a chymotrypsin-like fold (*19*, *20*). This proteinase activity is thought to be necessary for viral replication, and many researchers are focusing on it as a therapeutic target.

The C-terminal two-thirds of NS3 contains conserved amino acid motifs predictive of nucleoside triphosphatase (NTPase, EC 3.6.1.15) and RNA helicases, which belong to the DEAD box protein family. In fact, some groups have demonstrated experimentally that this domain has RNA helicase activity and RNA-dependent NTPase activity (*21–27*).

Many RNA helicases and RNA-dependent NTPases from various organisms ranging from *Escherichia coli* to humans and viruses belong to the DEAD box protein family. These RNA helicases catalyze the unwinding of double-strand RNA and secondary (stem-loop) structures in a single-strand RNA, and are thought to be involved in RNA splicing, ribosome assembly, translational initiation, viral replication, and transcriptional processes (*28*, *29*). The helicases intrinsically possess NTPase activity, which hydrolyzes nucleoside triphosphates in the presence of RNA, and provides the energy source for unwinding. In the life cycle of HCV, NS3 helicase/NTPase is thought to unwind the secondary structure of (+)-strand genome or (–)-strand RNA, and to promote RNA synthesis by NS5B RNA polymerase. Therefore, NS3 helicase/NTPase, as well as NS3 protease, is an attractive target for the development of anti-HCV drugs.

Previously, we reported the establishment of a high-throughput screening system for HCV helicase inhibitors involving SPA technology, and a purification method for the HCV NS3 helicase involving an immunoaffinity column with rabbit polyclonal antibodies specific to NS3 (*30*). We obtained highly purified full-length NS3 protein, from which DNase and RNase were eliminated, and which was suitable for the mesurement of RNA helicase

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activity using this purification procedure. However, the amount of protein was insufficient for mass screening and a few impurities were present in the sample. Therefore, we attempted to improve the expression and purification step.

Here, we describe the expression and purification of the full-length NS3/4A complex, and also the characterization of its helicase activity using the SPA helicase system.

MATERIALS AND METHODS

Plasmid Constructions—Plasmid pQEtPRO-His, which was used to express a truncated protease domain (amino acids 1027–1207 of an HCV polyprotein) containing a 6xHis tag derived from the vector at the C terminus, was constructed by PCR amplification using a sense primer, 5′-GCCGCATGCATGCGCCCATCACGGCCTACTCC-3′, containing an *Sph*I site, an anti-sense primer, 5′-CGGAGATCTGGACCGCATAGTGGTTTCCAT-3′, containing a *Bgl*II site, and pUNS2D34D (*30*) as a template, and then the PCR-generated fragment was subcloned at the *Sph*I site and *Bgl*II site of pQE70 (Qiagen).

Baculovirus transfer vector pAcHLTNS34A, which was used to prepare the recombinant baculovirus expressing the NS3/4A protein, was constructed as follows: the 2.1-kb DNA fragment encoding the NS3-4A region (amino acids 1027–1711 of a HCV polyprotein) and the stop codon was amplified by PCR using a sense primer (5′-GCTGAATTCGCGCCCATCACGGCCTACTCC-3′) containing an *Eco*RI site, an anti-sense primer (5′-GCTCTGCAGTTAGCACTCTTCCATTTCATCGAAC-3′) containing a *Pst*I site and the stop codon, and pUNS2D34D as a template. The PCR-generated fragment was inserted into the *Eco*RI site and *Pst*I site of pAcHLT-A (PharMingen).

Preparation of the Truncated Protease Domain—The *E. coli* strain JM109 was transformed with pQEtPRO-His and then grown in an LB medium containing ampicillin. The expression of recombinant proteins was induced by adding 0.4 mM isopropyl-β-D-thiogalactopyranoside (IPTG) for 2 h at 30°C.

The truncated protease domain was purified using a gel filtration column (Superdex 75 pg 16/60, Amersham Pharmacia Biotech) after nickel column chromatography (Amersham Pharmacia Biotech). The purified proteins were kept in aliquots at –80°C until use.

Expression of NS3/4A Protein with a Recombinant Baculovirus System—Transfer vector pAcHLTNS34A was cotransfected with linearized AcNPV DNA (PharMingen) into Sf21 cells to obtain recombinant baculovirus BVHis-NS34A.

Sf21 cells, cultured in TNM-FH medium (Gibco BRL) supplemented with 10% fetal calf serum (FCS), were infected with the recombinant baculovirus. After 72 h culture, the cells were harvested and washed once with phosphate-buffered saline (PBS). The cell pellet was stored at –80°C until use.

Purification of Recombinant NS3/4A Protein—A membrane fraction was prepared as described previously (*30*), except that the solubilization was performed with nickel column starting buffer (20 mM Na-phosphate, pH 7.8, 500 mM NaCl, 0.1% Triton X-100, 10% glycerol) containing 0.9% Triton X-100 (final concentration, 1%). The solubilized suspension was centrifuged (8,700 ×*g*, 10 min). The supernatant was collected, passed through a 0.2 μm filter (Whatman), and then applied to a nickel column (HiTrap chelating, 1 ml, Amersham Pharmacia Biotech) equilibrated with the starting buffer. The column was washed with 10 column volumes of starting buffer containing 50 mM imidazole. Bound proteins were eluted with a linear gradient of imidazole (50 to 500 mM). Fractions of the eluate containing NS3/4A protein were pooled, and then dialyzed against 50% glycerol buffer (50% glycerol, 10 mM Tris-HCl, pH 7.5, 0.01% 2-mercaptoethanol, 0.01% Triton X-100) overnight at 4°C. A portion of the dialyzed sample was diluted four-fold with poly (U)-Sepharose 4B column starting buffer (20 mM Tris-HCl, pH7.5, 25 mM NaCl, 0.1% Triton X-100, 10% glycerol), so that the final concentration of glycerol was 10%, and then applied to a poly (U)-Sepharose 4B column (1 ml, Amersham Pharmacia Biotech) equilibrated with starting buffer. The column was washed with five column volumes of starting buffer, and then washed with same volume of starting buffer containing 10% of elution buffer (starting buffer containing 1 M NaCl). Bound proteins were eluted with 10 column volumes of elution buffer and the fractions containing NS3/4A protein were pooled, dialyzed against 50% glycerol buffer overnight at 4°C, and then stored at –80°C in aliquots.

Proteins were separated on PAGEL (ATTO, 10% or gradient gels of 5 to 20%) following Laemmli's method, and detected using a silver staining kit (Amersham Pharmacia Biotech). Western blot analysis was carried out using mouse anti NS3 monoclonal antibodies (Biodesign) or mouse anti NS4A monoclonal antibodies (The Research Foundation for Microbial Diseases of Osaka University, Biken), anti mouse IgG antibodies (Jackson), and an Enhanced Chemiluminescence System (Amersham Pharmacia Biotech).

Helicase Assay with the SPA System—Standard helicase substrates (RNA/DNA hetero duplex) were prepared as described previously (*30*). DNA/DNA substrates were prepared in the same way as the standard substrates. The template RNA and DNA were synthesized by Sawady Technology (Tokyo, Japan). The release strand DNA (referred to as annealed oligo. specific radioactivity >1,700 Ci/mmol) was purchased from Amersham Pharmacia Biotech. The sequences are shown below. Template RNA: 5′-ACGUAGGUUCUGAGGGUGGCGGUACUAACGUC-3′, 32mer; Template DNA: 5′-ACGTAGGTTCTGAGGGTGGCGGTACTAACGTC-3′, 32mer; Annealed oligo: 5′-TAGTACCGCCACCCTCAGAACC [^{3}H]T25–3′, 47mer.

The M13DNA/DNA substrate contained in the Helicase [^{3}H] SPA Enzyme Assay System (Amersham Pharmacia Biotech) was used.

Standard helicase reactions were carried out according to the manufacturer's manual for the Helicase [^{3}H]SPA Enzyme Assay System, unless otherwise indicated. The reaction mixture (50 μl), comprising 5 μl of purified NS3/4A protein, 1 μl (0.8 nM) of substrate (40 fmol, 6,250 cpm/pmol), 25 mM MES-KOH, pH 6.0, 0.01% BSA, 2 mM ATP, and 1.5 mM $MnCl_2$, was incubated at 37°C for 30 min in OptiPlate (Packard). The reaction was terminated by adding 10 μl of stop/capture reagent containing 0.9 pmol of capture oligo (Amersham Pharmacia Biotech), followed by incubation at 37°C for 15 min.

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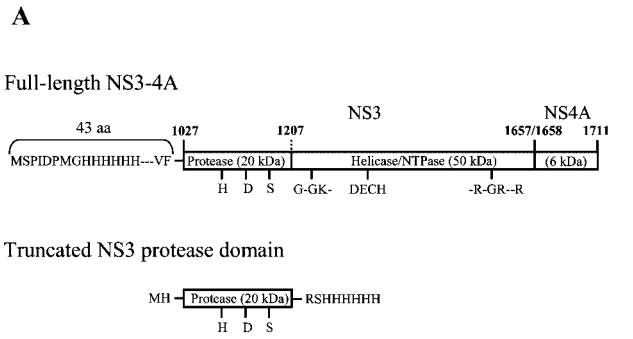

Fig. 1. **Expression and partial purification of the HCV NS3/4A complex.** (A) Schematic diagram of the constructs expressing the HCV NS3-4A protein and truncated protease domain. H, D, and S represent the catalytic triad amino acid residues His, Asp, and Ser, respectively, for serine protease activity. G-GK-, DECH, and -R-GR--R are the three consensus motifs required for helicase and NTPase activities. (B) CBB-stained SDS-polyacrylamide gel. A membrane fraction (lane 1) was prepared from BVHisNS34A-infected Sf21 cells, and purified by nickel column chromatography as described under "MATERIALS AND METHODS." Lanes: 2, flow-through; 3, 50 mM imidazole wash; 4–11, eluted fractions 1–8.

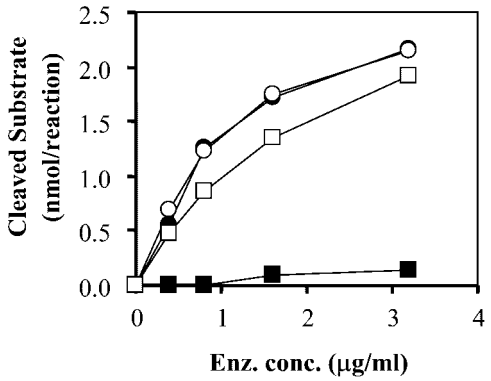

Fig. 2. **Protease activity of partially purified NS3/4A complex toward a peptide substrate corresponding to the NS5A-NS5B junction.** The purified truncated protease domain (solid squares and open squares) and partially purified NS3/4A complex (solid circles and open circles) were serially diluted, from 3.2 to 0.4 μg/ml, with 50 mM Tris-HCl pH 7.5, 30 mM NaCl, 1% Triton X-100, 10 mM DTT, 5 mM $CaCl_2$, and then used for measurement of protease activity in the presence (open squares and open circles) or absence (solid squares and solid circles) of the cofactor NS4A peptide as described under "MATERIALS AND METHODS." The amounts of cleaved substrates calculated based on the un-cleaved substrates were plotted against the concentration of the enzyme.

All experiments were performed in triplicate. Helicase activity was calculated as the % of unwinding (the percentage of the SPA count for annealed oligo unwound by the enzyme as to that of that unwound by heat denaturation).

Measurement of Protease Activity—The synthesized peptide substrate, which contains the cleavage site at the NS5A-5B junction, dansyl (Dns)-GEAGDDIVPCSMSYTWTK-Dns, and the synthesized cofactor peptide, which corresponds to amino acids 18 to 40 of the HCV NS4A protein, LTTGSVVIVGRIILSGRPAIIPD, were used. The peptide substrate and cofactor peptide were dissolved in DMSO to 10 mM and 20 mM as stock solutions, respectively. The synthesized peptide, Dns-GEAG, was used as the internal standard, and dissolved in DMSO to 10 mM as a stock solution. The reaction mixture (100 μl) contained various concentrations of purified protein, 100 μM cofactor peptide or 0.5% DMSO, 10 μM internal standard peptide, 25 μM substrate, 50 mM Tris-HCl, pH7.5, 30 mM NaCl, 1% Triton X-100, 10 mM DTT, and 5 mM $CaCl_2$. The reaction was started by the addition of the substrate, performed for 1h at 37°C, and stopped by the addition of 10 μl of 1% TFA. The reaction solution was analyzed with a HPLC (Shimazu LC-4A) and a fluorescence HPLC monitor (Shimazu RF-530). Protease activity was calculated using the height of the substrate peak corrected as to that of the internal standard peak.

RESULTS

Expression and Partial Purification of the HCV Full-length NS3/4A Complex—In this experiment, we intended to express the NS3-4A region (amino acids 1027 to 1711 of a HCV polyprotein), and to add a hexa histidine tag at its N-terminus to facilitate purification. Therefore, we subcloned the DNA fragment encoding the NS3-4A region into transfer vector pAcHLT-A. As a result, it was expected that 43 amino acids including the hexa histidine tag would be added at the N-terminus of the expressed protein (Fig. 1A). A recombinant baculovirus, BVHisNS34A, was generated and the NS3/4A protein was expressed in insect cells as described under "MATERIALS AND METHODS." As expected, NS3 formed a complex with NS4A, localized in the membrane fraction of insect cells infected with BVHisNS34A (Fig. 1B, lane 1). As the first purification step, the membrane fraction was applied to a nickel column. Since no detectable NS3/4A band was observed for the flow-through fraction, it is thought that almost all of the NS3/4A protein was bound to the column (Fig. 1B, lane 2). With a 50 mM imidazole wash, most of the impurities were removed from the column (Fig. 1B, lane 3). The NS3/4A protein was eluted in the range of 230 mM to 365 mM imidazole (Fig. 1B, lanes 7–10). Judging from the CBB stained gel, the purity was more than 80%. Eluted fractions 4 to 8 were pooled and dialyzed against 50% glycerol buffer.

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Table 1. **Comparison of protease activity between the NS3/4A complex and truncated protease domain.**

Enzyme	NS4A peptide	Specific activity (37°C for 1 h)	
		(mmol substrate/mg protein)	(mol substrate/mol enzyme)
Full 6His-NS3/4A	–	15.74	1,197
	+	15.26	1,160
Truncated protease-6His	–	0.00	0
	+	10.69	214

Evaluation of NS3/4A Complex Formation by Measurement of NS3 Protease Activity—To confirm that NS3 formed a complex with NS4A in this fraction, we evaluated the possibility of cleaving the peptide substrate containing an NS5A/5B junction. We used the truncated protease domain as the control enzyme. The truncated protease domain showed substrate cleavage in the presence of the cofactor peptide, but no activity in its absence (Fig. 2), while the NS3/4A protein cleaved the substrate irrespective of the presence or not of the cofactor peptide (Fig. 2). Since the lysate of the insect cells infected with the wild-type baculovirus (AcNPV) showed no cleavage activity toward the peptide substrate (data not shown), the cleavage activity observed in the eluate obtained on nickel column chromatography was thought to be due not to the proteases derived from host cells, but to the expressed NS3/4A protein. These findings suggested that NS3 formed a complex with NS4A. Furthermore, this complex showed 5-fold higher specific activity than the truncated protease domain (Table 1). Next, we performed the helicase assay using this NS3/4A sample as described under "MATERIALS AND METHODS." Although the helicase activity was ATP-dependent, no divalent cation-dependency was observed (data not shown). We concluded that this was due to the contamination by RNase and thus further purification was necessary.

Further Purification of the HCV Full-length NS3/4A Complex—The NS3/4A protein was further purified with poly (U)-Sepharose as described under "MATERIALS AND METHODS." We evaluated the purity by silver staining and Western blot analysis (Fig. 3A, B).

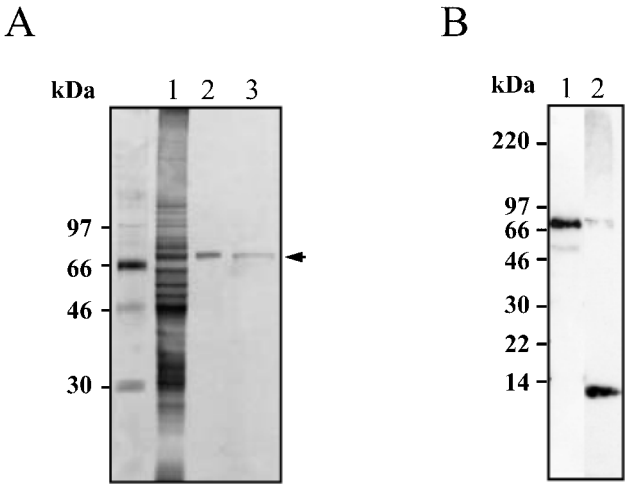

Fig. 3. **Analysis of the poly (U)-Sepharose affinity-purified NS3/4A complex.** (A) Silver-stained SDS-polyacrylamide gel. Lanes: 1, the membrane fraction; 2, eluate from the nickel column; 3, eluate from the poly (U)-Sepharose column. (B) Western blot analysis with HCV NS3-specific (lane 1) or NS4A-specific (lane 2) monoclonal antibodies of the poly (U)-Sepharose affinity purified material.

As shown in Fig. 3A, there was a single major band corresponding to 70 kDa for the poly (U)-Sepharose eluate (lane 2). Western blot analysis was carried out with anti-NS3 or anti-NS4A mouse monoclonal antibodies. In the case of anti-NS3 antibodies, a major band corresponding to 70 kDa and a faint band corresponding to 56 kDa were detected (Fig. 3B, lane 1). Considering the sizes of the polypeptides, the 56-kDa protein was thought to be a proteolytic product of NS3. Western blot analysis with anti-NS4A antibodies gave a major band, which corresponded to smaller than 6 kDa, and a faint band, which corresponded to slightly larger than in the case of NS3 (Fig. 3B, lane 2). It was suggested that this faint band corresponded to a 76-kDa NS3-4A protein, which remained uncleaved at the NS3/4A junction. Since this protein was not observable in the silver-stained gel, the content in the eluate was expected to be extremely low, if any at all.

From these data, we concluded that NS3 and NS4A formed a complex in the eluate obtained on poly (U)-Sepharose chromatography. Therefore, we attempted to use this enzyme for further experiments.

Characterization of the Helicase Activity of the HCV Full-length NS3/4A Complex with the SPA System—We next determined, using the SPA system, the optimal reaction conditions for the helicase activity of the HCV full-length NS3/4A complex, using the RNA/DNA. At first, the optimal concentrations of divalent cations were investigated. The helicase reactions were performed in the presence of various concentrations (0 to 8 mM) of either $MgCl_2$ or $MnCl_2$. As shown in Fig. 4A, the highest activity was observed at 1.5 to 3 mM (for $MnCl_2$), and 3 mM (for $MgCl_2$). $MnCl_2$ was more efficient than $MgCl_2$ under all conditions tested. The reaction conditions were further examined in the presence of 1.5 mM $MnCl_2$. Under the conditions of 1.5 mM $MnCl_2$ and 4 mM ATP, the optimal pH was 6.0 (Fig. 4B). Finally, the optimal concentration of ATP was determined in the presence of 1.5 mM $MnCl_2$ at pH 6.0. The helicase activity was maximal at concentrations higher than 1 mM (Fig. 4C). On the basis of the results obtained and shown above, the optimal conditions were determined and used in all subsequent experiments.

Next, we investigated the requirement of the HCV NS3/4A complex for nucleoside triphosphates (NTPs) other than ATP. With the standard substrate, the enzyme could utilize all NTPs and dNTPs, but showed less selectivity as to GTP and dGTP than the others (Fig. 5).

To determine whether or not the HCV full-length NS3/4A complex could act on the DNA template, two additional duplex substrates were prepared and used for the helicase assay as described under "MATERIALS AND METHODS." These substrates possessed the same release

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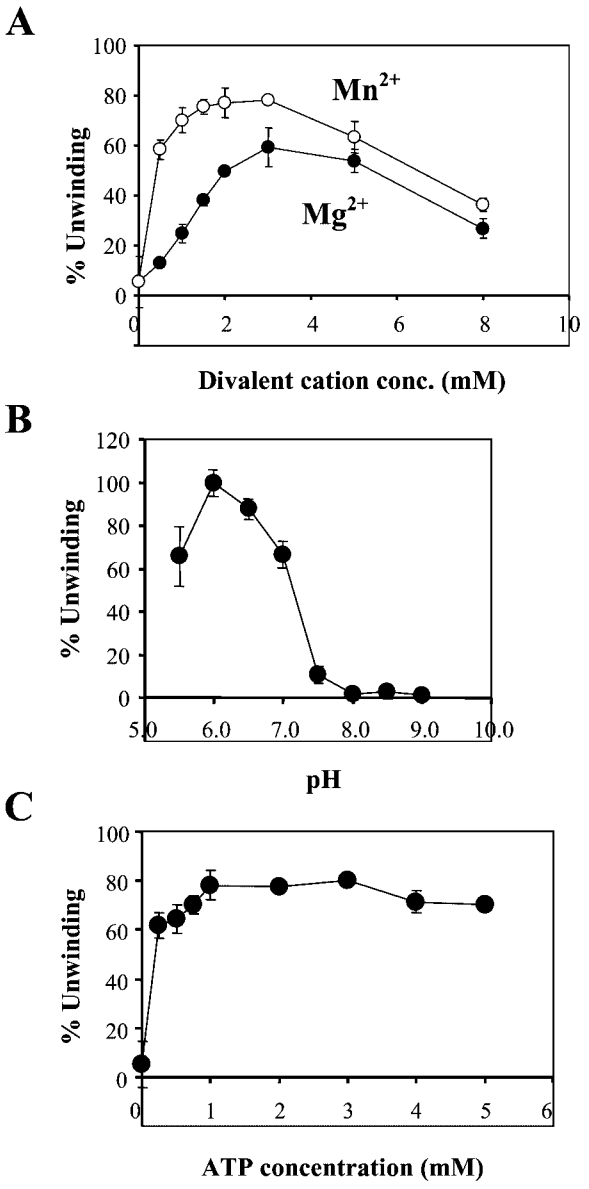

Fig. 4. **Optimization of the helicase reaction conditions for the HCV NS3/4A complex using the SPA system.** The activity was quantified by determining the percentage of unwinding with 0.8 nM standard substrate, as described under "MATERIALS AND METHODS." Each condition was tested in triplicate and the data are presented as means ± SD. (A) Determination of the concentrations of two different divalent cations, Mn^{2+} and Mg^{2+}, was performed in 25 mM MES (pH 6.0) with 4 mM ATP. (B) The optimal pH was determined in 25 mM buffer at the indicated pH with 1.5 mM $MnCl_2$ and 4 mM ATP. The buffers were as follows: pH 5.5 and 6.0, MES-KOH; pH 6.5 and 7.0, MOPS-KOH; pH 7.5–9.0, Tris-HCl. (C) Determination of ATP concentrations was performed in 25 mM MES (pH 6.0) with 1.5 mM $MnCl_2$.

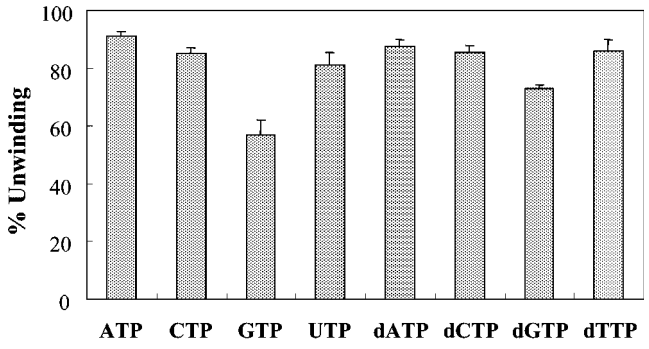

Fig. 5. **Requirement of the HCV NS3/4A complex helicase activity for NTPs and dNTPs.** The activity was measured under the standard reaction conditions as described under "MATERIALS AND METHODS." The indicated NTP was present in the reaction at 2 mM. The data are presented as means ± SD.

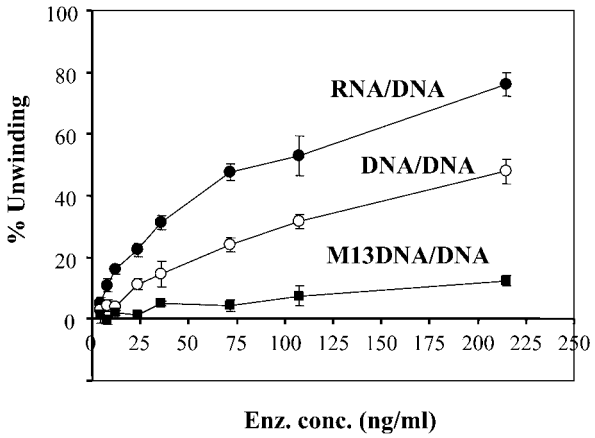

Fig. 6. **Substrate specificity of the HCV NS3/4A complex helicase.** Each substrate was prepared as described under "MATERIALS AND METHODS." The purified NS3/4A complex was serially diluted, from 215 ng/ml to 4 ng/ml, with 50% glycerol buffer and then incubated under standard reaction conditions with each substrate as described under "MATERIALS AND METHODS." The data are presented as means ± SD.

strand, only each template strand was different. The results shown in Fig. 6 indicate that the rate of unwinding of substrates increased with the enzyme concentration, and that most of the substrates were unwound at 11 ng/well of the enzyme with the RNA/DNA substrate. The standard substrate (RNA/DNA) containing the RNA template was the most preferable for the full-length NS3/4A protein. The enzyme could also act on the DNA template strand, which was similar to the RNA template in length and sequence, but almost completely did not recognize the M13DNA/DNA substrate.

DISCUSSION

The NS3 (p70) protein of HCV is a member of the DEAD box protein family, which includes numerous RNA helicases and RNA-dependent NTPases in prokaryotic and eukaryotic cells, and viruses. The DEAD box protein family consists of three subfamilies, DEAD proteins, DEAH proteins (*28*), and DEXH proteins (*29*, *31*). These subfamilies contain eight highly conserved domains. The first (AXXGXGKX), referred to as the A motif (*32*), is involved in ATP binding (*33*). The fifth (LDEAD), referred to as the B motif (*32*), is thought to be involved in ATP binding and/or ATP hydrolysis (*34*). The eighth (XRXGRXXR) is a basic domain, which possibly interacts with RNA. The HCV NS3 protein is classified into the DEXH protein subfamily based on the conserved motifs

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(*22*). Recently, mutational studies on (*35*, *36*) and the X-ray crystal structures of the HCV NS3 helicase domain were reported (*37–39*), and the roles of these conserved domains were elucidated.

When starting a project to search for helicase inhibitors, we intended to use not a helicase domain but a full-length NS3/4A complex, which was presumed to be a native form in HCV-infected cells, as an enzyme source. In a previous study, we used insect cells infected with recombinant baculovirus, which expresses a polyprotein comprising from amino acid 813 in NS2 to amino acid 1,723 in NS4B as a starting material. To eliminate the influence of insufficient cleavage at the NS2/NS3 junction by the NS2/3 protease on the expressed amount of the NS3/4A protein, we intended in this experiment to express the NS3-4A region (amino acids 1027 to 1207 of a HCV polyprotein). As a result, the expression level was improved because the NS3 protein was detected as a visible band in the CBB stained gel after a single preparation step for a membrane fraction (Fig. 1B, lane 1). The final protein amount obtained with the improved method was 35-fold more (about 14 μg per 75 cm^2 flask) than that with the immunoaffinity column method reported previously (0.4 μg per 75 cm^2 flask). This value corresponded to 933 μg of protein/liter of culture and is consistent with that reported by Sali *et al.* (*40*).

Since a hexa histidine tag was added to the N-terminus of NS3, we purified NS3 by nickel column chromatography and examined if the partially purified sample formed a complex with NS4A. While the truncated HCV protease domain required the presence of the cofactor NS4A peptide for the cleavage of the substrate peptide corresponding to the NS5A/NS5B junction, full-length NS3 could cleave it without the cofactor peptide (Fig. 2). Since it has been demonstrated that cleavage of the NS5A/NS5B junction was enhanced in the presence of NS4A (*41*), our results suggest that NS4A associated and was co-purified with NS3. The specific activity of our enzyme (1,160 mole-substrate per mole-enzyme) was about 8-fold higher than that reported by Sali *et al.* (148.3 mole-substrate per mole-enzyme, recalculated using reported K_m and V_{max} values with 25 μM substrate and 7 nM enzyme) (*40*). This discrepancy may be due to the different HCV strain used in the study (1b *versus* 1a by Sali *et al.*) or the different assay temperature (37°C *versus* 30°C by Sali *et al.*). The former is probable because according to their results, the NS3 protease activity at 40°C was at most twice as high as that at 30°C (*40*).

We successfully obtained a highly purified NS3/4A complex, which was suitable for measurement of helicase activity with poly (U)-Sepharose as the second step. The helicase activity of our prepared NS3/4A complex was optimal at pH 6.0 and pH 6.5. This profile differs from the pH optimum of 7.0 previously reported for a NS3 helicase domain (*27*) and a full-length NS3 (*42*, *43*). On the other hand, the results for a helicase domain reported by Gwack *et al*. are similar to ours (*25*).

With respect to the requirement of divalent cations, our enzyme preferred Mn^{2+} to Mg^{2+} at all concentrations tested.

The NS3/4A complex could utilize all NTPs and dNTPs (Fig. 5). Regarding NTPs, our results are consistent with those reported by Morgenstern *et al*. for the NS3/4A complex expressed in COS cells (*44*). Since it has been reported that for the BVDV NS3 RNA helicase, the efficiency of utilizing NTPs and dNTPs other than ATP was quite low (*45*), this phenomenon appears to be an inherent property of the HCV NS3 RNA helicase.

As has been reported by other groups (*25*, *27*, *35*), the NS3/4A complex could act on a DNA template. However, our finding that this enzyme could not unwind the M13DNA/DNA substrate is in contrast with the result reported by Heilek *et al*. (*35*). In our case, the enzyme might have been dispersed away from a release strand rather than an increase in the local concentration of the enzyme as they described, since the M13DNA template had extra sequences compared to the DNA template. On comparison between the RNA/DNA and DNA/DNA substrates, our enzyme was found to act on the former more effectively than on the latter. However, Pang *et al.* reported the opposite (*46*). It is thought that this discrepancy is due to the different assay methods (single-cycle conditions for Pang *et al.* and multiple cycle conditions for us). Pang *et al.* pre-incubated the NS3 and substrates to form a functional complex, and used a large excess of NS3 enzyme over the substrate (ratio of 70:1). Moreover, they initiated reactions by the simultaneous addition of ATP and a trap oligonucleotide. However, we did not perform pre-incubation of the NS3/4A and substrate. We initiated the helicase reaction by adding the reaction buffer containing ATP, $MnCl_2$, and the substrate, but not containing a trap oligonucleotide, to wells to which various concentrations of the enzyme had already been added, and then incubated the reaction mixtures for 30 min. Under these reaction conditions, it is thought that the enzyme can rebind to the substrates and unwind them during the reaction. Moreover, the molar ratio of NS3/4A to substrate under our conditions is in the range of 1:15.7 to 3.5:1, and this is standard for general enzymatic assays. While Pang *et al.* focused on the transient kinetics at each step of the helicase reaction, which was composed of the binding of the enzyme to the substrates and the unwinding of the substrates under unusual conditions, we examined a standard steady-state helicase reaction under physiological conditions.

The improvement of the expression level will enable us to supply sufficient NS3/4A complex for extensive screening for helicase inhibitors. Moreover, information obtained on characterization of this enzyme will be useful for evaluating selected inhibitors. The combination of high-throughput screening described here with a rational drug design using the X-ray crystal structure of single chain NS3-4A (*47*) is expected to accelerate the development of helicase inhibitors for anti-HCV drugs.

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