JOURNAL OF VIROLOGY, Nov. 1997, p. 8416–8428 Vol. 71, No. 11
0022-538X/97/$04.00+0

Biochemical Properties of Hepatitis C Virus NS5B RNA-Dependent RNA Polymerase and Identification of Amino Acid Sequence Motifs Essential for Enzymatic Activity

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Received 23 April 1997/Accepted 4 August 1997

The NS5B protein of the hepatitis C virus (HCV) is an RNA-dependent RNA polymerase (RdRp) (S.-E. Behrens, L. Tomei, and R. De Francesco, EMBO J. 15:12–22, 1996) that is assumed to be required for replication of the viral genome. To further study the biochemical and structural properties of this enzyme, an NS5B-hexahistidine fusion protein was expressed with recombinant baculoviruses in insect cells and purified to near homogeneity. The enzyme was found to have a primer-dependent RdRp activity that was able to copy a complete in vitro-transcribed HCV genome in the absence of additional viral or cellular factors. Filter binding assays and competition experiments showed that the purified enzyme binds RNA with no clear preference for HCV 3′-end sequences. Binding to homopolymeric RNAs was also examined, and the following order of specificity was observed: poly(U) > poly(G) > poly(A) > poly(C). An inverse order was found for the RdRp activity, which used poly(C) most efficiently as a template but was inactive on poly(U) and poly(G), suggesting that a high binding affinity between polymerase and template interferes with processivity. By using a mutational analysis, four amino acid sequence motifs crucial for RdRp activity were identified. While most substitutions of conserved residues within these motifs severely reduced the enzymatic activities, a single substitution in motif D which enhanced the RdRp activity by about 50% was found. Deletion studies indicate that amino acid residues at the very termini, in particular the amino terminus, are important for RdRp activity but not for RNA binding. Finally, we found a terminal transferase activity associated with the purified enzyme. However, this activity was also detected with NS5B proteins with an inactive RdRp, with an NS4B protein purified in the same way, and with wild-type baculovirus, suggesting that it is not an inherent activity of NS5B.

The hepatitis C virus (HCV) is the major cause of transfusion-associated hepatitis and accounts for a significant proportion of hepatitis cases worldwide (for a review, see reference 23). Most, if not all infections become chronic, and about 60% of patients develop liver disease with various clinical outcomes ranging from an asymptomatic carrier state to chronic active hepatitis and liver cirrhosis, which is strongly associated with the development of hepatocellular carcinoma (for reviews, see references 11 and 23).

HCV is a member of the family *Flaviviridae* (38). Characteristic for this group of viruses, the genomic RNA contains a single long open reading frame (ORF) which in HCV is translated as a polyprotein of about 3,010 amino acids (for reviews, see references 4 and 47). Upstream of the coding region is a 341-nucleotide nontranslated region (NTR) that is important for translation of the viral ORF (reference 21 and references therein). At the 3′ end of the viral genome a ca. 230-nucleotide NTR was identified (29, 55). It is composed of a rather variable sequence of about 38 nucleotides following the stop codon of the polyprotein, a polyuridine tract of variable length, and a 98-nucleotide sequence, which is highly conserved among various HCV isolates (29, 56, 59). By analogy to other plus-strand RNA viruses, the 3′ NTR is assumed to play an important role in viral RNA synthesis.

Recombinant expression systems have been used to identify HCV polyprotein cleavage products and have established the following order within the genome (from the amino to the carboxy terminus): NH_2-C-E1-E2-p7-NS2-NS3-NS4A-NS4B-NS5A-NS5B-COOH (14, 18, 19, 31, 35, 50, 57). This polyprotein is cleaved co- and posttranslationally by two classes of proteinases (for reviews, see references 4 and 47). Processing of the structural proteins core (C), envelope protein 1 (E1), E2, and the p7 region is mediated by host signal peptidases. In contrast, maturation of the nonstructural (NS) region is accomplished by two viral enzymes: the NS2-3 proteinase, cleaving at the NS2-3 junction, and the NS3/4A proteinase complex, responsible for processing at all the remaining sites (4, 47).

Studies of HCV so far have been hampered by the lack of efficient tissue culture systems, by the lack of an infectious cDNA clone, and by the very low levels of protein expression in infected tissues. Therefore, most of our knowledge comes from studies with heterologous expression systems. Until now, they were focused on the characterization of the structural proteins as well as the mechanisms of polyprotein expression and maturation, and they culminated in the determination of the X-ray crystal structure of the NS3 proteinase (28, 33). In contrast, very little is known about the mechanisms of virus replication. As deduced by analogy to other plus-strand RNA viruses like poliovirus, alphaviruses, or some plant viruses (1, 45, 51), limited biochemical analyses of recombinant HCV proteins, and some tissue culture experiments, replication of HCV is assumed to occur in membrane-associated replication complexes. Within these, the genomic plus strand RNA is transcribed into minus-strand RNA, which in turn can be used as a template for synthesis of progeny genomic plus strands. At

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least two viral proteins appear to be involved in this reaction: the NS3 protein, which carries in the carboxy terminal two-thirds a nucleoside triphosphatase/RNA helicase (16, 22, 27, 52, 53), and the NS5B protein, which is a membrane-associated phosphoprotein with an RNA-dependent RNA polymerase activity (RdRp) (6, 24). While the role of NS3 in RNA replication is less clear, NS5B obviously is the key enzyme responsible for synthesis of progeny RNA strands. Using recombinant baculoviruses to express NS5B in insect cells, Behrens et al. (6) identified two enzymatic activities associated with it: a primer-dependent RdRp and a terminal transferase (TNTase) activity. Apart from this, up to now no detailed information about the biochemical and structural properties of NS5B has been available.

In this report, we describe a simple method which allows the rapid purification of an NS5B-His_6 fusion protein. We show that the purified enzyme has a highly active, primer-dependent RdRp activity which is able to copy an in vitro-transcribed full-length HCV RNA in the absence of additional viral factors. We characterized RNA binding and the template requirements for efficient polymerization, and we used site-directed mutagenesis to identify four amino acid sequence motifs crucial for RdRp activity. Finally, we present evidence that HCV NS5B has no intrinsic TNTase activity.

MATERIALS AND METHODS

Cells and viruses. Sf9 cells, High5 cells, and linearized baculovirus DNA were purchased from Clontech (Heidelberg, Germany). The cells were grown in suspension in Grace's insect medium (Gibco BRL, Eggenstein, Germany) supplemented with 10% fetal calf serum (FCS). For protein expression, 2×10^7 to 4×10^7 High5 cells were infected at a multiplicity of infection of about 10 with recombinant baculoviruses in a total volume of 7.5 ml of FCS-free medium for 1 h at room temperature. After the cells were seeded on a 175-cm^2 dish, 7.5 ml of medium supplemented with 10% FCS was added and the cells were incubated for 72 h at 27°C.

Plasmid constructions. Standard recombinant DNA techniques were used to generate all the constructs (48). All HCV fragments were cloned from a chronically infected patient and belong to genotype 1b (5). The basic transfer vector pBac9 was purchased from Clontech. To facilitate the insertion of HCV sequences, the multiple-cloning site of this vector was modified to contain only the restriction sites for *Bam*HI, *Sma*I, *Eco*RI, *Nco*I, *Spe*I, and *Pac*I. All the HCV fragments were inserted between the *Nco*I site and the *Spe*I site, with the ATG codon in the engineered *Nco*I site serving as the start codon for translation and termination occurring at the authentic stop codon. Plasmid pBac5B was obtained by insertion of a 900-bp *Nco*I-*Sfi*I PCR fragment and a 929-bp *Sfi*I-*Spe*I fragment in a three-factor ligation into the modified pBac9 restricted with *Nco*I and *Spe*I. The resulting plasmid contained the complete NS5B gene and included 54 nucleotides of the 3′ nontranslated region. NS5B proteins with six histidine residues at their amino termini were translated from plasmid pBac/N-His. This construct was derived from the modified pBac9 by insertion of two complementary oligonucleotides, introducing a start codon and six histidine codons in frame with the ATG codon of the *Nco*I site, between the *Bam*HI site and the *Nco*I site of the parental vector. Carboxy-terminal fusion proteins with six histidine residues were generated from plasmid pBac/C-His, which was derived from the modified pBac9 by insertion of two complementary oligonucleotides, introducing six histidine codons and a stop codon, between the *Spe*I site and the *Pac*I site of the vector. Plasmid pBac/N-His5B directing the expression of a complete NS5B protein fused amino terminally to six histidine residues was obtained by insertion of a 900-bp *Nco*I-*Sfi*I fragment and a 929-bp *Sfi*I-*Spe*I fragment into pBac/N-His restricted with *Nco*I and *Spe*I. Plasmid pBac/C-His5B directing the expression of a complete NS5B protein fused carboxy terminally to six histidine residues was generated by the same strategy with pBac/C-His as the vector. However, in this case, the *Sfi*I-*Spe*I fragment was generated by PCR with oligonucleotide primers allowing an in-frame fusion of the last amino acid of NS5B with the six histidine residues.

Site-directed mutagenesis was done by PCR as described by Ho et al. (20). All fragments were cloned into pBac/C-His5B. In the case of nucleotide exchanges in the 5′ half of the NS5B gene, a 553-bp *Hpa*I-*Sfi*I PCR fragment was inserted into pBac/C-His5B, whereas in the case of substitutions in the 3′ half of the NS5B gene, an 870-bp *Sfi*I-*Spe*I PCR fragment was exchanged. Amino-terminal deletions of NS5B were generated by PCR, and *Nco*I-*Sfi*I fragments were inserted into pBac/C-His5B. Carboxy-terminal deletions were obtained by insertion of *Sfi*I-*Spe*I-restricted PCR fragments into pBac/N-His5B. All transferred PCR fragments were completely sequenced with IRD-41 labeled primers (MWG, Ebersbach, Germany) and a model 4000 DNA sequencer (Li-Cor, Lincoln, Neb.). An overview of all NS5B proteins expressed with the individual constructs is given in Fig. 1. Plasmid pBSK/lacZ contains a 3,736-bp *lacZ* fragment isolated from plasmid pCH110 (Pharmacia) and inserted via *Hin*dIII-*Bam*HI into pBSK− (Stratagene, Heidelberg, Germany). Plasmid pAT1-9604 is a pBR322 derivative containing a modified promoter of T7 RNA polymerase upstream of a full-length HCV genome (32). Plasmid pBSK9286-9604/7 was obtained by insertion of an HCV fragment corresponding to the 3′-terminal 319 nucleotides of the HCV genome into the *Sma*I site of pBSK−.

Generation of recombinant baculoviruses. A total of 10^6 Sf9 cells per 35-cm^2 dish seeded 1 h before transfection were washed twice with 1.5 ml of FCS-free medium. Then 1 μg of transfer plasmid was mixed with 0.2 μg of linearized BacPAK6 DNA (Clontech) in a total volume of 50 μl, and after the addition of 50 μl of Lipofectamine (Gibco BRL), diluted 1:1.5 with water, the liposome-DNA mixture was incubated for 10 min at room temperature. After dropwise addition to the cells and a 5-h incubation at 27°C, 1.5 ml of medium supplemented with 10% FCS was added. The cells were incubated for 5 days at 27°C, and half of the supernatant was used for amplification on Sf9 cells. For plaque purification, the Sf9 cells were infected with a serial dilution of virus-containing FCS-free medium and the cell monolayers were overlaid with 0.5% SeaPlaque GTG agarose (FMC BioProducts, Oldendorf, Germany). Well separated plaques were punched out with a Pasteur pipette, and the virus was amplified on Sf9 cells.

Purification of NS5B from infected cells. A total of 4×10^7 High5 cells infected as described above were scraped off the plate and centrifuged, and the cell pellet was washed once with phosphate-buffered saline. All subsequent steps were performed at 4°C, and all buffers contained 1 mM phenylmethylsulfonyl fluoride and 4 μg of leupeptin per ml (Sigma, Deisenhofen, Germany). The appropriate buffer system was deduced from recent reports (6, 46). The cells were resuspended in 1 ml of lysis buffer I (LBI) (10 mM Tris-HCl [pH 7.5], 10 mM NaCl, 1.5 mM $MgCl_2$, 10 mM 2-mercaptoethanol [2-ME]) and incubated for 30 min. After a 10-min centrifugation at $10,000 \times g$, the supernatant (S1) was removed, the pellet was resuspended in 1 ml of LBII (20 mM Tris-HCl [pH 7.5], 300 mM NaCl, 10 mM $MgCl_2$, 0.5% Triton X-100, 20% glycerol, 10 mM 2-ME) and the suspension was sonicated five times for 10 s (at an output control setting of 2) at 7°C with a Branson 450 Sonifier and a cup-horn with a cooling device. After a 10-min centrifugation at $10,000 \times g$, the supernatant (S2) was removed, the pellet was resuspended in 1 ml of LBIII (20 mM Tris-HCl [pH 7.5], 500 mM NaCl, 10 mM $MgCl_2$, 2% Triton X-100, 10 mM imidazole, 50% glycerol, 10 mM 2-ME), and the suspension was sonicated five times for 20 s at an output control setting of 3. After a 10-min centrifugation at $10,000 \times g$, the supernatant (S3) was applied to a Ni-nitrilotriacetic acid spin column (Qiagen, Hilden, Germany), preequilibrated with LBIII, and centrifuged for 5 min at $500 \times g$. The column was washed once with 500 μl of LBIII and twice with 500 μl of nuclease S7 buffer (LBIII diluted 1:4 with 20 mM Tris-HCl [pH 7.5]–10 mM $MgCl_2$–10 mM imidazole–1 mM $CaCl_2$–10 mM 2-ME). To remove nucleic acids bound to the column matrix or the immobilized protein, the column was washed with 500 μl of S7 buffer supplemented with 30 U of nuclease S7 (Boehringer, Mannheim, Germany). For optimal digestion, the columns were centrifuged at low speed to achieve a total nuclease incubation time of 20 min at room temperature. The column was washed three times with 500 μl of LBIII containing 50 mM imidazole, and bound protein was eluted with 200 μl of LBIII containing 250 mM imidazole and 2 mM EGTA. Purified NS5B was stored in small aliquots at −70°C for up to 3 months without significant loss of RdRp activity. $NS4B^{C\text{-}His}$ was prepared in the same way, except that S2, which contained the majority of this protein, was applied to the affinity column. Purified proteins were quantified by a modification of the method of Lowry (42). To quantify NS5B proteins contained in S3, serial dilutions were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Gels were stained with Coomassie brilliant blue, and the amounts of NS5B were determined by densitometry scanning and comparison with a dilution series of bovine serum albumin of known concentration analyzed on the same gel.

Protein gel electrophoresis and Western blot analysis. Proteins were separated by SDS-PAGE (11% polyacrylamide) and electrotransferred to a polyvinylidene difluoride membrane (Polyscreen; NEN, Bad Homburg, Germany) with a semidry-transfer cell as described previously (8). The filters were incubated with antibodies directed against the amino-terminal region of NS5B (amino acids 2420 to 2626 of the polyprotein) at a dilution of 1:2,000, and bound antibody was detected by chemiluminescence with CDP-star as specified by the manufacturer (Du Pont NEN). Prestained molecular weight markers were purchased from Bio-Rad (Munich, Germany) and contained myosin, β-galactosidase, bovine serum albumin, ovalbumin, carbonic anhydrase, soybean trypsin inhibitor, lysozyme, and aprotinin.

Preparation of RNAs for in vitro assays. For the full-length RNA, plasmid pAT1-9604 was restricted with *Xba*I immediately downstream of the HCV insert and nucleotides corresponding to linker sequences were removed by treatment with mung bean nuclease as specified by the manufacturer (Biozym, Oldendorf, Germany). DNA was extracted twice with phenol and once with chloroform and recovered by ethanol precipitation. To obtain RNAs corresponding to the 3′-terminal sequence of the HCV genome and ending with the authentic 3′ end of the viral genome, DNA templates were generated by PCR with Vent polymerase (Biolabs, Schwalbach, Germany), plasmid pBSK9286-9604/7 as the template, an oligonucleotide hybridizing to plasmid sequences upstream of the T7 promoter,

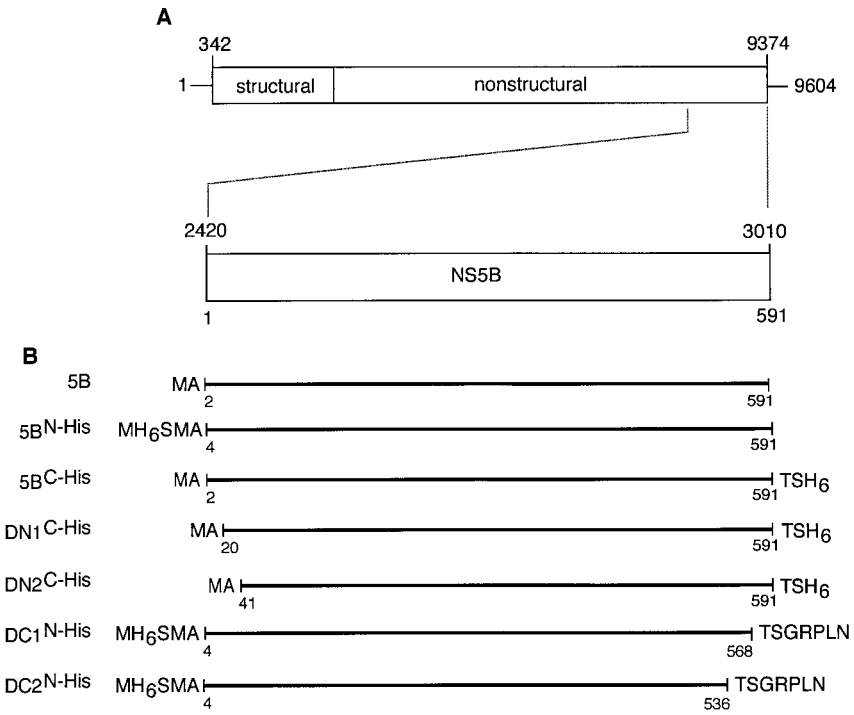

FIG. 1. HCV genome structure and expression constructs. (A) A schematic presentation of the HCV polyprotein with the structural protein encoded in the amino-terminal quarter followed by the nonstructural protein is shown. The 5′ and 3′ nontranslated regions are indicated by the thin lines. A detailed view of the NS5B protein (amino acids 2420 to 3010 of the polyprotein) is drawn below the polyprotein. (B) Summary of the HCV expression constructs used in this study. Numbers below the lines refer to the first and last amino acids of the expressed NS5B sequence. Heterologous amino acids at the amino and carboxy termini of the expressed proteins are given in the single-letter code. The nomenclature for each polypeptide is given to the left.

and a primer corresponding to the 27 3′-terminal nucleotides of the HCV insert. The PCR fragments were purified by preparative agarose gel electrophoresis, and about 1 μg of the eluted DNA was used for in vitro transcription with T7 RNA polymerase (Promega, Heidelberg, Germany) as specified by the manufacturer. After treatment with DNase, transcripts were precipitated with isopropanol, and their integrity was analyzed by agarose gel electrophoresis. Radiolabeled RNAs were generated in the analogous way with [α-^{32}P]UTP, and the integrity of the transcripts was determined by denaturing PAGE. Homopolymeric RNA substrates were purchased from Pharmacia, and 12-mer RNA oligonucleotides were purchased from MWG (Ebersberg, Germany). To prepare primer-template mixtures, equal volumes of homopolymer (0.4 μg/μl) and RNA oligonucleotide (4 pmol/μl) were mixed, denatured for 2 min at 95°C, and incubated for 5 min at 37°C. Then 2 μl of this primer-template mixture was used for an RdRp assay as described below. For 5′-end labeling, RNAs were dephosphorylated with shrimp alkaline phosphatase (Amersham, Braunschweig, Germany) and radiolabeled with [γ-^{32}P]ATP and T4 polynucleotide kinase (Amersham) as specified by the manufacturer. Ribonucleic acid homopolymers were heterogeneous in size as determined by denaturing PAGE, and concentrations were therefore expressed as micrograms per milliliter. RNA molecular weight standard (purchased from Gibco BRL) contained a mixture of RNA fragments of 240, 1,350, 2,370, 4,400, 7,460, and 9,490 nucleotides.

Enzymatic assays. RdRp assays and TNTase assays were done essentially as described recently (6). In brief, cell extract or purified protein was incubated with 0.5 μg of HCV RNA or various homopolymeric RNAs in a total volume of 25 μl containing 20 mM Tris-HCl (pH 7.5), 5 mM $MgCl_2$, 1 mM dithiothreitol, 25 mM KCl, 1 mM EDTA, 10 to 20 U of RNasin (Promega), 2 to 10 μCi of [α-^{32}P]nucleoside triphosphate (NTP) (3,000 Ci/mmol; Amersham), and 500 μM (each) remaining nucleotides. For the full-length HCV RNA template, the concentration of the radioactive nucleotide was adjusted to 10 μM with the same, nonradioactive nucleotide. Where indicated, dactinomycin (Sigma) was included at a final concentration of 50 μg/ml. Samples were incubated for 2 h at 22°C, and the reactions were terminated by the addition of 100 μg of calf thymus DNA and 1 ml of 10% trichloroacetic acid–0.5% tetrasodium pyrophosphate (TCA–PP_i). After a 30-min incubation at 4°C, the samples were filtered through GF/C glass microfiber filters (Whatman). The filters were washed five times with 1% TCA–0.1% $NaPP_i$, and bound radioactivity was measured after addition of Rotiszint 2200 (Roth, Karlsruhe, Germany) in a liquid scintilation counter (Beckman Instruments, Palo Alto, Calif.). When RNAs were analyzed by gel electrophoresis, the samples were digested for 30 min at 37°C with 100 μg of proteinase K in a total volume of 100 μl of PK buffer (150 mM NaCl, 50 mM Tris-HCl [pH 7.5], 0.5% [wt/vol] SDS, 10 μg of tRNA) and extracted with phenol-chloroform and nucleic acids were precipitated with isopropanol. RNAs were analyzed on denaturing 6% polyacrylamide–8 M urea gels at an acrylamide/bisacrylamide ratio of 29:1. TNTase activity was determined in the same way, except that 100 pmol of oligo(U) was used per assay and the nonradioactive nucleotides were omitted. Labeled RNA oligonucleotides were separated on denaturing 15% polyacrylamide–8 M urea gels at an acrylamide/bisacrylamide ratio of 38:2.

Nuclease digestions and denaturing gel electrophoresis. Single-stranded RNAs were digested as described previously (3). In brief, precipitated nucleic acids were dissolved in RPA (8% formamide, 10 mM HEPES [pH 7.5], 5 mM EDTA, 350 mM NaCl) and digested with 40 μg of RNase A per ml and 700 U of RNase T1 per ml (Boehringer). After 1 h at 22°C, the samples were incubated for 30 min at 37°C with 100 μg of proteinase K in PK buffer and extracted with phenol-chloroform, and nucleic acids were precipitated with isopropanol. RNAs were dissolved in RNase-free water and analyzed on 1% formaldehyde–agarose gels (48) or dissolved directly in denaturing sample buffer and analyzed on denaturing 6% polyacrylamide–8 M urea gels.

RNA filter-binding studies. RNA filter-binding studies were done by the method of Oberste and Flanegan (41). A standard binding reaction mixture contained 100 ng of purified NS5B and different amounts of a ^{32}P-labeled RNA homopolymer or in vitro transcript in 50 mM HEPES (pH 7.5), 7 mM $MgCl_2$, and 5 mM 2-ME in a total volume of 100 μl. After a 1 h incubation at 22°C, the reaction solutions were diluted to 1 ml with TE (10 mM Tris-HCl [pH 7.5], 1 mM EDTA) and filtered through 0.45-μm-pore-size nitrocellulose filter disks (BA 85; Schleicher & Schuell, Dassel, Germany) which had been boiled for 30 min in TE prior to use. After the filters were washed with 5 ml of TE, bound radioactivity was counted in Rotiszint 2200 in a liquid scintilation counter.

Nucleotide sequence accession number. The nucleotide and amino acid sequence of the NS5B used in this study were submitted to the EMBL nucleotide sequence database and can be retrieved under accession no. Z97730.

RESULTS

Expression of enzymatically active NS5B proteins in insect cells. To study the biochemical properties of the HCV NS5B protein, different recombinant baculoviruses were constructed (Fig. 1). Bac5B directs the expression of an NS5B protein which, due to the engineered *Nco*I site, differs from the au-

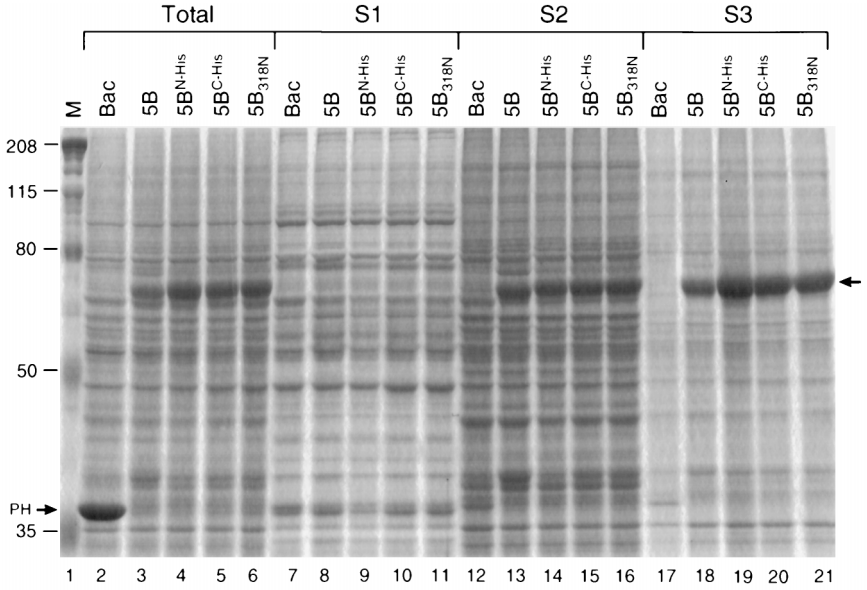

FIG. 2. Enrichment of NS5B proteins in subcellular fraction S3. Pellets of cells infected with the wild-type baculovirus (Bac) or any of the recombinant viruses directing the expression of NS5B, NS5B$^{N\text{-}His}$, NS5B$^{C\text{-}His}$, or the NS5B$^{C\text{-}His}$ protein carrying an amino acid substitution at position 318 ($5B_{318N}$) were extracted with buffers containing increasing concentrations of detergent, glycerol, and salt, and 1/100 of each fraction (S1, S2, or S3) was analyzed by SDS-PAGE (11% polyacrylamide). For comparison, 1/200 of total cell lysate of each infection (Total) was analyzed in parallel (lane 2 to 6). The positions of protein molecular mass standards (in kilodaltons) are given on the left. The arrow on the right indicates the position of the NS5B proteins, and the arrow on the left refers to the polyhedrin (PH).

thentic protein by the presence of the methionine start codon and an alanine residue instead of a serine residue at position 1 (see Materials and Methods). Bac5B$^{N\text{-}His}$ allows the expression of an NS5B protein which carries a hexahistidine affinity tag at the amino terminus, and Bac5B$^{C\text{-}His}$ directs the synthesis of an NS5B carrying the affinity tag at the carboxy terminus. As the negative control, we used a recombinant virus directing the expression of an NS5B$^{C\text{-}His}$ protein in which the first aspartic acid residue of the GDD motif, assumed to be the active site of the polymerase (see below), was replaced by an asparagine residue. These recombinant viruses were used to infect High5 cells in parallel with the wild-type baculovirus, and RdRp activity was measured in cell lysates. In pilot experiments, we had found that in contrast to most cellular proteins, NS5B is poorly solubilized in buffers containing low or moderate concentrations of detergent, glycerol, and salt but was extracted efficiently from the cell pellet with buffers containing high concentrations of these components. We exploited this property to partially purify the NS5B proteins by first using low-stringency buffers in which most cellular proteins could be solubilized. Under these conditions, the majority of NS5B proteins remained in the pellet but could be eluted in a subsequent step with a high-stringency buffer (see Materials and Methods). As shown in Fig. 2, most of the cellular proteins were eluted from the cell pellet with buffers containing low or moderate concentrations of Triton X-100, glycerol, and NaCl (supernatants 1 and 2 [S1 and S2], respectively). In contrast, all NS5B proteins eluted efficiently only with the high-stringency buffer and therefore could be enriched in S3, whereas the majority of polyhedrin expressed by the wild-type baculovirus remained in the cell pellet.

To analyze whether NS5B proteins, enriched in S3, had an RdRp activity, polymerase assays were performed and the activities were determined by measuring the incorporation of radioactivity into a homopolymeric substrate. As summarized in Table 1, both the parental NS5B protein and the fusion proteins had clearly detectable polymerase activities which were not inhibited by dactinomycin. The highest incorporation was obtained with the NS5B protein carrying six histidine residues at the carboxy terminus, whereas the activity of the amino-terminal fusion protein was reduced about threefold compared to the parental protein. No incorporation above background was found with the $5B_{318N}$ protein, in agreement with the assumption that the GDD motif is essential for polymerase activity (see below). The reason why the heterologous sequence at the carboxy terminus enhanced RdRp activity but had an inhibitory effect when present at the amino terminus is not clear but could be explained by an effect on polypeptide folding.

Purification of an enzymatically active HCV NS5B protein. Since the carboxy-terminal fusion protein had the highest enzymatic activity, it was used for all subsequent analyses. To further purify this protein, the S3 subcellular fractions containing the majority of this protein or the NS5B mutant were applied to an affinity column. After several washes, cellular DNA and RNA bound to the matrix or the NS5B proteins were digested with micrococcal nuclease, and after several washes, bound protein was eluted with imidazole. As shown in

TABLE 1. RdRp activities of NS5B proteins enriched in S3

Enzyme	Amt of GMP (pmol) incorporated per mg of 5B-protein per 2 h[a]
5B	48.5
5B$^{N\text{-}His}$	15.7
5B$^{C\text{-}His}$	69.3
$5B_{318N}$	<0.4

[a] mean values from duplicates determined by a standard polymerase assay with a serial dilution of each S3 fraction and 1 μg of poly(C)-oligo(dG) as the substrate in the presence of dactinomycin and 5 μCi of [α-^{32}P]GTP; the background, as determined with S3 of wild-type baculovirus, is ca. 0.4 pmol. The amounts of NS5B proteins in each fraction were determined by densitometry scanning as described in Materials and Methods.

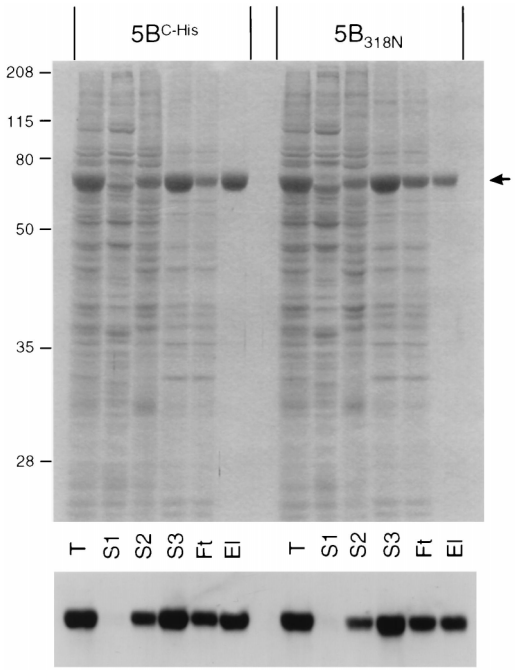

FIG. 3. Purification of NS5B. Total cell lysates (T) (1/200) and each fraction obtained by the purification scheme described in Materials and Methods (1/100) were analyzed by SDS-PAGE, and proteins were detected by Coomassie brilliant-blue staining (top panel) or by Western blotting and chemiluminescence with an antibody directed against the amino-terminal NS5B domain (bottom panel). Numbers on the left refer to the sizes of marker proteins (in kilodaltons). The arrow in the upper panel marks the position of NS5B proteins. Ft, column flowthrough; El, eluate.

Fig. 3, in 5B$^{C\text{-}His}$, more than 50% of the protein contained in S3 bound to the column and was recovered in the eluate. In 5B$_{318N}$, binding was less efficient, suggesting that the amino acid exchange affected the overall folding of the protein, rendering the affinity tag less accessible to the Ni^{2+} ions of the resin. However, even in this case, sufficient amounts of highly purified protein could be eluted from the column under nondenaturing conditions (Fig. 3). As judged from the Coomassie blue-stained protein gel, the purified proteins were homogeneous and no additional proteins were detected.

To determine the quality of the purified proteins in terms of contamination with cellular nucleic acids or polymerases and to evaluate the NS5B RNA polymerase activities in more detail, equal amounts of all fractions of both proteins were each tested in a standard polymerase assay in the absence of dactinomycin by using an RNA template corresponding to the last 319 nucleotides of the HCV genome (Fig. 4A). This RNA carries 74 nucleotides of linker sequence at its 5′ end and terminates with the authentic 3′ end at position 9604 of our HCV isolate. We chose this RNA because it (i) may represent the RNA substrate used to prime the initiation of minus-strand RNA synthesis and (ii) most probably has a highly ordered structure, which should allow "copy-back" (*cis*) priming, which has been shown to be the preferred reaction in NS5B-dependent RNA synthesis (6). As shown in Fig. 4B, an RNA corresponding in size to the input RNA was generated with S1 of the parental 5B$^{C\text{-}His}$ and, to a lesser extent, with 5B$_{318N}$, suggesting that this RNA was radiolabeled by cellular terminal transferases or by polymerases with low processivities (lanes 2 and 12, respectively). A reproducible pattern of RNAs shorter and longer than the input RNA was obtained with all other fractions of 5B$^{C\text{-}His}$, with the purified protein giving the highest incorporation (lane 6). As inferred from the signals obtained with S2, S3, and the flowthrough of the inactive 5B$_{318N}$, cellular polymerases were detected in these fractions but no longer with the eluted proteins, indicating that the NS5B proteins purified by this method were virtually free from contaminating cellular enzymes (compare lanes 9 and 10 with lane 11). To test for contaminating cellular RNAs in the individual fractions, the assays were performed without exogenous HCV RNAs in parallel (Fig. 4B, right panel). While for both proteins in S3 and the flowthrough of the column, cellular RNAs were detected which could be used as substrate for the NS5B RdRp and cellular enzymes, no signal was obtained with the eluted proteins, indicating that the cellular nucleic acids had been removed by our purification scheme. Interestingly, the strong signals obtained with S3 and the flowthrough fractions of 5B$^{C\text{-}His}$ in the absence of exogenous RNA (lanes 14 and 15) were not detected when HCV-RNA was added (lanes 5 and 6), indicating that the viral RNA was a preferred substrate for the RdRp or that the amounts of HCV-RNA added to the reaction mixtures were much larger and therefore competed the much smaller amounts of endogenous contaminants.

Using different preparations of 5B$^{C\text{-}His}$ and HCV-RNA substrates corresponding to the 3′ end of the viral genome, we reproducibly obtained a series of RNA fragments migrating faster than the input RNA and a heterogenous population of RNA molecules which were longer (Fig. 4B, lane 6). Several explanations can be given for this pattern. (i) Substrate RNA or the reaction products are cleaved at specific sites (e.g., preferred single-stranded regions) by RNases present in the enzyme preparation. (ii) The majority of products are not generated by a copy-back mechanism but, rather, by short RNA molecules, still present in the enzyme preparation or in the substrate RNA, which hybridize to preferred single-stranded sites and serve as primers for the RdRp. (iii) The molecules are generated by a copy-back mechanism but on small incomplete transcripts which are not denatured under these conditions of gel electrophoresis (a similar phenomenon has been described recently [6]). (iv) Due to alternative structures, copy-back initiates at various sites. However, even in this case, the resulting products should be larger than the input substrate. To differentiate between these possibilities, several experiments were carried out. First, a radiolabeled substrate RNA was incubated with large amounts of purified 5B$^{C\text{-}His}$, and after 2 h of incubation, the integrity of the RNA was analyzed. No degradation was detected, suggesting that very minor, if any, RNase contamination was present in the enzyme preparation (data not shown). Second, when the input RNA was purified by denaturing PAGE, the distinct products smaller than the input RNA were no longer detected and the majority of radioactivity was incorporated into products larger than the monomer (Fig. 4C, compare lane 2 with lane 3). The heterogeneity of products observed in this case could be due to only partial denaturation (duplex and copy-back RNAs are very difficult to denature), to aberrant migration, to alternative copy-back priming, or to premature termination of elongation. Third, in the case of molecules generated by a copy-back mechanism, the template and product should be double stranded and protected from RNase digestion but covalently linked by an RNase-sensitive, single-stranded loop. Alternatively, if synthesis is primed by exogenous RNAs, the resulting products should be fully protected and no change of the pattern would be expected. When the products of the RdRp reaction were treated with RNases under conditions where cleavage of only single strands occurs, all fragments smaller than the input were fully protected and no change of the pattern was observed (Fig.

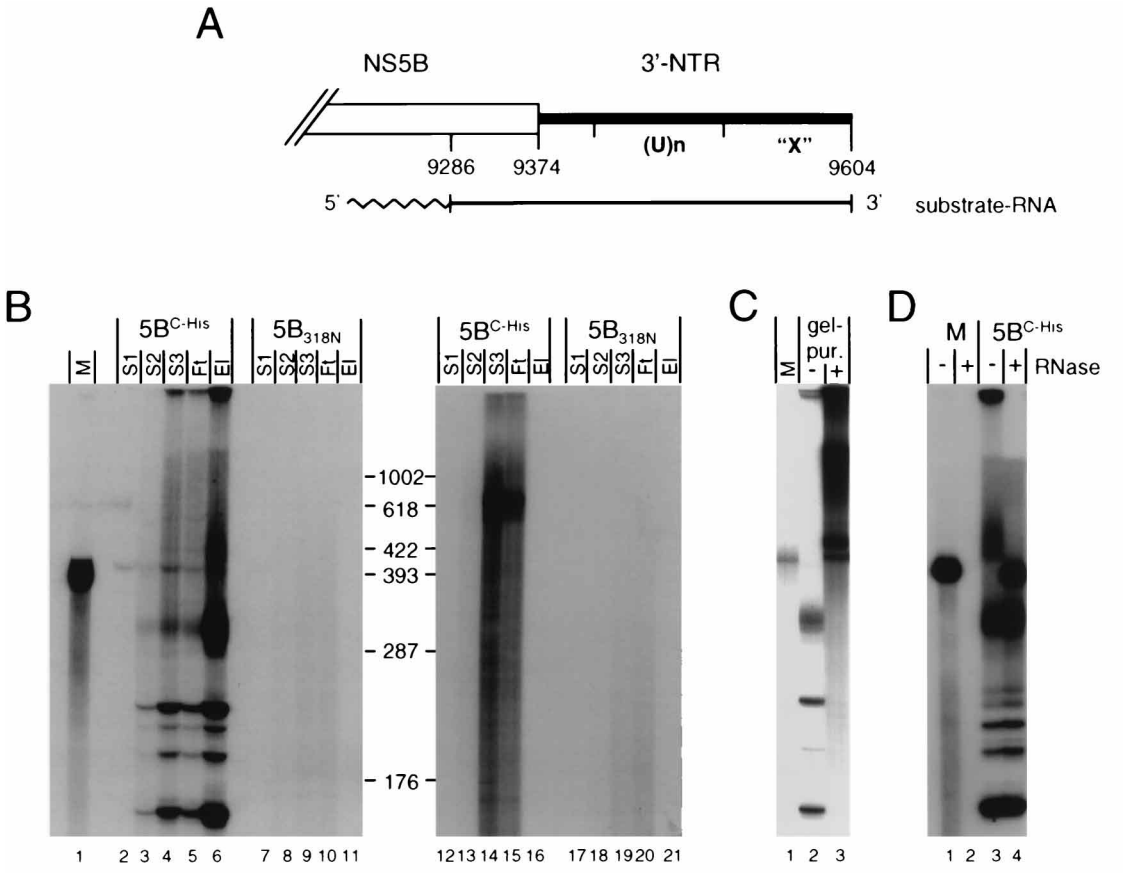

FIG. 4. Detection of RdRp activities in subcellular fractions S1 to S3, the column flowthrough (Ft), and the eluate (El). (A) Structure of the substrate RNA used for the analysis. Part of the NS5B ORF and the 3′ NTR with the polyuridine tract [$(U)_n$] and the X tail are shown at the top. The region corresponding to the RNA substrate is drawn below, with the wavy line on the left indicating the heterologous linker sequence at the 5′ end. Numbers refer to nucleotide positions of our HCV isolate. (B) Equal volumes of each fraction of either $NS5B^{C\text{-}His}$ or $NS5B_{318N}$ (Fig. 3) were used for an RdRp assay under standard conditions in the presence or absence of HCV-specific RNA (left and right panels, respectively). To obtain a size marker with the identical sequence, the HCV 3′-RNA was radiolabeled by in vitro transcription and analyzed by denaturing PAGE in parallel (M, lane 1). The positions of RNA size markers are given between the panels (in nucleotides). (C) Comparison of patterns obtained with unpurified HCV 3′-RNA or the same RNA after purification by preparative PAGE (gelpur.). Authentic marker RNA radiolabeled by in vitro transcription with [^{32}P]UMP is shown in lane 1. (D) Characterization of the RNA products by RNase digestions. RdRp assays were performed under standard conditions with unpurified HCV 3′-RNA. Products were analyzed in parallel with the same RNA radiolabeled by in vitro transcription by denaturing PAGE either directly (lanes 1 and 3) or after digestion with RNases under high-salt conditions (lanes 2 and 4).

4D, compare lane 3 with lane 4) whereas under the same conditions the radiolabeled input RNA was completely degraded (lane 2). In contrast, most of the products larger than the input RNA had disappeared and an RNA fragment of the approximate size of the template was detected consistent with the idea that these molecules were generated by a copy-back priming mechanism (lane 4). In summary, these results suggest that the RNAs smaller than the template were synthesized from primers present mainly in the input RNA whereas the larger products were generated by a copy-back mechanism.

Activity of the NS5B RdRp on full-length HCV RNA. To characterize the activity of purified $5B^{C\text{-}His}$ on its natural RNA and to estimate the reaction kinetics, a full-length HCV genome with authentic 5′ and 3′ termini was used as the substrate in a standard RdRp assay. Aliquots were taken from the mixture at several time points, and after inactivation of the enzyme by the addition of PK buffer and proteinase K, all the samples were analyzed by electrophoresis through a denaturing agarose gel. To test for RNases present in the NS5B preparation and to generate size markers with identical sequences, the input RNA was labeled in a standard reaction containing only [α-^{32}P]GTP and no nonradioactive nucleotides (Fig. 5A, lane 1). As shown in lanes 2 to 11, synthesis of complementary RNA proceeded linearly, and products significantly larger than the template (approximately 20 kb) were detected after about 2 h. During the next 4 h, the amount of products increased whereas no significant change of the pattern was found. The presence of a smear rather than a distinct band could be due to premature termination or might reflect nascent RNA chains. In addition to these major products, several RNA fragments shorter than genome length were found. These products could represent molecules initiated from RNA primers present in the input RNA or by copy-back on incomplete transcripts.

To determine the size of the reaction products more precisely, RNAs obtained after 20, 120, and 240 min of incubation were treated, in parallel with the input RNA, with RNases under conditions which allow cleavage of only single-stranded, but not double-stranded, RNAs. As shown in Fig. 5B, under these conditions the single-stranded input RNA was completely degraded. In case of the RdRp reaction products, after a 20-min incubation, newly synthesized RNA had an average length of about 2.5 kb and increased in length up to about 10 kb for the 2-h incubation. In summary, these results suggest that (i) in this in vitro system the majority of products are

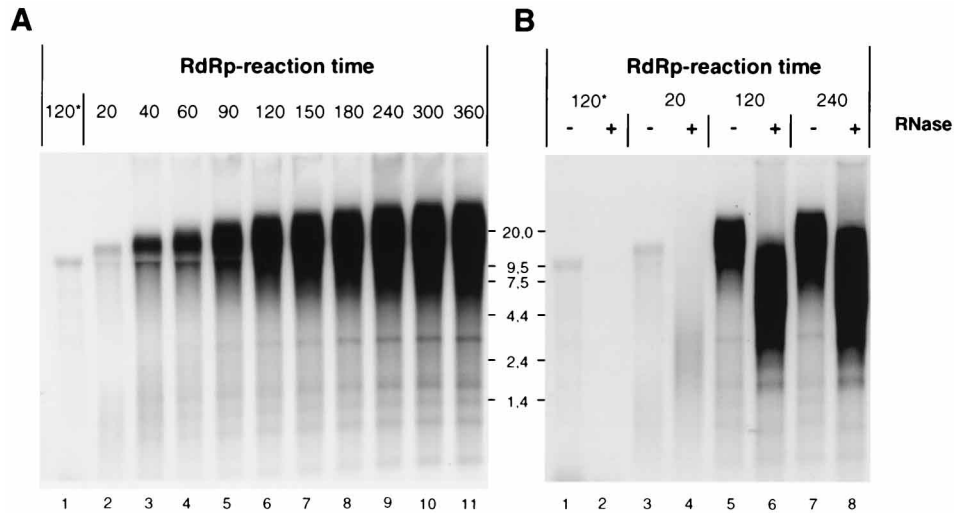

FIG. 5. Activity of purified NS5B$^{C\text{-}His}$ on in vitro-transcribed HCV full-length RNA. (A) An RdRp reaction was performed under standard conditions, and aliquots were withdrawn at the indicated times (minutes). Reactions were stopped by the addition of SDS-containing buffer and proteinase K, and the samples were analyzed by denaturing formaldehyde-agarose gel electrophoresis. To obtain a size marker of identical sequence, the in vitro-transcribed full-length HCV RNA was radiolabeled in a 2-h RdRp reaction with [α-^{32}P]GTP as the only nucleotide (lane 1). (B) Full-length HCV RNA radiolabeled with [α-^{32}P]GTP alone (lanes 1 and 2) or in a standard reaction performed for the indicated times was digested with RNases under high-salt conditions and analyzed by formaldehyde-agarose gel electrophoresis in parallel with the untreated samples (lanes 1, 3, 5, and 7). Numbers between the panels indicate the positions of RNA size markers (in kilobases). Note that the position of the 20-kb marker is only an approximation because it was deduced from a standard curve established with the RNA markers on the same gel.

generated by a copy-back priming mechanism and (ii) NS5B is able to copy a full-length or nearly full-length genome (i.e., the size of the template minus the 3′ sequences required for intramolecular priming) in the absence of additional viral or cellular cofactors. Whether this is due to a high processivity or falloff and reinitiation remains to be determined.

RNA binding properties of purified NS5B. Besides having a polymerase activity, NS5B also must be able to bind to RNA. We were particularly interested in the specificity of RNA binding and therefore performed competition experiments. Assuming that binding might be specific for the HCV 3′-RNA, it should be competed only with a homologous RNA but not with a heterologous one. Thus, purified 5B$^{C\text{-}His}$ was incubated with a constant amount of radiolabeled HCV 3′-RNA (Fig. 6A) and increasing amounts of homologous nonlabeled competitor RNA or increasing amounts of a 419-nucleotide heterologous *lacZ* RNA. Protein-RNA complexes were collected on nitrocellulose filters, and bound radioactivity was measured by liquid scintillation counting. As shown in Fig. 6A, binding of 5B$^{C\text{-}His}$ to the viral RNA was competed both with the homologous RNA and with the heterologous RNA. Furthermore, binding to the heterologous radiolabeled *lacZ* RNA also could be competed with both RNAs, showing that NS5B can bind to RNA but that at least in this in vitro system, the binding is nonspecific.

To exclude the possibility that RNA binding was due to a cellular protein present in the NS5B preparation, binding assays were performed with an NS4B$^{C\text{-}His}$ purified in the same way (see below). Assuming that a cellular contaminant would bind nonspecifically to the affinity column, we expected to find such a protein in every preparation irrespective of the nature of the HCV protein. However, the NS4B protein bound less than 5% of the amount of RNA bound by 5B$^{C\text{-}His}$, demonstrating that the RNA binding we had measured was due to NS5B itself (data not shown).

To simplify all subsequent analyses, we attempted to set up in vitro systems with homopolymeric substrate RNAs. As a first step in this direction, we characterized the binding capacity of purified 5B$^{C\text{-}His}$ to different ribohomopolymers. 5B$^{C\text{-}His}$ (100 ng) was incubated with increasing amounts of radiolabeled RNA homopolymers, and bound radioactivity was determined as described above. As a negative control, 100 ng of bovine serum albumin was used for RNA binding assays in parallel and the amount of RNA bound to this protein was subtracted as the background. As shown in Fig. 6B, poly(U) was bound with the highest efficiency whereas binding to poly(A) and in particular poly(C) was much less efficient. While binding to these homopolymers could be saturated, binding to poly(G) could not, which is most probably due to aggregates forming with high concentrations. In summary, these data demonstrate that NS5B binds to RNA homopolymers with the following order of specificity: poly(U) > poly(G) > poly(A) > poly(C).

Activity of NS5B on homopolymeric templates. Having analyzed the RNA binding of NS5B to homopolymeric substrates, we next wanted to know whether there was a correlation between the efficiency of RNA binding and the efficiency with which this RNA could be used as a template for the RdRp. Furthermore, by using RNA homopolymers with or without annealed RNA oligonucleotide primer, we sought to characterize the primer dependence of this enzyme more precisely because ribohomopolymers cannot form intramolecular hairpins serving as initiation sites. Different substrates were incubated with purified 5B$^{C\text{-}His}$ in the presence of the corresponding radiolabeled nucleotide under standard conditions, and incorporated radioactivity was collected after TCA precipitation on fiberglass microfiber filters and measured by liquid scintillation counting. As shown in Table 2, in the absence of RNA primers almost no incorporation was found for poly(A), poly(G), and poly(U) whereas a low activity could be detected for poly(C). Even in the presence of complementary RNA primers, poly(G)-oligo$(C)_{12}$ and poly(U)-oligo$(A)_{12}$ still could not be used as substrates. In contrast, for poly(A), addition of the oligo$(U)_{12}$ primer dramatically increased RNA synthesis, and the synthesis was maximal when poly(C)-oligo$(G)_{12}$ was used as the substrate. It should be noted that poly(C)-oligo$(dG)_{12–18}$ was also accepted as a substrate (Table 1), show-

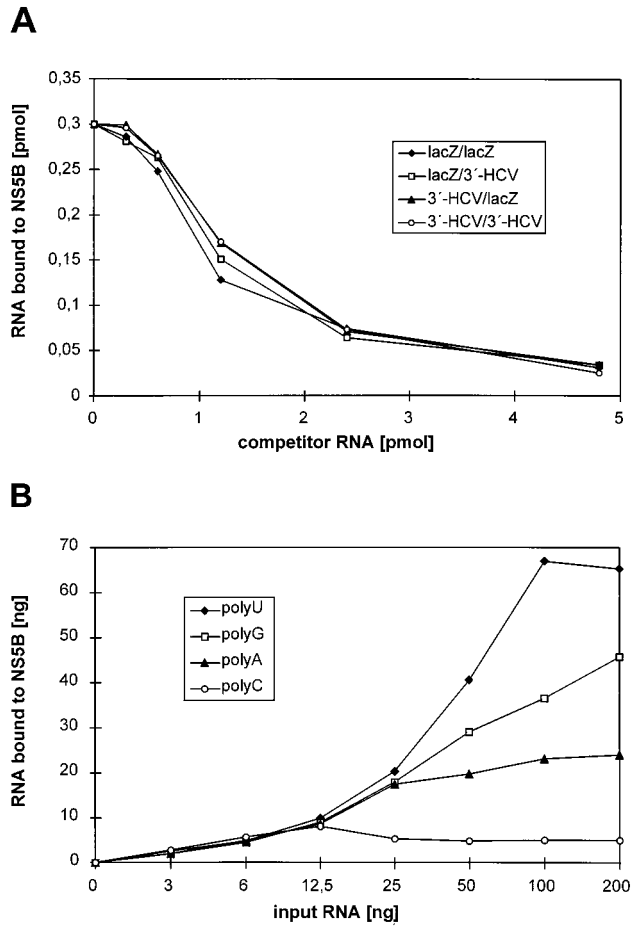

FIG. 6. RNA binding properties of purified $5B^{C\text{-}His}$. (A) A 100-ng sample of protein (corresponding to ca 1.5 pmol) was incubated with 0.3 pmol of a radiolabeled *lacZ* RNA and increasing amounts of nonlabeled *lacZ* RNA or increasing amounts of nonlabeled HCV 3′-RNA. Alternatively, the same amount of enzyme was incubated with radiolabeled HCV 3′-RNA and increasing amounts of nonlabeled *lacZ* RNA or increasing amounts of nonlabeled HCV 3′-RNA. Bound RNA-protein complexes were collected on nictrocellulose filters, and the radioactivity was measured by liquid scintillation counting. (B) A 60-ng sample of $5B^{C\text{-}His}$ was incubated with increasing amounts of radiolabeled RNA homopolymers, and bound radioactivity was determined in the same way.

ing that NS5B RdRp also can use DNA as a primer for RNA synthesis.

Interestingly, there was an inverse correlation between RNA binding and RdRp activity for the different substrates. While poly(U) was bound very efficiently, it could not be used as a template for the polymerase, whereas the most efficient template [poly(C)] was bound the most weakly. Although we cannot exclude the possibility that the apparent differences with which the homopolymers were used as templates were due to different K_m values for individual NTPs present at low concentrations in the reactions, the results suggest that strong binding interferes with processivity. In summary, these results demonstrate the primer dependence of NS5B RdRp and identify poly(C)-oligo$(G)_{12}$ as the most efficient substrate.

Mutation analysis of NS5B RdRp. To identify amino acid sequence motifs which might be crucial for the NS5B RdRp, sequence alignments between an HCV NS5B consensus sequence and various reverse transcriptases (RTs) and viral RdRps, as described by Poch et al. (44), were performed. By using this approach, four sequence motifs could be identified (Fig. 7A). Motif A, characterized by an invariant aspartic acid residue, most probably is involved in nucleotide binding and catalysis, while motif B, characterized by an invariant glycine residue, probably is involved in template and/or primer positioning. Motif C, the GDD motif which is a hallmark of most polymerases, appears to be important for NTP binding and catalysis. Interestingly, within motif D, which also seems to be involved in NTP binding and catalysis, for all RTs and nearly all viral RdRps, a lysine residue is found whereas an invariant arginine residue is present at this position in all the HCV isolates.

TABLE 2. Activity of purified $NS5B^{C\text{-}His}$ on homopolymeric substrates

Substrate	Incorporation (10^3 dmp)[a]
Poly(A)	0.9
Poly(A)-oligo$(U)_{12}$	3,290
Poly(C)	68.8
Poly(C)-oligo$(G)_{12}$	10,978
Poly(G)	0.6
Poly(G)-oligo$(C)_{12}$	2.2
Poly(U)	0.8
Poly(U)-oligo$(A)_{12}$	0.8

[a] Incorporation of radioactivity in a 2-h standard reaction with 400 ng of RNA homopolymer or a mixture of 400 ng of homopolymer plus 4 pmol of RNA oligonucleotide, 5 μCi of the corresponding nucleotide, and 250 ng of purified $NS5B^{C\text{-}His}$.

To analyze the importance of these motifs for enzymatic activity, single conservative and nonconservative amino acid substitutions according to the suggestions of Bordo and Argos (9) were introduced into the $NS5B^{C\text{-}His}$ gene and proteins expressed with recombinant baculoviruses were purified as described for the parental $5B^{C\text{-}His}$. As shown in Fig. 7B, lanes 1 to 21, for all proteins the expression levels and purities were comparable to those of the wild type (lane 22). To analyze whether the substitutions had an effect on RNA binding, constant amounts of purified proteins were incubated with the radiolabeled HCV RNA corresponding to the 3′ end of the viral genome and bound RNA was measured as described above. As summarized in Table 3, none of these amino acid exchanges had a detectable influence on RNA binding. To measure the RdRp activities of all these NS5B proteins, standard reactions were performed with poly(C)-oligo$(G)_{12}$ as the substrate and incorporation of radioactivity was determined by TCA precipitation as described above. With the exception of $5B_{220N}$, all substitutions within motif A were inactive (Table 3). Similarly, all amino acid exchanges within motif B had a drastic effect on the RdRp. While substitution of the conserved threonine residue at position 287 by cysteine reduced the activity to about 4% of the parental NS5B, substitution of the nonconserved threonine at position 286 by valine had an even more drastic effect. All other substitutions within motif B led to a complete inactivation of the enzyme. Within motif C, all substitutions of the first aspartic acid residue of the GDD motif completely destroyed enzymatic activity. Conservative amino acid exchanges of the second aspartic acid residue were less deleterious, as was the case for the substitutions of the glycine residue in the same motif. The most surprising phenotype was observed for the substitution of arginine at position 345 by lysine, which created an enzyme with an activity higher than that of the wild type. Interestingly, a lysine residue was found in all RTs and nearly all viral RdRps, but an arginine residue was found invariantly in all HCV isolates.

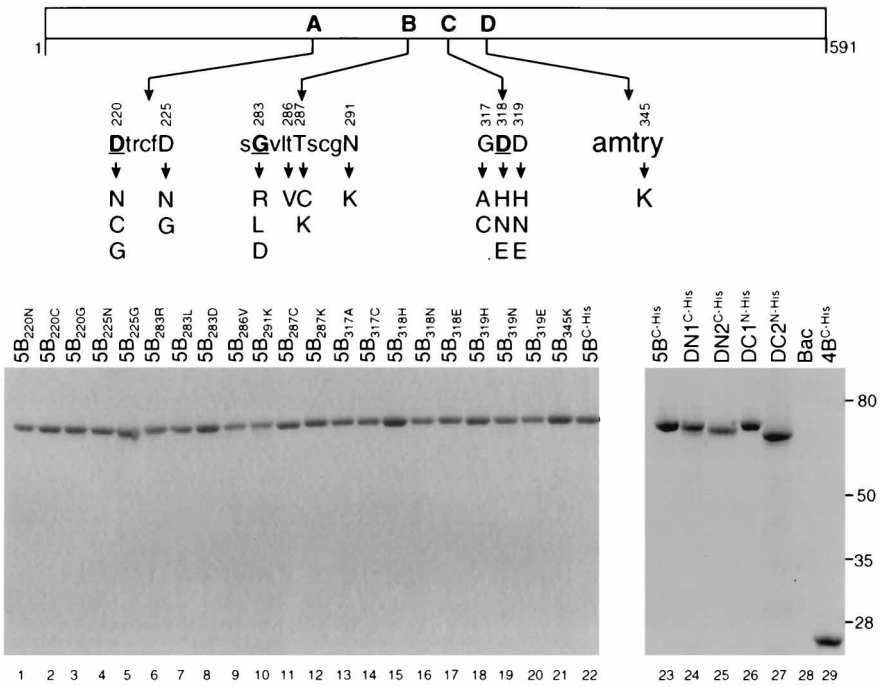

FIG. 7. Expression and purification of NS5B proteins carrying different amino acid substitutions or terminal deletions and NS4B$^{C\text{-}His}$. A schematic presentation of NS5B indicating the positions of conserved amino acid motifs A to D potentially important for RdRp activity is given at the top. Arrows point to the primary sequences of the respective motifs that are highly conserved among most HCV isolates. Capital letters indicate amino acid residues that are highly conserved among plus-strand RNA virus enzymes, and underlined and bold capital letters refer to residues that are highly conserved among all viral polymerases. Numbers above the letters refer to the amino acid position of NS5B; arrows below the letters point to substituting residues. The result of an analysis of purified NS5B proteins and NS4B$^{C\text{-}His}$ by SDS-PAGE is shown below. About 0.5 to 1 μg of each purified NS5B protein or NS4B was analyzed by SDS-PAGE, and the proteins were detected by staining with Coomassie blue. Numbers to the right refer to the sizes of molecular mass standards (in kilodaltons). Bac refers to an affinity column eluate prepared from a lysate of cells infected with wild-type baculovirus and subjected to the identical purification scheme as for all other NS5B proteins.

To map the minimal domain required for the RdRp activity, a series of amino- or carboxy-terminal deletions was constructed. While amino-terminal deletions fused carboxy terminally to the His_6 affinity tag could be expressed to high levels, carboxy-terminal deletions could be expressed efficiently only when the affinity tag was fused to the amino terminus (data not shown). All truncated proteins could be enriched in S3. However, when subjected to affinity chromatography, only NS5B proteins lacking 19 or 40 amino-terminal residues could be purified (Fig. 7B, lanes 24 and 25) whereas proteins lacking 83 or more amino acids at the amino terminus no longer bound to the affinity matrix (data not shown). Similarly, purification of NS5B proteins lacking 23 or 55 carboxy-terminal residues still bound to the column (Fig. 7B, lanes 26 and 27) whereas proteins truncated by 83 or more amino acids at the carboxy terminus did not, suggesting that removal of longer regions from the termini of NS5B had a drastic effect on the overall folding of the protein. On the other hand, the fact that the small deletions still could be purified under nondenaturing conditions suggested that the overall folding of these proteins was not grossly disturbed. As summarized in Table 3, all truncated NS5B proteins still bound the HCV 3′ RNA. Surprisingly, deletion of only 19 residues from the amino terminus severely reduced the polymerase activity, which was completely abolished when 40 residues were removed. Truncations from the carboxy terminus were less deleterious. While DC1$^{C\text{-}His}$ was clearly impaired, the RdRp activity of DC2$^{C\text{-}His}$ was reduced to only about 42% of the wild-type activity, indicating that for DC1$^{C\text{-}His}$, structural alterations are the primary reason for the low activity. In summary, these results show that sequences at the termini are dispensable for RNA binding, and they suggest that carboxy-terminal sequences are less important for the RdRp activity of NS5B than are residues at the amino terminus.

Lack of correlation between the RdRp activity and TNTase activity. For several viral replicases including HCV, copurifications of host-encoded TNTases or detection of a replicase-inherent TNTase activity have been described (2, 6, 12, 36, 39). Therefore, we wanted to know whether the NS5B we had purified had a similar transferase activity. In the first set of experiments, we analyzed whether a TNTase copurified with 5B$^{C\text{-}His}$ by testing all different fractions generated during the enzyme purification in a TNTase assay. Equal volumes of each fraction were added to a TNTase reaction mixture containing a 12-mer oligouridine RNA substrate and [α-^{32}P]GTP. To test for contaminating RNAs, TNTase assays were performed without a substrate in parallel. As shown in Fig. 8, no reaction products were obtained when the substrate was omitted (lanes 2 to 6). When oligo$(U)_{12}$ was added, a very low transferase activity was observed in fractions S2 and S3, in the column flowthrough, and with the purified 5B$^{C\text{-}His}$ adding 1 GMP residue to the RNA oligomer (Fig. 8, lanes 8 to 11). The apparent migration difference between the radiolabeled substrate and the reaction product, corresponding to 2 nucleotides, is due to the presence of the 5′ phosphate group of the marker (lane 1), which is missing in the oligo$(U)_{12}$ RNA substrate (data not shown). Using the purified protein, we found that the TNTase accepts all four NTPs with a slight preference for UTP but, in all cases, adds only 1 nucleotide to the 3′ end of the input RNA (data not shown).

To further characterize this activity and to analyze whether the same amino acid sequence motifs essential for RdRp ac-

TABLE 3. Substitutions within NS5B and their effects on RdRp activity, RNA binding, and TNTase activity

Motif[a]	5B protein	Mutation	RdRp activity (pmol/mg/2 h)[b]	RNA binding[c]	TNTase[d]
	$5B^{C\text{-}His}$	None	1,748	+	+
A	$5B_{220N}$	220D→N	2	+	+
A	$5B_{220C}$	220D→C	0	+	+
A	$5B_{220G}$	220D→G	0	+	+
A	$5B_{225N}$	225D→N	0	+	+
A	$5B_{225G}$	225D→G	0	+	+
B	$5B_{283R}$	283G→R	0	+	+
B	$5B_{283L}$	283G→L	0	+	+
B	$5B_{283D}$	283G→D	0	+	+
B	$5B_{286V}$	286T→V	18	+	+
B	$5B_{287C}$	287T→C	74	+	+
B	$5B_{287K}$	287T→K	0	+	+
B	$5B_{291K}$	291N→K	0	+	+
C	$5B_{317A}$	317G→A	214	+	+
C	$5B_{317C}$	317G→C	142	+	+
C	$5B_{318H}$	318D→H	0	+	+
C	$5B_{318N}$	318D→N	0	+	+
C	$5B_{318E}$	318D→E	0	+	+
C	$5B_{319H}$	319D→H	0	+	+
C	$5B_{319N}$	319D→N	156	+	+
C	$5B_{319E}$	319D→E	133	+	+
D	$5B_{345K}$	345R→K	2,661	+	+
–	$DN1^{C\text{-}His}$	Δ1–19	8	+	+
–	$DN2^{C\text{-}His}$	Δ1–40	0	+	+
–	$DC1^{N\text{-}His}$	Δ567–591	244	+	+
–	$DC2^{N\text{-}His}$	Δ537–591	734	+	+

[a] Altered amino acid sequence motif within NS5B (44).

[b] Picomoles of GMP incorporated per milligram of purified NS5B protein per 2 h as determined in a standard reaction with 250 ng of NS5B, 1 μCi of [α-^{32}P]GTP, and poly(C)-oligo(G) (0.4 μg/4 pmol) as the substrate; mean values from duplicates with at least two different enzyme preparations subtracted for the background (5 pmol).

[c] Binding of purified NS5B proteins to HCV 3′ RNA (9286 to 9604) under standard conditions with 100 ng of purified enzyme and 0.3 pmol of RNA. +, RNA binding comparable to that of the wild-type enzyme (±10%).

[d] Determination of TNTase activity as described in the legend to Fig. 8. +, an amount of the $(U)_{12}$-GMP product comparable to that of the parental $5B^{C\text{-}His}$.

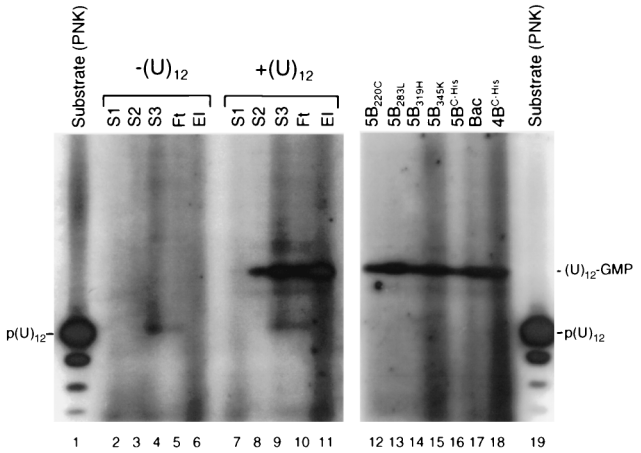

FIG. 8. Detection of a TNTase activity. Equal volumes of each subcellular fraction (S1 to S3), the column flowthrough (Ft), or the eluate (El) of $5B^{C\text{-}His}$ (lanes 2 to 11) were used for a TNTase assay in the absence or presence of oligouridylic acid $[(U)_{12}]$ as a substrate. Reaction products obtained with 350 ng of each mutant protein (lane 12 to 15) or $NS4B^{C\text{-}His}$ (lane 18) or 2.5 μl of an eluate prepared in the same way with wild-type baculovirus infected cells (lane 17) are shown in the right panel. RNAs were analyzed by denaturing PAGE. $(U)_{12}$, radiolabeled by in vitro phosphorylation with polynucleotide kinase (PNK) and [γ-^{32}P]ATP, was used as a size standard $[p(U)_{12}$; lanes 1 and 19]. The position of the reaction products is indicated $[(U)_{12}$-GMP].

tivity are also important for the TNTase activity, all NS5B substitutions and deletions were tested in the same way (Table 3). A selection of completely inactive RdRp mutants in motifs A to C and the lysine substitution at position 345 are shown in lanes 12 to 15. None of these substitutions abolished or enhanced the TNTase activity, suggesting that different amino acid motifs or protein domains of NS5B are responsible for the TNTase activity or that this activity is due to a cellular protein copurifying in minute amounts with $5B^{C\text{-}His}$. To differentiate between these possibilities, lysates from cells infected with either wild-type baculovirus or a recombinant virus directing the expression of an $NS4B^{C\text{-}His}$ protein were subjected to the same purification scheme (Fig. 7B, lanes 28 and 29). As shown in Fig. 8, about the same activity was found with the eluate prepared from the wild-type baculovirus or with purified $4B^{C\text{-}His}$ (lanes 17 and 18), suggesting that the TNTase activity we observed with NS5B was due to a contaminating cellular protein present in minute amounts in the enzyme preparation.

DISCUSSION

In this study, we investigated some of the biochemical properties of the NS5B RdRp of HCV and identified by site-directed mutagenesis 4 amino acid motifs essential for polymerase activity. In agreement with previous observations for HCV and other viruses such as poliovirus, flaviviruses, and the plant virus brome mosaic virus (7, 15, 24, 46), the enzyme was found to be associated with intracellular membranes but could be solubilized with buffers containing high concentrations of salt and detergent. By exploiting this property and by using an affinity-tagged NS5B, we were able to develop a simple and rapid purification scheme which enabled us to obtain sufficient amounts of pure enzyme to examine some of its biochemical properties. The purified protein had an RdRp activity and was able to copy a full-length or near-full-length HCV genome in the absence of any viral or cellular cofactor, indicating a high processivity. In agreement with a recent report (6), we found that initiation of RNA synthesis was dependent on a primer which could be RNA or DNA. Furthermore, the enzyme bound to and acted on RNA templates without detectable specificity. These properties are largely reminiscent of findings for other viral RdRps purified from native and recombinant sources like the ones from poliovirus, encephalomyocarditis virus, rhinovirus, Dengue virus, or brome mosaic virus (36, 40, 43, 46, 49, 54). However, distinct differences exist, and they invite a comparison of these enzymes.

(i) All these enzymes require a primer for initiation of RNA synthesis. This primer can be provided by the 3′ end of the input RNA when folding onto itself or by an exogenous RNA molecule. In poliovirus, which has a 3′ homopolymeric poly(A) tail with an average length of about 60 nucleotides, an oligo(U) primer is required in vitro for initiation of RNA synthesis, generating a full-length minus strand (34, 40, 43). Interestingly, this primer can be substituted by a host cell factor, assumed to be a terminal uridylyl transferase (13, 17), which can add several uridine residues to the 3′ end of the genome, generating an RNA molecule which can form a 3′-terminal loop structure. Consequently, molecules primed by this mechanism have twice the length of the genome (17). As described here and in a recent report (6), the HCV NS5B RdRp also can initiate RNA synthesis on RNA templates by a copy-back mechanism but

does not require an exogenous primer or host factor. Probably, this is not a particular property of the enzyme, but, rather, the heteropolymeric 3′ end of the HCV RNA is sufficient to form a stable structure, allowing RNA synthesis by a copy-back mechanism.

(ii) At least under in vitro conditions, most RdRps have no specificity for the viral template. One of the exceptions to this rule is the BMV RdRp, which acts only on homologous RNA templates (46). However, this enzyme is a complex of two viral and at least five cellular proteins, and it is not clear whether specificity is mediated by the RdRp itself or by one or several other proteins residing in this complex. Another exception is the encephalomyocarditis virus RNA polymerase. By using competition experiments and highly purified $3D^{pol}$ expressed in *Escherichia coli*, specific binding of this enzyme to an RNA fragment corresponding to about the last 135 nucleotides of the genome was found and was shown to be dependent on the presence of the 3′ poly(A) tail (10). Finally, poliovirus $3D^{pol}$ binds to viral and nonviral RNAs equally well, but the affinity of binding to nonviral RNAs is about five times lower, suggesting a sequence specificity (41). For the HCV NS5B polymerase, we were unable to find specific binding to the 3′ end of the viral genome. However, given that binding may depend on specific RNA structures, it is possible that they will form only with longer templates. It is also possible that additional viral or cellular proteins are required for specificity as such or for modulation of NS5B in a way to create specificity. Alternatively, both the viral RNA and NS5B might be concentrated in a subcellular compartment (membrane-associated replicase complex) largely excluding heterologous RNAs, so that even a rather nonspecific enzyme would preferentially copy the viral template.

(iii) As far as they have been examined, the RdRps operate with high processivities and can copy RNA templates several thousand nucleotides long. In this respect, the best-examined example is poliovirus $3D^{pol}$, for which, depending on the reaction conditions, elongation rates between 300 and 1250 nucleotides per min were found (40, 58). For HCV NS5B, due to the complex pattern obtained with the full-length RNA, we have so far not attempted to determine elongation rates. However, as deduced from the experiment in Fig. 5, the enzyme appears to copy the template with much lower rates. Whether this is inherent to our NS5B or due to the reaction conditions is not known. Alternatively, additional cellular or viral factors may be required to enhance the polymerase activity. For example, with poliovirus it was shown that 3AB, when added to purified $3D^{pol}$, stimulated oligo(U)-primed poly(U) synthesis about 100-fold (30). For HCV, Behrens et al. (6) found that after expression of an NS2-5B polyprotein fragment in insect cells, the RdRp activity of this NS5B was much higher than that of NS5B expressed without further NS proteins. Either the NS5B released from the precursor by NS3-mediated proteolytic processing is more active than NS5B with an engineered methionine start codon or one or more of the HCV NS proteins may function as a cofactor for RdRp activity.

(iv) Using different RNA homopolymers, we found that NS5B binds to these RNAs with different specificities, and the order observed was poly(U) > poly(G) > poly(A) > poly(C). Interestingly, when using these homopolymers as templates in a primer-dependent reaction, the inverse order was found, with poly(C)-oligo$(G)_{12}$ used most efficiently and poly(U)-oligo$(A)_{12}$ virtually not used. These results suggest that preferential binding of the polymerase to the primer and low-level binding to the template correlates with high levels of RNA synthesis whereas low-level binding to the primer and tight binding to the template correlates with low levels of synthesis. A similar inverse correlation between the specificity of binding to RNA homopolymers and their use as templates for the RdRp has been described for poliovirus (41). Probably, in both cases tight binding of the polymerase to the template may slow movement on the template and inhibit RNA synthesis.

(v) Numerous studies have shown that nucleic acid polymerases show fundamental structural and mechanistic similarities which are reflected by distinct sequence motifs (for a review, see reference 26). Using linear sequence alignments of HCV NS5B with RTs and viral RdRps as a basis for site-directed mutagenesis experiments, we identified four amino acid motifs which appear to play a pivotal role in polymerase activity. Nearly all substitutions in motif A, probably involved in NTP binding and catalysis and characterized by an invariant aspartic acid residue, led to a complete inactivation of enzymatic activity. Similarly, amino acid replacements within motif B, characterized by an invariant glycine residue, drastically reduced or completely abolished the RdRp activity. Substitutions in motif C had a more differential effect. While changes of the absolutely conserved aspartic acid residue at position 318 completely destroyed the enzymatic activity, in most cases substitutions of the less highly conserved Gly-317 and Asp-319 residues were tolerated, although the enzymatic activities of these enzymes were reduced about a factor of 8 to 13 compared to the wild type. A similar tolerance of the glycine residue within the GDD motif has been described for poliovirus $3D^{pol}$, where it was shown that a single change of the glycine to alanine or serine resulted in RNA polymerases with levels of enzyme activity between 5 and 20% of wild-type levels (25). The most interesting observation we made was the conservative substitution within motif D, which enhanced enzymatic activity by about 50% compared to the wild type. Interestingly, an invariant lysine residue was found at this position in all RTs and RdRps. As inferred from the three-dimensional structure of human immunodeficiency virus RT, this lysine, together with the highly conserved carboxylates of motifs A and C, is directly involved in catalysis (26, 37). The reason why an arginine residue which reduced enzymatic activity is found at this position in all HCV isolates is not clear but might provide one possible explanation for the apparently low replication levels of HCV in vivo (23). However, it should be noted that we measured RdRp activity with purified NS5B only in the absence of other viral proteins. As described above, enzymatic activity could be modified by cellular and viral cofactors and compensate for the lower activity exerted by this less favorable residue. In summary, the results of our mutation study identified four amino acid sequence motifs essential for RdRp activity. Since these four motifs are crucial elements of the so-called polymerase module, it is likely that the corresponding module of NS5B has structural similarities to those described for other polymerases (26).

(vi) In addition to the RdRp activity, a TNTase activity was found to be associated with the purified NS5B which adds a single nucleotide to the 3′ end of the input RNA. While a similar observation has been reported recently (6), the transferase activity described here most likely is due to a cellular enzyme for two reasons. First, all of the 21 mutants tested had about the same TNTase activity irrespective of the level of the RdRp activity. Second, about the same level of activity was found with an $NS4B^{C\text{-}His}$ or with lysates of cells infected with wild-type baculovirus and purified in the same way. This does not necessarily exclude an NS5B-inherent TNTase activity, but in this case the TNTase would be inactive with our purified enzyme. Furthermore, since all RdRp mutants still retained transferase activity, an NS5B-inherent TNTase should reside in a functional domain different from the one responsible for

RdRp activity. For poliovirus $3D^{pol}$, it was shown that the highly purified enzyme expressed in *E. coli* has a TNTase activity (39). However, this activity differs from the one described here in several respects. First, of all four NTPs, only ATP was accepted as a substrate for the transferase reaction. Second, depending on the acceptor, the enzyme added 9 to 18 nucleotides to the 3′ end of the input RNA. Third, mutations inactivating the polymerase also abolished the TNTase activity. On the other hand, there are several examples of copurification of cellular transferases with viral replicases. In the flavivirus West Nile virus, cellular terminal adenylyl and uridylyl transferases were associated with partially purified replicase complexes (15). Since these enzymes could not label West Nile virus RNA, it was concluded that they play no specific role in RNA synthesis. For poliovirus, a host factor was copurified with viral replicase and later shown to be a terminal uridylyl transferase (2).

Although we are far away from a detailed understanding of the mechanisms underlying HCV replication and the cellular and viral factors required for these processes, the availability of sufficient amounts of purified NS5B and the preliminary characterization of its biochemical properties should provide the basis for further studies in this direction. Furthermore, given the knowledge about the structure and function of other polymerases and the possibility that at least some of these properties will be common to all these enzymes (the polymerase module), NS5B also provides an attractive target for the development of an effective antiviral therapy. It is obvious that this enzyme plays an important role in the HCV life cycle.

ACKNOWLEDGMENTS

We are grateful to Axel Roos for preparing the NS5B substitutions, Trevor Wilkinson for a critical reading of the manuscript, and Jan Oliver Koch for stimulating discussions.

This work was supported by a grant from the Bundesministerium für Forschung und Technologie (01 KI 9653/9) and a grant from Roche Products Ltd., Welwyn Garden City, United Kingdom.

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