Modulation of the Hepatitis C Virus RNA-dependent RNA Polymerase Activity by the Non-Structural (NS) 3 Helicase and the NS4B Membrane Protein*

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The hepatitis C virus (HCV) nonstructural protein 5B (NS5B) is believed to be the central catalytic enzyme responsible for HCV replication but there are many unanswered questions about how its activity is controlled. In this study, we reveal that two other HCV proteins, NS3 (a protease/helicase) and NS4B (a hydrophobic protein of unknown function), physically and functionally interact with the NS5B polymerase. We describe a new procedure for generating highly pure NS4B, and use this protein in biochemical studies together with NS5B and NS3. To study the functional effects of the protein-protein interactions, we have developed an in vitro replication assay using the natural noncoding 3′ regions of the respective positive ((+)-3′-untranslated region) and negative ((−)-3′-terminal region) RNA strands of the HCV genome. Our studies show that NS3 dramatically modulates template recognition by NS5B and changes the synthetic products generated by this enzyme. The use of an NTPase-deficient mutant form of NS3 demonstrates that the NTPase activity (and thus helicase activity) of this protein is specifically required for these effects. Moreover, NS4B is found to be a negative regulator of the NS3-NS5B replication complex. Overall, these results reveal that NS3, NS4B, and NS5B can interact to form a regulatory complex that could feature in the process of HCV replication.

Hepatitis C virus (HCV) is a major pathogen of parenterally transmitted non-A, non-B hepatitis (1) and often causes the development of malignant chronic disease, including liver cirrhosis and hepatocellular carcinoma (2). With nearly 3% of the population of the world infected with HCV and no protective vaccine available at present, this disease has emerged as a serious global health problem since the virus was first identified (3, 4). HCV is a positive-stranded RNA virus with a genome of ~9400 bp. This genomic RNA initially directs the synthesis of at least 10 structural and nonstructural viral proteins (5). Following that, it is utilized by the viral RNA-dependent RNA polymerase (the nonstructural protein 5B; NS5B) as template to generate a complementary negative-stranded RNA. Once synthesized, the negative strands are transcribed into new molecules of positive-stranded genomic RNA, which in turn provide additional templates for viral protein synthesis as well as genomic RNA for the production of progeny virus (5, 6). However, the molecular events that mediate this process remain largely unclear.

Several attempts to dissect the mechanistic details of the viral replication cycle have been reported to date. The focal point of such investigations has been NS5B, which possesses an RNA-dependent RNA polymerase (RdRp) activity and is believed to be the key enzyme catalyzing HCV RNA synthesis (7–14). Its crystal structure reveals that it contains the classical finger, palm, and thumb subdomains of the polymerases with the unique feature of a more fully enclosed active site tunnel (15–17), and a recent report by Bressanelli and colleagues (18) has provided additional information about the complex of NS5B with ribonucleotides. Recombinant NS5B protein from different sources has been shown to replicate a range of natural and synthetic RNA templates, both in a primer-dependent and primer-independent fashion (19–25). Because NS5B does not discriminate against nonviral RNA templates in vitro, signal sequences present in the 3′-terminal regions of the genomic and antigenomic RNA as well as viral and/or cellular factors most likely confer the specificity of NS5B for viral replication in vivo. Furthermore, localization of the replication complexes to specific cellular compartments might also impose a degree of selectivity for the viral RNA. Recently, it has been suggested that NS5B guides the replication complex to the ER membrane (26).

Even though recent evidence has indicated that NS5B associates with other HCV viral proteins, including NS3, NS4A, and NS5A (27, 28), the direct influence of these proteins on the NS5B RdRp activity has been uncertain, with the exception that a recent report has indicated that NS5A modulates NS5B activity (28). NS3 possesses a serine protease domain in the NH2-terminal one-third of the protein and an NTPase/helicase domain that resides in the COOH-terminal 500 amino acid residues (5). In vitro analyses suggest that dimerization or oligomerization of NS3 is necessary for optimal helicase activity (29–31). Its helicase activity has been shown to be indis-
pensable for in vivo propagation of the virus, strongly suggesting that NS3 is a component of the replication complex (32). However, the mechanism of action of NS3 in promoting replication is not known.

The relatively hydrophobic protein NS4B is also supposed to interact with the replication complex. However, this 30-kDa protein is the least understood of the HCV proteins and has no known function. It is tightly associated with the endoplasmic reticulum membrane (33) and has been implicated in the phosphorylation of NS5A (34). It has the ability to transform NIH3T3 cells in cooperation with the Ha-ras oncogene (35) and recently it has been reported that NS4B inhibits the cellular translation apparatus (36). It has also been suggested that NS4B associates with NS3 via its interaction with NS4A (37). However, direct evidence for an interaction with NS4A has been lacking.

In this study, we investigated the possibility that NS3 and NS4B constitute part of an HCV replication complex. We have obtained direct biochemical evidence for physical interactions between NS3 and NS5B as well as between NS3 and NS4B. Moreover, the potential influence of NS3 and NS4B proteins on the priming activity of the viral polymerase was examined. Specifically, the 3′-terminal ends of the viral genome, namely the positive-strand (+3′)-UTR and negative-strand (−3′)-TR, were used as templates in an in vitro replication system, in which the effects of NS3 and NS4B on NS5B were monitored. Our data demonstrate that both proteins modulate the NS5B RdRp activity in quite distinct ways.

EXPERIMENTAL PROCEDURES

Plasmid Construction—The sequences corresponding to the 3′ ends of the plus and minus RNA strands of HCV were PCR amplified from the plasmids pHU304xT (38) and pACTEVEc2 UTR (kindly provided by Dr. Giuseppe Ciaramella, Pfizer, Sandwich, Kent, United Kingdom), using the following primers: 3′-TTR forward, 5′-ggagctccgggcccagctgccgac-3′, and 3′-UTR reverse, 5′-cggggggcccggccaggtgctgac-3′ for the plus strand; 5′-UTR forward, 5′-ggagctccgggcccagctgccgac-3′, and 5′-UTR reverse, 5′-ggagctccgggcccagctgccgac-3′ for the minus strand. The products from these PCR reactions were then cloned into the TA-vector (Inviogene). The positive clones were digested with BglII/HindIII and the fragments were then cloned in the pHST07 vector containing the δ ribozyme (38) inserted in the NotI/EcoRI sites, giving pHST7(+3′)UTR and pHST7(−3′)TR. The sequence corresponding to the (−3′)-TR was PCR amplified from the clone pHU304xT. The PCR primers were designed to introduce a T7 RNA polymerase promoter in the correct orientation. The PCR fragment was subsequently cloned in the BglII/EcoRI restriction sites of the pcDNA vector (Promega), giving pc(−5′)-TR. The plasmid pcNS3 contains the full-length NS3 coding region from genotype 1b cloned into the KpnI-EcoRI sites of the pcDNA vector under the control of the T7 promoter. The NS3 region was obtained via PCR using pHU304 (kind donation from Dr. Giuseppe Ciaramella, Pfizer) as template and the following two primers: forward, 5′-gggacccggttcagggggccggtc-3′, and reverse, 5′-gggacccggttcagggggccggtc-3′. Underlined nucleotides represent the restriction sites used for the cloning and the initiation and stop codons are in boldface. Underlined nucleotides represent the restriction sites used for the cloning.

Synthesis and Purification of Recombinant NS4B Protein—For the bacterial expression of recombinant NS5B, BL-21 Escherichia coli cells were transformed with pET21a5B (a kind gift of Dr. Helen Lavender, Pfizer), which encodes the consensus amino acid sequence of NS5B as published in Lohmann et al. (39). Transformed E. coli cells were grown at 37 °C in LB medium containing 100 μg/ml ampicillin to an optical density of 0.6 at 600 nm. Isopropyl-1-thio-β-D-galactopyranoside (Sigma) was added to a final concentration of 0.5 mM and the bacterial cells were grown for four additional hours at 22 °C and then pelleted by centrifugation and stored at −20 °C. For purification of NS5B, the bacterial pellets were resuspended in lysis buffer containing 50 mM Tris-HCl, pH 8, 400 mM NaCl, 0.05% n-octyl-β-D-glucopyranoside, 1% Triton X-100, 10 mM 2-mercaptoethanol, and 10% glycerol and loaded onto a Talon cobalt-based immobilized metal affinity chromatography column (Talon™, Clontech). The recombinant NS5B protein was eluted using a gradient of 50–500 mM imidazole. The fractions with the highest NS5B concentration were pooled together, dialyzed against a buffer containing 50 mM Tris-HCl, 400 mM NaCl, and 20% glycerol, and passed through a second Talon column. Again a gradient of 60–500 mM imidazole was used to elute the protein, and the fractions with the highest NS5B concentration were pooled together, dialyzed against a buffer containing 50 mM Tris-HCl, 150 mM NaCl, and 20% glycerol, and concentrated using a Centricon tube (Amicon).

Preparation of RNA Templates—For the preparation of unlabeled RNA templates for RdRp reactions, 100 μg each of the plasmids pHS7(+3′)UTR and pHS7(−3′)TR were linearized with EcoRI and transcribed in vitro using T7 RNA polymerase (New England Biolabs) in the manufacturer’s buffer supplemented with 25 mM MgCl₂, which proved in our system to be the concentration for optimal δ ribozyme function. The transcription reaction was carried out for 3 h at 37 °C. Subsequently, the reaction was loaded onto a 10% native polyacrylamide gel to separate the cleaved RNA from the precursor RNA and the δ ribozyme. The cleaved RNA was visualized by UV shadowing and eluted from the gel in 500 μl of RNA extraction buffer comprising 0.5 mM ammonium acetate, 0.05% SDS, and 2 mM EDTA, shaken for 4 h at 37 °C. The supernatants were collected via a 10-kDa centrifugal filter, and the pellet was washed with 70% ethanol, dried, and resuspended in diethyl pyrocarbonate-treated H₂O.

3P-Labeled marker RNAs were prepared using pHST7(+3′)UTR and pHST7(−3′)TR cleaved with NotI (see Fig. 3). The run-off transcripts prepared using these templates were 6 nucleotides longer than the RNAs generated by RdRp activity on the ribozyme-cleaved RNA templates.

In Vitro RdRp Incorporation Assay—In the standard assay, 15 pmol of purified NS5B protein were incubated with 3 pmol of RNA template in a 40-μl reaction mixture containing 20 mM Tris-HCl, pH 7.5, 5 mM MgCl₂, 25 mM KCl, 25 mM NaCl, 40 units of RNase inhibitor (Promega), and 1 mM dithiothreitol for 10 min at 37 °C. When additional proteins were studied, varying amounts of purified NS3 or NS4B were preincubated with the RNA template for 10 min at 37 °C. The reactions were initiated by the addition of a mixture of NTPs, comprising 0.5 mM each of ATP, CTP, GTP, and 16 μM UTP with 10 μCi of [α-32P]UTP (800 Ci/mmol; ICN), and incubated for 1 h at 37 °C, unless otherwise stated. 0.5 μl of diethyl pyrocarbonate-treated water was subsequently added to the mixture to terminate the reaction. After completion of the reaction, the products were extracted with acidic phenol/chloroform, precipitated with 2.5 volumes of 5 M ammonium acetate/isopropyl alcohol (1:5), and washed with 70% ethanol. The pellet was dried, resuspended in a denaturing buffer contain-
ing 95% formamide, 10 mM EDTA, 20 mM Tris-HCl, pH 8, heated at 95 °C for 5 min, and then resolved on an 8% urea, 5% polyacrylamide gel. After electrophoresis, the gels were dried and exposed to x-ray film.

In control experiments, the incorporation products were incubated with protease K (1 mg ml⁻¹) for 30 min before samples were phenol extracted and subjected to gel electrophoresis.

**Prime-dependent UMP Incorporation**—In this assay, 3 pmol of (+)-3′-UTR were first hybridized with 30 pmol of the following RNA oligos, p3UTR, 5′-ACAGAUICGCAAGA-3′, and p3STR, 5′-GCAGCCCCUCGUA-3′, respectively, in a buffer containing 40 mM PIPES, pH 6.4, 1 mM EDTA, pH 8, 0.4 mM NaCl, 80% formamide. The hybridization mixture was denatured at 95 °C for 5 min and incubated overnight at 50 °C. The RNA hybrids were precipitated overnight in 2 volumes of absolute ethanol and utilized for a typical NSSB RdRp assay as described above.

**Single-stranded DNA Binding Experiments**—Binding experiments were performed using a Beacon fluorescence polarization system (Pan Vera). Substrates (0.5 mM) were composed of a 5′-fluorescein-labeled 15-mer DNA of the sequence (dT) 15. Binding buffer consisted of 50 mM MOPS-K+, pH 7.0, and 50 mM EDTA. Binding of NS3 was measured by the change in polarization (mP). Binding data were fitted to a hyperbola using the program Kaleidograph (Synergy Software).

**ATPase Assays**—Spectrophotometric ATPase assays were performed using 10 mM enzyme, 0.05 mM poly(U), 50 mM MOPS, pH 7.5, 5 mM ATP, 10 mM MgCl₂, 4 mM P-enolpyruvate, 0.7 mg ml⁻¹ NADH, 10 units ml⁻¹ pyruvate kinase/lactate dehydrogenase, 0.1 mg ml⁻¹ BSA, and 10 mM NaCl at 37 °C. Oxidation of NADH was followed at 340 nm for 30 s (NSS) or 5 min (NS3 + NS4B).

**Northern Blot Analysis**—The NSSB RdRp reaction products were subjected to 5% 8 M urea-PAGE, transferred onto a nylon membrane (Hybond). After electrophoresis, the gels were dried and exposed to x-ray film. Northern hybridization was carried out overnight in 10 ml of aqueous solution containing 1 x NaCl, 1% SDS, 74.5 mM NaH₂PO₄, 25 mM Na₂HPO₄, 0.1 mM phosphate buffer, pH 6.4. The membrane was dried for 2 hours at 80 °C and exposed to a source of ultraviolet irradiation (254 nm) at 0.15 J/sq cm.

**Far-Western blotting analysis** was performed as described previously (41). More specifically, purified recombinant NS3 helicase and NSB, BSA, and poly(A)-binding protein fractions were subjected to SDS-PAGE on a 12.5% polyacrylamide gel and electrotransferred to a nitrocellulose membrane. The membranes were washed with buffer A (10 mM HEPESS-KOH, pH 7.5, 80 mM KCl, 1 mM EDTA, and 1 mM-mercaptoethanol) and incubated with 6 μg guanidine HCl for 15 min at 4 °C and then sequentially with 1.5, 0.75, 0.38, 0.19, and 0.09 μg guanidine HCl for 5 min each to renature the proteins. The membrane was subsequently blocked for 1 h at 4 °C with 5% nonfat dry milk and 3% BSA in buffer A containing 0.05% Nonidet P-40. The in vitro translated [35S]Met-labeled HCV NS5B, NS3, or eIF4A proteins were incubated with the membrane in buffer A containing 3% nonfat dry milk and 0.05% Nonidet P-40 overnight at 4 °C. Unbound proteins were removed by washing three times with buffer A containing 1% nonfat dry milk and 0.05% Nonidet P-40. Protein binding was detected by autoradiography. In experiments to control for RNA-mediated binding, 2 μg of RNAse A were added to each 40 μl of [35S]Met-labeled protein 15 min prior to adding the entire mixture to the blotted membrane.

**Enzyme-linked Immunosorbent Assay**—The enzyme-linked immunosorbent assays (42) were performed by immobilizing 0.5 μg of each target protein in 50 mM NaHCO₃, pH 9.6, per microtiter well plate overnight at 4 °C. After blocking with 1% phosphate-buffered saline, pH 7.5, containing 1% gelatin for 2 h at 37 °C, each protein ligand was incubated within the appropriate wells for 1 h at room temperature at a concentration of 5 μg ml⁻¹ in phosphate-buffered saline, pH 7.5, with 0.2% gelatin and subsequently analyzed for binding using an anti-poly-His antibody (Santa Cruz Biotechnology). In control experiments, the first antigen was omitted from the immobilization step. Incubation with each antibody was carried out in the same solution overnight at 4 °C.

**RESULTS AND DISCUSSION**

Purification of NS Proteins—At the outset of this work, recombinant NS4B was not available as a research reagent, and we therefore decided to develop a new procedure for generating this protein. Full-length NS4B protein was tagged at the amino terminus with a hexahistidine sequence and produced using a recombinant baculovirus system in Sf9 insect cells. After 36 h post-infection, NS4B became detectable as a prominent protein in total cell lysates, and was found to accumulate to high levels 48 h later (data not shown). The recombinant protein was purified using a Ni-NTA-Sepharose column and eluted using an imidazole gradient. The 30-kDa protein was further purified on a HQPoros perfusion chromatography column, and the peak fractions were then run on a second Ni-NTA-Sepharose column. Western blot analysis confirmed that this protein was recognized by an anti-poly-His antibody (Fig 1A). NSSB was also generated as a hexahistidine fusion protein in E. coli, and purified using repeated steps of cobalt affinity chromatography (Fig 1B, lane 1). NSSB was prepared according to a previously published procedure (40) (Fig 1B, lane 2).

**NSS3 Interacts with NS4B and NSSB in Vitro**—We investigated the potential interactions between the three HCV non-structural proteins using a combination of techniques. First, we performed far-Western protein blotting analysis. In this assay, purified recombinant full-length HCV NSSB protein, NS4B protein, and NS3 helicase protein were subjected to SDS-PAGE and transferred to a nitrocellulose membrane. Proteins on the membrane were denatured, renatured, and incubated with [35S]-labeled, in vitro synthesized HCV NS3 protein (Fig 2A) or...
Fig. 2. Interactions of HCV NS proteins 5B, 3, and 4B. A and B, far-Western protein blotting assays. Recombinant proteins were separated on 12.5% SDS-polyacrylamide gels and electrophoresed onto nitrocellulose membranes. The proteins on the membranes were denatured and renatured, and the membranes were then incubated with in vitro synthesized [35S]Met-labeled NS3 (A) and NS5B (C) proteins. Control experiments were performed in which the proteins were preincubated with RNase A (panels B and D), and where [35S]Met-labeled eIF4A was used as the potential ligand (E). Protein binding was detected by autoradiography. F, enzyme-linked immunosorbent assay analyses for interactions between NS3 and either NS5B or NS4B. NS3 was immobilized on a microtiter plate and allowed to react with either poly-His-NS5B or poly-His-NS4B, both of which were detected by an anti-poly-His antibody. In the control experiments marked as C on the x axes, NS3 was omitted from the immobilization step. Further controls were run using poly(A)-binding protein and BSA. As previously established, NS5B is able to initiate RNA synthesis on several specific and nonspecific templates, both in a primer-dependent and primer-independent manner. However, it has been shown that NS5B most likely uses only GTP or ATP as the initiating nucleotide. Interestingly, these are the 5′-terminal nucleotides of plus and minus strand RNA, respectively (19–21, 23–25, 44).

The ability of NS3 to interact with NS4B or NS5B was further assessed by means of a sandwich enzyme-linked immunosorbent assay procedure. NS3 was immobilized independently in microtiter wells and allowed to react either with NS4B or NS5B, which were in turn detected using an anti-poly-His polyclonal antibody. This analysis confirmed the physical interactions between NS3 and NS5B and between NS3 and NS4B (Fig. 2F). The above experiment was also performed with NS4B and NS5B immobilized independently in microtiter wells and the interacting NS3 protein was detected using an anti-NS3 antibody (data not shown). As with the other experiments, NS3 was found to bind to both NS5B and NS4B. In control enzyme-linked immunosorbent assay experiments, poly-His-tagged eIF1, poly-His-tagged eIF4A, and poly-His-tagged eIF4E-binding domain of eIF4G were substituted for the poly-His-tagged NS proteins (Fig. 2F). Again, no positive signals were obtained with these control proteins.

Fig. 2G summarizes the above findings by indicating the physical interactions between the respective NS proteins and RNA. It also points out that additional NS proteins (5A and 4A) may contribute to the activities of the replication complex. Because there is evidence that at least NS5B and NS3 function as oligomers (31, 43), uncertainty remains about the stoichiometry of the respective components of this complex.

Initiating RNA Synthesis at Accurately Synthesized 3′ Ends—As previously established, NS5B is able to initiate RNA synthesis on several specific and nonspecific templates, both in a primer-dependent and primer-independent manner. However, it has been shown that NS5B most likely uses only GTP or ATP as the initiating nucleotide. Interestingly, these are the 5′-terminal nucleotides of plus and minus strand RNA, respectively (19–21, 23–25, 44).

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Analysis of the RNA Products Generated by NS5B—We examined RNA synthesis catalyzed by the purified NS5B protein in a radioactive nucleotide incorporation assay using the new 3′
region RNA templates. No product was detectable when the (+)-3‘-UTR was used as template (data not shown). In contrast, NS5B generated the correct length positive strand product when provided with the ribozyme-cleaved (−)-3‘-TR RNA as template (Fig. 4A). This product is 383 nucleotides long and therefore runs somewhat faster than the T7 run-off transcript (lane T) that was used as a marker. The latter transcript was generated using the DNA template indicated in Fig. 3 after cleavage at the 3′ end with NsiI (and was therefore not exactly equivalent in length to the ribozyme-cleaved RNA product used as the template for NS5B). NS5B also generated a further product with very distinct mobility (marked b in Fig. 4A), together with a relatively weak product (marked a) that was not always readily visible in the gels. To ascertain whether the two major bands were genuine products of NS5B polymerization activity (as opposed to terminal transferase activity), we performed RNase protection analysis experiments (data not shown). When the products were hybridized with unlabeled (−)-3′-TR RNA, both bands were protected against RNase treatment, thus confirming the presence of the labeled, complementary strand in the RNAs synthesized by NS5B.

Because product b was observed to migrate faster than the template size RNA, one possibility was that it represents a premature terminated product of NS5B polymerase. However, a similar observation by Behrens et al. (8) led us to investigate whether product b is a folded RNA possibly generated by copy-back extension beyond the single-length template. The major and minor folded products resulting from copy-back extension beyond the single-length template are indicated by b and a, respectively. nt, nucleotide.

Further work confirmed that our NS5B preparation does not exhibit terminal transferase activity. Unlike polymerase activity on the RNA templates described in this work, any terminal transferase activity would be expected to manifest itself in the absence of a full complement of nucleotides. However, no labeling of template RNA was observed under such conditions, in contrast to the clearly identifiable generation of radiolabeled products seen when all four nucleotides were present (Fig. 5).

There is evidence that previous reports of terminal transferase activity associated with NS5B were in reality attributable to contamination from the host organism used to synthesize the protein (39).

**Modulation of NS5B Activity by NS3 and NS4B**

**Fig. 3.** Ribozyme constructs for generating (+)-3′-UTR and (−)-3′-TR RNA. Schematic representation of the constructs used for the generation of the templates for the incorporation assay. The two regulatory regions, namely (+)-3′-UTR (pHST7(+3′UTR; panel A) and (−)-3′-TR (pHST7(−3′TR; panel B), were cloned upstream of the δ ribozyme (38). Both regions were under the control of the T7 promoter. The cleavage site of the δ ribozyme is indicated by an arrow and the first three template nucleotides used to initiate RNA synthesis are underlined. The T₁ site for (+)-3′-UTR is a U and for (−)-3′-TR a C. The NotI and EcoRI restriction sites that were used for cloning and linearization of the vectors are indicated.

**Fig. 4.** Characterization of the products of RdRp activity. Comparison of the mobility of the NS5B RdRp products using (−)-3′-TR as template under alternative electrophoresis conditions. A, the NS5B RdRp products were separated on a 5% 8 M urea polyacrylamide gel. B, the NS5B RdRp products were subjected to highly denaturing 10% 8 M urea polyacrylamide gel electrophoresis at a temperature of ~80 °C. T denotes loading with the 32P-labeled template RNA generated by in vitro transcription of the DNA construct for (−)-3′-TR (Fig. 3B), whereas P denotes loading with the 32P-labeled products generated by NS5B acting on the unlabeled RNA template. The difference in the sizes of the respective RNAs is because of the fact that T represents a normal T7 run-off transcript synthesized after linearization of the corresponding plasmid with NotI, whereas the template used in the RdRp assay is created via cleavage with the δ ribozyme. The major and minor folded products resulting from copy-back extension beyond the single-length template are indicated by b and a, respectively. nt, nucleotide.
behavior of the $h_1$ and $h_2$ products, indicating that these are not RNA-protein complexes (Fig. 6A, lane 17, and B, lane 16).

**Do the Primer-initiated Products Differ?**—In the above experiments, we relied upon 3'-end-dependent initiation of replication to generate the observed RNA products. We next examined whether priming the reactions using short oligoribonucleotides complementary to the 3'-most segments of the RNA templates would change the nature and/or abundance of the products. More specifically, when a 14-nucleotide RNA oligo complementary to the (+)-3'-UTR (Fig. 7A) or the (-)-3'-TR template (Fig. 7B) was used in the RdRp assay, the newly synthesized high molecular mass RNA products were still observed. However, with the (-)-3'-TR template there was an increase in the amount of template-length (+) single-stranded copy RNA (running at the position of the 389-nucleotide RNA band) generated upon priming with the oligo (Fig. 7B). This suggests that there could be some communication between the initiation and termination sites on the RNA, perhaps mediated by NS5B oligomerization.

However, less total RNA product was formed in the presence of an RNA oligo. This was most likely because of the oligo-primed reaction being less efficient, and competing with 3'-end-dependent RNA synthesis. A potential explanation for the relatively poor priming by oligo primers can be found in the structure of NS5B, which includes a unique β-hairpin in the thumb subdomain that protrudes toward the active site of the enzyme. This may function as a selective mechanism that favors initiation at a single-stranded 3' end (19).

**Modulation of NS5B Function Is Specifically Dependent on NS3 NTPase/Helicase Activity**—The full modulatory effect of NS3 was only observed in the presence of a high concentration of ATP (5.5 mM) (Fig. 6A, lanes 14 and 16, B, lanes 12 and 14, and C) and the modulation was ATP concentration-dependent. The yield of the high molecular mass multimer products increased with increasing ATP concentration until a maximum intensity was reached at 5.5 mM ATP and above (Fig. 6C). Because the T$_{1}$ nucleotides for (+)-3'-UTR and (-)-3'-TR are U and C, respectively, the amount of ATP should not affect the initiation of RNA synthesis. Given that the helicase activity is coupled to ATP hydrolysis, it was important to determine whether this dependence of the modulation of the NS5B activity on ATP was linked to the helicase activity of NS3. To explore this relationship further, we used a mutant form of NS3 (NS3m), which is deficient in ATPase activity. The NS3m protein has mutations in the NTPase motif that inactivate both ATPase driven unwinding activity of the helicase domain.

**NS4B Is an Antagonistic Modulator of NS5B Activity**—We had also observed that NS4B is capable of forming a complex with NS3, and therefore wanted to know if this constitutes a further modulatory activity. The results of further replication assays revealed that NS4B suppresses the effect of NS3 on NS5B, preventing the formation of the additional high molecular mass product that is generated by virtue of extension of RNA synthesis beyond the full-length of the complementary...
**FIG. 6.** **NS3 protein modulates NS5B activity.** A, 18 pmol of purified NS5B were tested with different amounts of purified NS3 in the 
\[^{32}\text{P}\]\text{-UTP} incorporation assay using the (+)3'-UTR RNA as template. Except where indicated, reactions were carried out with ATP at a final concentration of 5.5 mM. Lane 1, \[^{32}\text{P}\]-labeled (+)3'-UTR RNA generated by \textit{in vitro} transcription; lane 2, negative control RdRp reaction with NS5B protein but without RNA template; lane 3, negative control RdRp reaction with no NS5B protein; lane 4, negative control RdRp reaction with NS3 protein and RNA template but without NS5B protein; lane 5, positive control RdRp reaction with (+)3'-UTR RNA as template and NS5B; lanes 6–13, NS5B RdRp reactions with increasing amounts of NS3 full-length protein (0.75, 1.5, 3, 6, 9, 12, 15, and 18 pmol, respectively); lane 15, NS5B RdRp reactions with NS3 helicase protein; lanes 12 and 14, NS5B RdRp reactions with NS3 or NS3 helicase, respectively, except that these reactions were carried out with ATP at a final concentration of 0.5 mM. Lane 17 shows the result of incubation of the RNA products from the experiment of lane 8 with proteinase K, followed by phenol extraction. Positions of RNA molecular mass markers are indicated on the left-hand side of the gel.

B, 18 pmol of purified NS5B were tested as in panel A except that (-)3'-TR RNA was used as template. Except where indicated, reactions were carried out with ATP at a final concentration of 5.5 mM. Lane 1, \[^{32}\text{P}\]-labeled (-)3'-TR RNA generated by \textit{in vitro} transcription; lane 2, negative control RdRp reaction with NS5B protein but without RNA template; lane 3, negative control RdRp reaction with NS5B protein and RNA template but without NS5B protein; lanes 5–11, NS5B RdRp reactions with increasing amounts of NS3 full-length protein (0.75, 1.5, 3, 6, 9, 12, 15, and 18 pmol, respectively); lane 13, NS5B RdRp reactions with NS3 helicase protein; lanes 12 and 14, NS5B RdRp reactions with NS3 or NS3 helicase, respectively, except that these reactions were carried out with ATP at a final concentration of 0.5 mM. Lane 16 shows the result of incubation of the RNA products from the experiment of lane 8 with proteinase K, followed by phenol extraction. RNA molecular mass markers are indicated on the left-hand side of the gel.

C, titration of the amount of ATP required for maximal enhancement of NS5B activity by NS3. ATP was present at concentrations of 5, 4, 3, 2, 1, and 0.5 mM, respectively (lanes 6–11). The controls (lanes 2–5) were the same as in panel A. D, Northern
on an 8 M urea, 5% polyacrylamide gel and electrotransfered to a nitrocellulose membrane. The membrane was subsequently hybridized against

reaction using (H11032/H11001-UTR RNA as template; [59x228]from 200 to 97 nM s

equimolar NS4B to NS3 reduced the rate of ATP hydrolysis

by an

-U TR RNA template. The molar ratio between the template and the oligoribonucleotide was 1:10. The single-copy product synthesis in the presence of the primer is indicated by a small arrow on the right-hand side of the gel in panel B.

strand (Fig. 9, lane 5). NS4B therefore acts as a regulator of the replication activity that restricts the formation of extended RNA products that are generated by copy-back priming. To investigate this property further, we examined whether NS4B affects NS3 ATPase activity per se. Using a standard ATPase assay (see “Experimental Procedures”), we found that NS4B acts as an inhibitor of the NS3 ATPase domain in the presence of poly(U). At 0.05 μM poly(U) and 10 nM NS3, the addition of equimolar NS4B to NS3 reduced the rate of ATP hydrolysis from 200 to 97 nM s⁻¹. Taken together, these results suggest that NS4B is a negative regulator of NS3 function, preventing it from modulating NS5B RdRp activity. This may be partially attributable to an inhibitory functional interaction between NS4B and the NS3 ATPase domain. Fig. 9 also illustrates another property of recombinant NS3, namely that its activity decreased with increasing age of the preparation. However, irrespective of the age of the NS3 preparation, the positive modulatory effect of NS3 and the antagonistic effect of NS4B were always evident.

Identification of New Modulators of NS5B—We conclude from the results reported here that NS3 and NS4B act as distinct types of modulator of NS5B function in the context of a putative replication complex. NS3 physically interacts with NS5B (Fig. 2G), acting to unwind intramolecular structure in the single-stranded RNA template and thus to facilitate both second strand synthesis and extension of the replication reaction beyond the first 5' end encountered by the polymerase via a copy-back or copy-over mechanism. The synthesis of products that represent multiples of the initial template molecule by NS5B has been described before (12). Whereas a complete analysis of these products is not within the scope of the present study, the results of RNase protection and Northern blotting experiments indicate that they comprise multiple copies of the positive and negative strand sequences represented by the templates. It will be important in future work to determine the exact structure and function of these complex products. As to their potential function, we note that hepatitis δ virus generates high molecular mass multimeric species of HDV RNA, which are in turn processed into monomeric and dimeric forms (45).

NS4B, on the other hand, may influence NS5B function indirectly by virtue of its interaction with NS3, and possibly also via direct binding to NS5B (Fig. 2G). Not only does NS4B partially inhibit the NTPase activity of NS3, but it also suppresses the modulatory effect of NS3. This latter influence manifests itself as the almost complete elimination of the high

FIG. 7. Primer-dependent RNA synthesis. Comparison between NS5B/NS3 RdRp primer-dependent and primer-independent replication. A, lane 1, 32P-labeled (+)-3'-UTR RNA generated by in vitro transcription; lane 2, negative control RdRp reaction containing only the (+)-3'-UTR RNA template; lane 3, negative control RdRp reaction containing only NS5B protein but without RNA template; lane 4, negative control RdRp reaction containing only NS3 protein and the template; lane 5, positive control RdRp reaction containing (+)-3'-UTR RNA template (3 pmol), NS5B protein (18 pmol), and NS3 protein (18 pmol); lane 6, same as lane 5, but with the addition of a 14-nucleotide RNA oligonucleotide complementary to the 3' end of the (+)-3'-UTR RNA template. The molar ratio between the template and the oligoribonucleotide was 1:10. B, lane 1, 32P-labeled (-)-3'-TR RNA generated by in vitro transcription; lane 2, negative control RdRp reaction containing only the (-)-3'-TR RNA template; lane 3, positive control RdRp reaction containing (-)-3'-TR RNA template (3 pmol), NS5B protein (18 pmol), and NS3 protein (18 pmol); lane 4, same as lane 3, but with the addition of a 14-nucleotide RNA oligonucleotide complementary to the 3' end of the (-)-3'-TR RNA template; the ratio between the template and the oligoribonucleotide was 1:10. The single-copy product synthesized in the presence of the primer is indicated by a small arrow on the right-hand side of the gel in panel B.

FIG. 8. Modulation of NS5B function is specifically dependent on NS3 NTPase/helicase activity. A, the effect of the wild type NS3 (NS3wt) on NS5B RdRp activity was compared with that of an ATPase-deficient NS3 mutant protein (NS3mt) using the [α-32P]UTP incorporation assay as described under “Experimental Procedures.” Lane 1, 32P-labeled (+)-3'-TR RNA generated by in vitro transcription; lane 2, positive control RdRp reaction containing (+)-3'-TR RNA template (3 pmol) and NS3 wt protein (18 pmol); lanes 3–5, NS5B RdRp products obtained with increasing amounts (5, 6, and 18 pmol, respectively) of NS3 wt; lane 6, negative control RdRp reaction containing (−)-3'-TR RNA template and NS3m but no NS5B; lanes 7–9, RdRp products obtained with increasing amounts (3, 6, and 18 pmol, respectively) of NS3m. B, far-Western analysis indicates that NS3m binds to NS5B with the same affinity as wild-type NS3. The procedure used was the same as that in Fig. 2A. C, NS3m was evaluated for binding to single-stranded DNA. In separate experiments, a fluorescein-labeled 15-mer was titrated with NS3m (open squares) or NS3 (filled circles) and the resulting change in fluorescence polarization was measured.

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Yet in the presence of NS3, the polymerase synthesizes a much greater amount of more complex multicopy products from both templates (Fig. 6). In the light of the observation that NS4B suppresses the NS3 effect on NS5B, it now seems more likely that the ratio between NS4B and NS3 may be a primary determinant of the relative abundance of the positive and negative strands. At the same time, it is important to note that under physiological solution conditions, NS3 is a less competent RNA helicase, and that NS4A is required to enhance this activity (46) (see also Fig. 2G).

Finally, it is evident that a full understanding of the replication cycle in HCV will require characterization of the physical and functional interactions between NS5B and the other proteins that modulate its function. Whereas other recent studies have indicated how NS5A and NS4A might participate in the replication process, the present study has provided insight into the potential roles of NS3 and NS4B. The modulatory influences are both positive and antagonistic in nature. Further work on the finely balanced interactions between these components should ultimately provide a meaningful model for the control of HCV replication. It will also be important to determine the relative abundance of the multicellular products, to what degree they are suppressed by the function of NS4B in infected cells, and thus to what extent they feature in the physiological HCV replication cycle.

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molecular mass multicopy RNA products (Fig. 9). Moreover, in the presence of NS4B, the NS5B-NS3 complex continues to show a greatly reduced capacity to generate even the single-length positive-sense copy of the (-3')-TR template. In other words, in the NS5B-NS3-NS4B complex, NS3 both blocks single-length complementary strand RNA synthesis (as manifested by NS5B alone) and shows itself incompetent to promote the modified synthesis pathway that generates the high molecular mass RNA products. Overall, it is possible that there is an interplay between NS3 and NS4B during HCV replication that allows for fine regulation of the RNA products generated by the polymerase. The balance of this interplay could potentially vary according to the stage of the HCV life cycle. We do not know the exact mode of action of NS4B. However, in one possible model its effect is at least partially related to its inhibitory effect on the NTPase activity that is associated with the NS3 helicase domain, and may also be partially mediated via a direct interaction between NS4B and NS5B. In the absence of a fully functional unwinding activity, NS3 may have a reduced capacity to deliver unstructured template RNA to the NS5B active site.

The preferential replication by purified NS5B of (-3')-TR RNA over (+)-3'-UTR RNA has been reported previously (12), although this previous study did not use HCV RNA templates bearing the correct 3'-ends. In itself, this observation seems to provide an attractive explanation as to how HCV might maintain an excess of positive over negative strand RNA in vivo. However, our results also clearly show that this preferential template selection is eliminated in the presence of NS3 (Fig. 6). This underlines the significance of the NS3 helicase activity within a replication complex together with NS5B. Given the availability of sufficient ATP to drive the unwinding reaction, NS3 modifies the kinetics and product profile of the HCV polymerase. As has also been noted by others (8), purified NS5B is capable of generating at least one folded RNA product that exceeds the length of the single template sequence (Fig. 4).
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Modulation of the Hepatitis C Virus RNA-dependent RNA Polymerase Activity by the Non-Structural (NS) 3 Helicase and the NS4B Membrane Protein
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