Selective Stimulation of Hepatitis C Virus and Pestivirus NS5B RNA Polymerase Activity by GTP*

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NS5B of the hepatitis C virus is an RNA template-dependent RNA polymerase and therefore the key player of the viral replicase complex. Using a highly purified enzyme expressed with recombinant baculoviruses in insect cells, we demonstrate a stimulation of RNA synthesis up to 2 orders of magnitude by high concentrations of GTP but not with ATP, CTP, UTP, GDP, or GMP. Enhancement of RNA synthesis was found with various heteropolymeric RNA templates, with poly(C)-oligo(G)12 but not with poly(A)-oligo(U)12. Several amino acid substitutions in polymerase motifs B, C, and D previously shown to be crucial for RdRp activity were tested for GTP stimulation of RNA synthesis. Most of these mutations, in particular those affecting the GDD motif (motif C) strongly reduced or completely abolished activation by GTP, suggesting that the same NTP-binding site is used for stimulation and RNA synthesis. Since GTP did not affect the overall RNA binding properties or the elongation rate, high concentrations of GTP appear to accelerate a rate-limiting step at the level of initiation of RNA synthesis. Finally, enhancement of RNA synthesis by high GTP concentrations was also found with NS5B of the pestivirus classical swine fever virus, but not with the 3D polymerase of poliovirus. Thus, stimulation of RdRp activity by GTP is evolutionarily conserved between the closely related hepaciviruses and pestiviruses but not between these and the more distantly related picornaviruses.

The hepatitis C virus (HCV)1 is an RNA virus that causes acute and chronic liver disease (for reviews see Refs. 1–3). A high proportion of infected patients fail to clear the virus and contract chronic infection, which may lead to liver cirrhosis and, eventually, to hepatocellular carcinoma. Currently it is estimated that 100–200 million people worldwide suffer from a chronic HCV infection. Thus, HCV became a focus of intensive research worldwide.

Based on similarities of genome organization and virus particle structure with the flaviviruses like yellow fever virus and the animal pathogenic pestiviruses like the classical swine fever virus (CSFV), HCV has been classified as the separate genus Hepacivirus in the family Flaviviridae (4).

These viruses have in common an enveloped virus particle and a single-stranded RNA genome of positive polarity encoding a polyprotein that is cleaved by host cell signalases and viral proteinases. In the case of HCV, the genome has a length of ∼9600 nucleotides. Its single long open reading frame is flanked at the 5′- and 3′-ends by nontranslated regions of about 340 and 230 nucleotides in length, respectively. The 5′ nontranslated region is important for efficient translation of the viral polyprotein and functions as an internal ribosome entry site (5–7). The 3′ nontranslated region most likely required for RNA replication carries a 3′-terminal highly conserved sequence forming stable secondary and probably also higher order structures (8–11).

HCV polyprotein processing is accomplished by a combination of host and viral proteinases (for reviews, see Refs. 2, 12, and 13). At least 10 different cleavage products could be identified, which are aligned within the polyprotein as follows (from the amino to the carboxyl terminus): NH2-C-E1-E2-p7-NS2-NS3-NS4A-NS4B-NS5A-NS5B-COOH (14–21). The structural proteins C-E2 are the major constituents of the virus particle, whereas the nonstructural proteins 2–5B most likely are required for RNA replication.

While the mechanisms of polyprotein processing have been studied in great detail, our knowledge about HCV replication is scarce. This is essentially due to the lack of robust cell culture systems allowing efficient virus propagation and the lack of an animal model other than the chimpanzee. By analogy to other plus strand RNA viruses, it is assumed that after infection of the host cell the viral RNA is liberated into the cytoplasm and used for synthesis of the viral proteins. These, most likely in conjunction with cellular proteins, assemble into a membrane-associated replicase complex responsible for the multiplication of the viral RNA via a minus strand RNA intermediate. At least two viral proteins most likely are directly involved in this reaction: NS3 carrying in the carboxyl-terminal domain an NTPase/helicase activity (22–25) and NS5B, the RNA-dependent RNA polymerase (26–30).

In an attempt to set up appropriate in vitro systems permitting a detailed analysis of HCV replication, we have recently developed a simple expression and purification system allowing the production of large quantities of an enzymatically active NS5B protein carrying a carboxyl-terminal hexahistidine affinity tag (27). In agreement with reports by other groups, we observed with the purified protein a primer-dependent RNA polymerase activity able to processively copy homo- and heteropolymeric templates without detectable requirement for additional cofactors and without template specificity (26–30). On heteropolymeric templates, initiation of RNA synthesis is primed via a "copy-back" mechanism; i.e. 3′ terminal sequences fold back intramolecularly, creating a template-bound primer, which is elongated to generate an RNA product twice the length of the input template. Only in the case of homopolymeric...
templates or templates with a 3' homopolymeric tract, which cannot form intramolecular base pairings, the enzyme requires the addition of complementary exogenous primers (26–30).

Using an intensive mutation analysis, we identified four amino acid sequence motifs within NS5B crucial for enzymatic activity (27). These motifs, designated A–D (31), are located in the central NS5B domain and highly conserved between RdRps of plus strand RNA viruses. Motif A, most likely involved in NTP binding and catalysis, is most sensitive toward substitutions (27). Motif B probably is involved in template and/or primer positioning and characterized by an invariant glycine residue absolutely essential for enzymatic activity. The well-known motif C, according to the primary sequence also designated the GDD motif, is important for NTP binding and catalysis, with the first aspartic acid residue being least tolerant toward substitutions. In contrast, alterations affecting the glycine and the second aspartic acid residue are much better tolerated, leading to enzymes with still well detectable RdRp activities (27). Motif D, probably also involved in NTP binding and catalysis, for most RdRps and reverse transcriptases is characterized by a highly conserved lysine residue, whereas an arginine residue is found for most HCV isolates. Interestingly, a lysine substitution for the arginine increases RdRp activity by about 50% (27).

In this report, we continue our biochemical characterization of HCV NS5B. We demonstrate that RdRp activity is selectively stimulated by up to 2 orders of magnitude by high concentrations of GTP. We present evidence for the specificity of this effect and show that GTP-mediated RdRp activation is not due to an increase of RNA binding or the elongation rate, suggesting that high concentrations of GTP accelerate a rate-limiting step during the early phase of RNA synthesis. Finally, we found that NS5B of CSFV can be stimulated by GTP with comparable efficiency but not the 3D polymerase of poliovirus.

**EXPERIMENTAL PROCEDURES**

**Materials—**All radiolabeled nucleotides (3000 Ci/mmol) were purchased from Amersham Pharmacia Biotech. Phosphorylated ribonucleotides were from Roche Molecular Biochemicals at the highest quality available. GDP and GMP (dissodium salts) and heparin 6000 were obtained from Sigma, homopolymeric RNA templates (average length 400 nucleotides) from Amersham Pharmacia Biotech, and 12-mer RNA oligonucleotides from MWG-Biotech. Mung bean nuclease was purchased from Biolabs, T7 RNA polymerase from Epicenter, and RNaseA and RNase-free DNase from Promega.

**Construction of Plasmids and Generation of Recombinant Baculoviruses**—Construction of the plasmid used for expression of an NS5B (residues 2421–3010 of the HCV-BK polyprotein (32)) carrying a hexahistidine affinity tag at the carboxyl terminus has been described (27). A histidine affinity tag at the carboxyl terminus has been described (27).

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Expression and Purification of HCV and CSFV NS5B Polypeptides—The method we developed for purification of HCV NS5B carrying a carboxyl-terminal hexahistidine affinity tag is based on the observation that in contrast to most cellular and baculovirus proteins, NS5B is poorly soluble under physiological conditions. However, it can be efficiently extracted from cell lysates with buffers containing high concentrations of salt, glycerol, and detergent (27). Therefore, lysates of infected insect cells were repeatedly extracted with buffers containing increasing concentrations of these ingredients, and soluble proteins contained in the supernatants were discarded (Fig. 1, lanes 2 and 3). After three extractions, NS5B was strongly enriched in supernatant 3 (S3; lane 4) and used for further purification by affinity chromatography. To compare the biochemical properties of HCV NS5B with the analogous protein of the closely related pestivirus CSFV, we constructed two recombinant baculoviruses directing the expression of NS5B of the CSFV Alfort/187 strain (33) carrying a hexahistidine affinity tag either at the amino or the carboxyl terminus. Both proteins could be purified in an enzymatically active form using the method developed for HCV-5B.2 As exemplified for the amino-terminal fusion protein, the differential extraction of the cell lysate allowed a strong enrichment of the protein in supernatant 3, and it could be further purified by affinity chromatography (lanes 10 and 12, respectively). Because the expression level of this protein was much higher than the one of the carboxyl-terminal fusion protein, the amino-terminal fusion protein was used for the analyses described in this report. Purity of this protein was >90% as was the case for HCV-5B.

Stimulation of NS5B RdRp Activity by High Concentrations of GTP—We have recently described a detailed determination of the kinetic constants of HCV-5B (36). During the course of these experiments, we observed a biphasic titration curve for GTP. At low concentrations of GTP, the enzyme displays a standard Michaelis-Menten kinetic with a $K_m$ value for GTP (36) of $K_m = 0.3 \, \text{mM}$ at $37 \, \text{°C}$ and $K_m = 0.4 \, \text{mM}$ at $37 \, \text{°C}$. Using an in vitro transcribed HCV full-length RNA ~9600 nucleotides in length as template, a linear increase of RNA synthesis was observed up to $5 \, \text{mM}$ GTP (Fig. 2A). Stimulation of RNA synthesis was maximal at $10 \, \text{mM}$ GTP, whereas higher concentrations led to an inhibition of the enzyme. Taking the amount of radioactive incorporation at $10 \, \text{mM}$ GTP as reference (6.05 × 103 cpm), an ~40- and 55-fold stimulation of RdRp activity was found at 5 and 10 mM GTP, respectively. It should be noted that radioactive incorpora-

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2 V. Lohmann, and R. Bartenschlager, unpublished results.
ration at 10 μM GTP was rather low and somewhat variable. Therefore, stimulation rates obtained in different experiments varied between 50 and 100 (see below).

In agreement with the copy-back priming mechanism, analysis of the reaction products on denaturing formaldehyde-agarose gels revealed that up to 1 mM GTP the size of the majority of radiolabeled RNAs was larger than the input template. At GTP concentrations beyond 12.5 mM, the sizes of all radiolabeled RNAs were significantly reduced, and only RNA products about the size of the input template and short RNAs (500–1500 nucleotides) accumulated during the 2-h incubation period (Fig. 3B, lane 4). With higher GTP concentrations in addition to an increase of these products, RNAs smaller than the input became prominent. At GTP concentrations beyond 12.5 mM, the sizes of all radiolabeled RNAs were significantly reduced, and only RNA products about the size of the input template and RNAs 2500–500 nucleotides in length were found (lanes 9–11). This pattern indicated an inhibition of elongation or processivity at very high concentrations of GTP with template-sized RNA products being generated by "abortive" copy-back priming from the input template and the small RNAs either from RNA primers present in the NS5B or the RNA template preparation or, less likely, by de novo initiation. Only under conditions of lower GTP concentrations would the enzyme have been processive enough to copy the template completely during the 2-h incubation period.

Several possibilities could be envisaged for the RdRp inhibition at very high GTP concentrations: (i) a competitive inhibition because of the high excess of GTP over the other NTPs; (ii) impurities present in low amounts in the GTP preparation, or (iii) an insufficient concentration of Mg2+ due to the excess of GTP over Mg2+. Because the latter possibility was the most likely, the GTP titration was repeated, but this time for every GTP concentration a 12.5 mM excess of MgCl2 was used. Under these conditions, an almost identical stimulation of RdRp activity with a maximum at 12.5 mM GTP was found (not shown). At higher GTP concentrations, RNA synthesis still was reduced but to a lower extent, suggesting that inhibition of RdRp activity at high GTP concentrations in part was due to the excess of GTP over Mg2+. Attempts to overcome this inhibition by further increasing the Mg2+ concentration were unsuccessful, because the enzyme is strongly inhibited by high concentrations of MgCl2 (36).

To exclude the possibility that the observed effects were due to contaminants in the GTP preparation used, several batches as well as GTP preparations from other suppliers were tested including lithium and sodium salts. In all cases, comparable stimulation rates were found (not shown). In summary, these results show that RdRp activity of HCV-5B is stimulated by high concentrations of GTP and that the inhibition found at GTP concentrations >10 mM in part was due to an imbalance of the GTP:Mg2+ concentrations. Since a linear increase of RNA synthesis was found with GTP only up to 5 mM, most subsequent titrations were performed up to this value using a constant concentration of MgCl2 (12.5 mM) shown to be optimal for our purified enzyme (36).

Selectivity of RdRp Stimulation—To analyze whether stimulation of RNA polymerase activity could also be mediated by other NTPs, RdRp reactions were carried out with increasing concentrations of ATP, CTP, and UTP. As shown in Fig. 3A, none of these nucleotides could enhance RNA synthesis to the extent of GTP. Maximum stimulation was found with ATP (~4-fold), whereas only a 3-fold increase of enzymatic activity was observed with CTP and UTP (Fig. 3B). Analysis of the reaction products by agarose gel electrophoresis revealed that comparable with the results obtained with GTP, under conditions of high NTP concentrations products about the size of the input template and short RNAs (500–1500 nucleotides) accumulated during the 2-h incubation period (Fig. 3C, lanes 4, 8, and 12). Thus, efficient stimulation of RNA synthesis can only be achieved with GTP, whereas the inhibition of the enzyme is found with high concentrations of either NTP, further supporting the conclusion that it is due primarily to an imbalance of the GTP:MgCl2 concentrations.

Most Amino Acid Substitutions in NS5B Reducing RdRp Activity Also Affect GTP-mediated Stimulation of RNA Synthesis—The experiments described thus far suggest a direct stimulation of RdRp activity by high concentrations of GTP. How-

[FIG. 3. Lack of RdRp stimulation by ATP, CTP, and UTP. A, standard RdRp assays were performed with given concentrations of ATP (●), CTP (▲), UTP (○), or GTP (△). After a 2-h incubation, incorporation of radioactivity was determined by trichloroacetic acid precipitation and liquid scintillation counting. B, RdRp assays were performed as in A with given concentrations of ATP (●), CTP (▲), or UTP (○). 1⁄5 of the reaction mixture was subjected to trichloroacetic acid precipitation and liquid scintillation counting, and 4⁄5 was processed for denaturing formaldehyde-agarose gel electrophoresis (C). Radiolabeled products were visualized by autoradiography. In the case of ATP and UTP titrations, reactions were performed with [α-32P]CTP, while for the CTP titration [α-32P]UTP was used as the radiolabeled nucleotide. Numbers to the left refer to the sizes of RNA markers (nucleotides). Note the different scales of the ordinates in A and B.}
Selective Stimulation of HCV RdRp by GTP

TABLE I
Amino acid substitutions in NS5B and their effects on GTP-mediated stimulation of RNA synthesis

| NS5B protein | Amino acid substitution<sup>a</sup> | Motif<sup>b</sup> | RdRp activity<sup>c</sup> | Stimulation<sup>d</sup> |
|--------------|----------------------------------|-----------------|--------------------------|-------------------------|
| 5BB<sup>c</sup>-His | None |                 | 100 | 50–100 |
| 5BB<sup>c</sup>Thr<sup>c</sup>-Val | B | 2 | 50–100 |
| 5BB<sup>c</sup>Thr<sup>c</sup>-Cys | B | 8 | None |
| 5BB<sup>c</sup>Gly<sup>c</sup>-Ala | C | 10 | 3–6 |
| 5BB<sup>c</sup>Gly<sup>c</sup>-Cys | C | 5 | 6–14 |
| 5BB<sup>c</sup>Asp<sup>c</sup>-Asn | C | 0 | NA<sup>e</sup> |
| 5BB<sup>c</sup>Asp<sup>c</sup>-Asn | C | 8 | 10–20 |
| 5BB<sup>c</sup>Asp<sup>c</sup>-Glu | C | 5 | 3–6 |
| 5BB<sup>c</sup>Arg<sup>c</sup>-Lys | D | 175 | 50–100 |

<sup>a</sup> Amino acid substitution in the NS5B protein. Numbers refer to the amino acid residue of NS5B of our HCV isolate (EMBL nucleotide sequence database; accession no. 7.97730).

<sup>b</sup> Nomenclature of the polymerase motifs according to (31).

<sup>c</sup> Percentage of RdRp activity of each altered NS5B protein compared to the parental NS5B (5B<sup>c</sup>-His) using synthetic full-length HCV RNA template labeled at the 3′-end by abortive copy-back priming.

<sup>d</sup> Ratio of rpm obtained with NS5B at 5 mM and 10 μM GTP (cpm (10 μM)).

<sup>e</sup> Not applicable.

Even, since the NS5B preparations used in this study still contained very low amounts of contaminating cellular proteins, we could not exclude the possibility that the activation effect was related to a copurified contaminant. For example, a GTase in the enzyme preparation could lower the actual GTP concentration, although in this case we would have been unable to determine the Km for GTP (0.5 μM on heteropolymeric templates; Fig. 2A; Ref. 36). Alternatively, a terminal transferase activity, present in low amounts in the enzyme preparation (27), might be stimulated by GTP, generating primer-templates that might be used efficiently by NS5B. To exclude these possibilities, we tested a panel of mutations in polymerase motifs B and C with reduced RdRp activity and an arginine substitution in motif D that increased enzymatic activity (~1.5-fold (Table I and Fig. 4A; Ref. 27). A significant reduction of GTP-mediated stimulation was found with all substitutions affecting the GDD motif, whereas the mutation in motif D had no effect (Table I). Two opposite phenotypes were found with the mutations in motif B. While the valine substitution for the nonconserved threonine at position 286 did not affect stimulation by GTP, the cysteine substitution for the highly conserved threonine at position 287 completely abolished this property.

To further characterize the latter mutant, RdRp assays were performed in parallel with the parental enzyme and an NS5B, which was enzymatically inactive due to an asparagine substitution for the invariant 318 aspartic acid. Analysis of the reaction products by agarose gel electrophoresis revealed that RdRp activity of 5B<sup>c</sup>-Thr<sup>c</sup>-Val was severely impaired and not stimulable by GTP (Fig. 4B). Only RNA products corresponding to the input template and ~700 nucleotides in length were found, indicating a strong processivity or elongation defect. This pattern was similar to the one obtained with the parental NS5B at GTP concentrations of ~20 mM GTP (Fig. 2B), and it suggested that the ~10-kilobase RNA corresponds to the input RNA template labeled at the 3′-end by abortive copy-back priming. The small RNAs probably represent short copy-back primed molecules or molecules initiated from exogenous primers or by de novo synthesis. In summary, the results obtained with these altered NS5B proteins strongly argue against the contribution of a cellular contaminant in the enzyme preparation, and they suggest that high concentrations of GTP directly stimulate RNA synthesis by NS5B. Furthermore, the fact that all mutations affecting highly conserved residues assumed to contribute to the active site of the enzyme can no longer be or can only poorly be stimulated by GTP suggests that the same NTP-binding site is used by GTP for stimulation and incorporation. Evidence for Incorporation of the Stimulating GTP—In the experiments described so far, we always used a RNA template corresponding to the HCV genome. To analyze whether GTP-mediated stimulation was dependent on this particular template, a panel of hetero- and homopolymeric RNAs was characterized in the same way. Comparable stimulation rates were found with an ~400-nucleotide-long HCV RNA corresponding to the 3′-end of the genome and with an in vitro transcribed lacZ RNA (data not shown) showing that GTP enhances RNA synthesis on very different heteropolymeric templates. In the case of homopolymeric RNAs, a stimulation was only found with poly(C)-oligo(G)<sub>12</sub>, whereas no activation was observed with poly(A)-oligo(U)<sub>12</sub> even at 5 mM GTP (Fig. 5A) or UTP (data not shown), suggesting that the stimulating GTP has to be incorporated into the RNA chain.

To further substantiate this assumption, we analyzed whether stimulation of RNA synthesis also could be achieved with GMP or GDP. Therefore, RdRp assays were performed with increasing concentrations of GMP or GDP. As shown in Fig. 5B, instead of a stimulation of RNA synthesis a strong reduction was found with GMP and, in particular, with GDP, corroborating that the stimulating nucleotide also has to be incorporated and that GMP and GDP compete with GTP for the same nucleotide binding site.

GTP Most Likely Accelerates an Early Step of RNA Synthesis—Template-dependent synthesis of a nucleic acid by an RNA polymerase can be divided into at least four consecutive steps: binding of the enzyme to the template, initiation of RNA synthesis, elongation, and termination. To find out which of these steps might be affected by GTP, the following experiments were carried out.

To analyze whether GTP has an effect on RNA binding, a radiolabeled RNA corresponding to the HCV genome was incubated with purified NS5B in the presence of increasing GTP concentrations, and bound RNA was quantitated by liquid scintillation counting (Fig. 6A). Using the assay conditions given under “Experimental Procedures,” no influence of GTP on RNA binding was found. This result corroborates the finding that GTP does not stimulate RNA synthesis from the poly(A)-oligo(U)<sub>12</sub> template and that the stimulating nucleotide has to be incorporated into the RNA product.

Alternatively, GTP might increase the elongation rate. To validate this assumption, RdRp assays were carried out with the HCV genomic RNA and increasing concentrations of GTP. After an initial 5-min incubation of NS5B and template, the reaction was started by the addition of NTPs together with an excess of heparin. The latter was included to prevent further elongation of the HCV genomic RNA and increasing concentrations of GTP. The determination of the sizes of the reaction products was complicated by the fact that most RNAs were primed by a copy-back mechanism and therefore covalently linked to the template. Given the lengths of these molecules, the sizes of newly synthesized RNAs could not be accurately determined. Therefore, RNA products taken at different time points during the reaction were treated with RNases under high salt conditions, allowing the cleavage of only single-stranded but not double-stranded RNA. In this way, single-stranded loops at the 3′-end of the template connecting the input RNA and product are cleaved allowing a separation of template and newly synthesized RNA by denaturing formaldehyde-agarose gel electrophoresis. As shown in Fig. 6B, no significant difference of the lengths of RNA products was found when assays were performed with 50, 250, or 1000 μM GTP (lanes 1–3 and 7–9),...
Fig. 4. A single amino acid substitution in polymerase motif B of NS5B abolishes GTP-mediated stimulation of RNA synthesis. A, a schematic presentation of NS5B indicating the positions of polymerase motifs A–D is given at the top. The amino acid sequences of individual motifs highly conserved among HCV isolates are shown below. Capital letters indicate amino acid residues that are highly conserved among RdRps of plus strand RNA viruses, and underlined and boldface capital letters refer to residues that are highly conserved among all viral RdRps. Numbers above the letters indicate the amino acid positions of NS5B, arrows below the letters refer to substituting residues (see Table I). B, standard RdRp assays were performed with synthetic HCV genomic RNA and GTP concentrations given above the lanes (mM). After 2 h at 22 °C, samples were processed for denaturing agarose gel electrophoresis. Numbers between the gels refer to the sizes of RNA markers (nucleotides). 5B<sup>CTH</sup> refers to the parental NS5B with the carboxy-terminal hexahistidine affinity tag.

whereas a strong reduction of their sizes was found with higher GTP concentrations (lanes 4–6 and 10–12). Thus, high concentrations of GTP have two opposite effects on RdRp activity: a strong stimulation of RNA synthesis and a strong reduction of the elongation rate, the latter being due to the excess of GTP over Mg<sup>2+</sup>. In summary, these data demonstrate that stimulation of RdRp activity by GTP is not due to an enhancement of RNA binding or an increase of the elongation rate. Based on these exclusion criteria, high concentrations of GTP (up to −10 mM) appear to stimulate initiation of RNA synthesis.

GTP-mediated Stimulation of RNA Synthesis by NS5B of a Pestivirus but Not by 3D<sup>pol</sup> of Poliovirus—As described in the Introduction, HCV belongs to the genus Hepacivirus and is most closely related to the pestiviruses, a group of animal pathogenic viruses to which CSFV belongs. This close evolutionary relationship led us to speculate that GTP-mediated stimulation of RNA synthesis might be a biochemical trait conserved between these virus groups and raised the question of whether it would also be found with the more distantly related poliovirus. To address these questions, we analyzed CSFV-5B and 3D<sup>pol</sup> of poliovirus for their ability to be stimulated by high concentrations of GTP. As described above, the CSFV enzyme was purified from insect cells (Fig. 1), whereas 3D<sup>pol</sup> of poliovirus was expressed in Escherichia coli and purified as described elsewhere (34). GTP-mediated stimulation of RNA synthesis was measured for these two enzymes in parallel with NS5B using the HCV genome length in vitro transcript as described under “Experimental Procedures.” A strong stimulation was found both with NS5B of HCV and the analogous enzyme of CSFV (Fig. 7A). Enhancement of RNA synthesis for the pestiviral polymerase was linear between 0.1 and 5 mM GTP, and the stimulation rate was well comparable to the one of HCV NS5B. Only at low GTP concentrations did these enzymes behave somewhat differently in that RdRp activity of CSFV NS5B was lower but showed a stronger stimulation between 10 and 100 μM GTP. In contrast, no stimulation was found with 3D<sup>pol</sup> of poliovirus, both with the full-length synthetic HCV RNA template (Fig. 7A) and with poly(C)-oligo(G)<sub>12</sub> (data not shown). Attempts to detect a significant stimulation by varying reaction conditions (in particular MgCl<sub>2</sub> or MnCl<sub>2</sub> concentrations) were not successful (data not shown).

An analysis of the reaction products by agarose gel electrophoresis revealed that the RNAs generated by all three enzymes were larger than the input suggesting that copy-back priming is a common mechanism (Fig. 7B). However, the overall sizes of the products were somewhat different. Compared with HCV-5B, RNAs generated by CSFV-5B were shorter and products obtained with 3D<sup>pol</sup> were longer, indicating different elongation rates or processivities. Furthermore, in contrast to the GTP-mediated stimulation observed with the NS5Bs, the poliovirus enzyme was instead inhibited by high concentrations of GTP. At ≥2.5 mM, much lower amounts of RNA products were found, and these RNAs were shorter in size than the ones obtained at lower GTP concentrations (Fig. 7B; compare, for example, lane 14 with lane 18). This pattern was reminiscent of the one found with HCV-5B at GTP concentrations of ≥15 mM (Fig. 2B) and could similarly be explained at least in part by the excess of GTP over Mg<sup>2+</sup>. In summary, these results clearly show that RdRp activities of HCV and CSFV NS5B but not of poliovirus 3D<sup>pol</sup> are stimulated by high concentrations of GTP.

**DISCUSSION**

Studies aimed at elucidating the mechanism of HCV replication are limited by the lack of robust and reliable cell culture systems and animal models other than the chimpanzee. Therefore, we have begun to characterize the biochemical properties of NS5B RdRp, most likely the key player of the viral replicase complex. During the course of the determination of the K<sub>m</sub> value for GTP, we observed a strong stimulation of RNA synthesis by high concentrations of GTP. It is toward a detailed analysis of this effect that the present study was undertaken.

Using synthetic full-length HCV RNA and purified NS5B, we found a 50–100-fold stimulation of RNA synthesis by GTP. This stimulation was selective and was not obtained with ATP, CTP, UTP, GMP, or GDP. As shown by the reduced stimulation...
of several NS5B mutants, GTP most likely directly acts on NS5B and occupies the same binding site that is used for RNA synthesis. The stimulation was found with every heteropolymeric template tested and with poly(C)-oligo(G)₁₂ but not with the poly(A)-oligo(U)₁₂ homopolymer. All of these data taken together demonstrate a selective GTP-mediated stimulation of RNA synthesis by NS5B.

The way this stimulation is accomplished currently is not known. GTP has no effect on the overall RNA binding properties or the elongation rate. Based on these observations the acceleration of a rate-limiting step during initiation of RNA synthesis is the most likely explanation. In a simple model, GTP would bind to NS5B and induce an "initiation-competent state," e.g. by the induction or stabilization of a conformational change required for efficient initiation. The "activated" GTP-NS5B complex would then bind to the template and initiate RNA synthesis. Alternatively, NS5B might first bind to the template and then to GTP. In either case, according to this model template binding in the absence of GTP would be non-productive, i.e. NS5B binds to the RNA but in most cases falls off without initiation of RNA synthesis. Once the initiation is completed, the enzyme processively copies the template and is no longer stimulated by high concentrations of GTP.

In this context, it should be noted that an alteration or stabilization of the enzyme conformation by high concentrations of GTP has been suggested for 3Dpol of poliovirus (37). Using cross-linking of oxidized GTP to purified 3Dpol, it was found that NTP binding protects RdRp activity from heat denaturation (37). Interestingly, GTP protected the enzyme to a greater extent than the other three NTPs indicating a tighter binding. Although $K_m$ for GTP of 3Dpol is very low, high concentrations ($\geq 2$ mM) were required for stoichiometric cross-linking and maximum protection from heat denaturation (37). Two NTP binding sites were mapped, one near the amino terminus (residues 57–74) outside of the polymerase core region and the other in the central region overlapping with motif B (residues 266–286) (38). Only the amino-terminal NTP binding site was shown to be crucial for RNA replication in that a leucine substitution for the highly conserved lysine residue at position 61 completely abolished RdRp activity (39, 40). For NS5B, the results described here with the various mutations suggest that an NTP binding site in the core of the enzyme is required. As deduced from the x-ray crystal structure of 3Dpol (41), the invariant carboxylates of motifs A and C of NS5B would be clustered in the "palm" (for a review see Ref. 42). Since substitutions of these residues both reduce RdRp activity.

**Fig. 5.** Evidence that the stimulating nucleotide has to be incorporated into the RNA. A, RdRp assays were performed as described under "Experimental Procedures" using poly(C)-oligo(G)₁₂ (○) or poly(A)-oligo(U)₁₂ (●) and given concentrations of GTP. Incorporation of radioactivity was determined by trichloroacetic acid precipitation and liquid scintillation counting. Because of the isotope dilution, GTP concentrations higher than 1 mM were not tested with the poly(C)-oligo(G)₁₂ template. B, effect of increasing concentrations of GMP (●) and GDP (○) on RdRp activity using synthetic full-length HCV RNA and 1 mM GTP (see "Experimental Procedures").

**Fig. 6.** Stimulation of RNA synthesis by GTP is not due to an enhancement of RNA binding or an increase of the elongation rate. A, RNA binding assays were performed with a radiolabeled HCV genomic RNA in the presence of increasing concentrations of GTP. RNA-NS5B complexes were immobilized on nitrocellulose filters, and bound radioactivity was determined by liquid scintillation counting. B, influence of increasing GTP concentrations on the elongation rate. RdRp assays were performed as described under "Experimental Procedures" using the in vitro transcribed HCV genomic RNA. After the given incubation times, RNAs contained in the reaction mixtures were purified by SDS/proteinase K digestion and subjected to an RNase treatment under conditions allowing only cleavage of single-stranded RNA. Protected RNAs were analyzed by denaturing formaldehyde-agarose gel electrophoresis and radiolabeled products were visualized by autoradiography. Numbers to the right refer to the sizes of RNA markers (nucleotides).
Selective Stimulation of HCV RdRp by GTP

A

![Graph](image)

B

![Table](image)

Fig. 7. GTP stimulates RNA synthesis by HCV and CSFV NS5B but not by 3D\(^{pol}\) of poliovirus. A, RdRp assays were performed with the \textit{in vitro} transcribed HCV genomic RNA and HCV-5B (●), CSFV-5B (○), or 3D\(^{pol}\) (▲). After 2 h at 22 °C, incorporation of radioactivity was determined by trichloroacetic acid precipitation and liquid scintillation counting. B, analysis of the reaction products by denaturing formaldehyde agarose gel electrophoresis. Numbers between the gels refer to the size of RNA markers (nucleotides). GTP concentrations are given above the lanes (mM).

and stimulation by GTP, the NTP binding site in the core appears to be involved in both reactions. However, currently we cannot completely rule out the possibility that these amino acid substitutions caused structural alterations affecting an NTP binding site somewhere else in NS5B. Furthermore, we also found a mutant in motif B (5B\(_{306V}\)) with a very low RdRp activity that could be stimulated with an efficiency comparable with the wild type. However, this mutation is the only one affecting a nonconserved residue, suggesting that this particular substitution affected a property of the enzyme not directly involved in NTP binding and/or catalysis. It remains to be established whether NS5B has more than one NTP binding site and, if so, which of these is required for enzymatic activity and/or stimulation by GTP.

Another unresolved question is why the stimulation is selectively exerted by GTP. Perhaps the initiating nucleotide has to be a guanylate as is the case for many RNA polymerases like the one from bacteriophage T7 or the plus strand RNA virus BMV (43, 44). For example, the DNA-dependent RNA polymerases of \textit{E. coli} and T7 possess at least two distinct NTP-binding sites: one that recognizes only the initiating GTP in a dual function; it can interact with 3D pol (52), and it is an RNA-binding protein able to interact with the template-primer (53). Mutational ablation of RNA binding also reduces 3D\(^{pol}\) stimulation, suggesting that 3AB enhances interaction between the template and 3D\(^{pol}\) (53). An additional function of VPg is to act as an acceptor of UMP added by 3D pol to the OH group of a tyrosine residue within VPg in a template-dependent reaction (54). Uridylylated VPg then is used as the primer for transcription of viral RNA. Whether RNA synthesis by NS5B

of HCV we did not detect a stimulation by GMP, and the \(K_m\) for GTP was \(~100\)-fold lower than the one reported for BMV (50 \(\mu M\); Ref. 45), suggesting that a different mechanism is responsible for the stimulation of RNA synthesis by high concentrations of GTP.

Another possibility of how GTP stimulates RNA synthesis by NS5B would be an enhancement of the transition from initiation to elongation. For the well studied DNA-dependent RNA polymerases, it was shown that transcription can be functionally separated into several distinct steps: template binding, promoter localization, melting of the DNA to form a transcriptionally open complex, nucleotide substrate binding, formation of the first phosphodiester bond, abortive RNA synthesis, promoter clearance, and processive elongation and termination (for a review, see Ref. 46). A characteristic feature of these enzymes is the inefficient transition from initiation to elongation resulting in the synthesis of abortive products. These oligonucleotide products dissociate from the initiated complex when they are not elongated. A similar phenomenon has been described for the RdRp of BMV, resulting in the synthesis of an 8-mer oligonucleotide in a 10-fold molar excess over the full-length RNA product (47, 48). Interestingly, GTP appears to stabilize the RdRp-RNA interaction and in this way increases efficiency of RNA synthesis (49). Although a similar mechanism might be responsible for stimulation of RNA synthesis with NS5B, it should be kept in mind that BMV RdRp initiates RNA synthesis de novo, whereas NS5B, at least under the \textit{in vitro} conditions used here, is primer-dependent. If abortive RNA products would be made, then they probably will not be released but remain covalently bound to the 3'-end of the primer. On the other hand, currently we cannot exclude the possibility that in addition to a stimulation of primer-dependent RNA synthesis, a certain fraction of the RNA molecules synthesized at high GTP concentrations are initiated de novo. Further studies will be required to clarify these issues.

The relevance of RNA synthesis stimulation by GTP for the \textit{in vivo} situation currently is not known. The fact that unphysiologically high concentrations of GTP are required for maximal stimulation clearly suggests that a different mechanism may operate in the infected cell. One simple explanation would be that the effect we observe here is due to the enzyme preparation containing largely misfolded or thermosensitive proteins and that GTP binds to and activates or stabilizes these molecules and in this way increases the number of enzymatically active NS5B proteins. However, the results obtained with the panel of NS5B mutants and with the NS5B of CSFV suggest that this is not the case. Furthermore, the findings that GTP does not enhance poly(U) synthesis from the poly(A) template and that GMP inhibits RNA synthesis indicate that GTP does not act allosterically on RdRp but rather has to be incorporated into the RNA. An alternative explanation would be that high concentrations of GTP substitute for a function normally executed by a cellular or viral protein. For example, in the case of poliovirus it was shown that VPg and in particular the VPg precursor 3AB enhance RNA synthesis by 3D\(^{pol}\) 50–100-fold (50, 51). Similar to what we describe here for NS5B, RNA synthesis stimulated by 3AB is primer-dependent. 3AB has a dual function; it can interact with 3D\(^{pol}\) (52), and it is an RNA-binding protein able to interact with the template-primer (53).
Selective Stimulation of HCV RdRp by GTP

3Dpol is the VPg (or its precursor 3AB), which is uridylylated by are strictly primer-dependent. As described above, in the case responsible for initiation of RNA synthesis. All of these RdRps between hepaciviruses and pestiviruses and probably reflects the conservation of this biochemical trait be-

3Dpol in a template-dependent reaction (54). For pestiviruses but not by 3D pol is stimulated by high concentrations of GTP can be stimulated by a cellular or viral protein (14).

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