Promoter/Origin Structure of the Complementary Strand of Hepatitis C Virus Genome*

Received for publication, February 6, 2002, and in revised form, May 24, 2002
Published, JBC Papers in Press, May 30, 2002, DOI 10.1074/jbc.M201251200

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Hepatitis C virus (HCV) NS5B protein encodes an RNA-dependent RNA polymerase (RdRp). Sequences in the 3′ termini of both the plus and minus strands of HCV genomic RNA harbor the activity of a replication origin and a transcription promoter. There are unique stem-loop structures in both termini of the viral RNA. We found that the complementary strand of the internal ribosome-binding site (IRES) showed strong template activity in vitro. The complementary strand RNA of the HCV genome works as a template for mRNA and viral genomic RNA. We analyzed the promoter/origin structure of the complementary sequence of IRES and found that the first and second stem-loops worked as negative and positive elements in RNA synthesis, respectively. The complementary strand of the second stem-loop of IRES was an important element also for binding to HCV RdRp.

In this study, we analyzed the promoter/origin structure of the complementary sequence of IRES, and we determined the role of the complementary strand of the first and second stem-loops of IRES in RNA synthesis in vitro. This domain perfectly overlaps with those identified in the HCV replicon system in vivo (35).

EXPERIMENTAL PROCEDURES

Recombinant HCV RdRp—HCV NS5B protein truncated by 21 C-terminal amino acids with a His<sub>6</sub> tag was expressed in Spodoptera frugiperda (SF-21AE cells and purified as described previously (26). Purified HCV RdRp was stored at −25 °C in the presence of 50% glycerol.

RNA Templates—RNA templates designed from the complementary sequences of HCV IRES (cIRES) were synthesized with a MEGAscript T7 RNA polymerase kit (Ambion) (Fig. 1). The DNA templates were produced by PCR using the primer pairs listed in Table I. The sequence UGGC was added to the 3′ terminus of all the templates. The DNA templates were removed by digestion with DNase I after in vitro transcription, and all transcripts were purified by 6% PAGE, 7 μm urea for use as the templates of in vitro transcription by HCV RdRp, followed by successive phenol-chloroform extraction and ethanol precipitation. These RNA templates were resuspended in RNase-free water and stored at −80 °C until used.

Transcription in Vitro—Unless otherwise indicated, HCV RdRp activity was measured in 50 μl of standard transcription buffer, T×2(+) (20 mM Tris/HCl (pH 8.0), 100 mM KCl, 2.5 mM MnCl<sub>2</sub>, 50 μM ATP, 50 μM CTP, 5 μM UTP, 0.5 mM GTP, 1.85 Mβg2 of [α-32P]UTP, 10 pmol of RNA template, 25 μg/ml actinomycin D, 5 units of human placental RNase inhibitor (Nacalai Tesque, Japan), 1 mCi DTT, and 10 pmol of NS5B). For the single-round transcription assay, the reaction mixture without nucleotides was preincubated with 50 or 500 μM GTP at 29 °C for 30 min. Then 0.2 mg/ml heparin (Wako Chemicals, Japan) was added to the mixture, followed by ATP, CTP, and UTP, respectively, and the reaction mixture was further incubated at 29 °C for an additional 90 min. The reaction was stopped by extraction of 150 μl of Sepasol RNA II (Nacalai Tesque, Japan) and 40 μl of chloroform.

The radiolabeled RNA was washed with 70% ethanol, dried, and resuspended in formamide dye loading buffer and analyzed by electrophoresis on a 6% PAGE containing 7 μm urea. The radioactivity of the transcribed RNA was measured with a BAS-2000 image analyzer (Fuji Film), and the amount of transcribed RNA was calculated from the

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† The abbreviations used are: HCV, hepatitis C virus; UTR, untranslated region; IRES, internal ribosome entry site; RdRp, RNA-dependent RNA polymerase; DTT, dithiothreitol; cIRES, complementary strand IRES.

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amount of UMP in the transcripts. Each value was calculated from the average of at least three independent assays.

Gel Shift Assay—cIRES, SL234–1D, SL234, SL34, SL4, and SL0 were transcribed in vitro in 40 mM Tris/HCl (pH 7.6), 10 mM DTT, 0.5 mM each of ATP, CTP, and GTP, 50 mM UTP, 50 Ci of UTP (Amersham Biosciences), 10 g of DNA template, 20 units of human placental RNase inhibitor, and 50 units of T7 RNA polymerase (Toyobo, Japan) at 37 °C for 4 h. The transcribed RNA was purified by electrophoresis on a 6% PAGE containing 7 M urea. [32P]UMP-labeled RNA templates (20,000 cpm) were incubated in 20 mM Tris/HCl (pH 8.0), 100 mM KCl, 2.5 mM MnCl2, 5 units of human placental RNase inhibitor, 1 mM DTT, 2 mg/ml yeast tRNA (Roche Molecular Biochemicals), and 3.2 pmol NS5B with or without 0.5 mM GTP (G+) in 20 mM Tris/HCl (pH 8.0), 100 mM KCl, 2.5 mM MnCl2, 25 µg/ml actinomycin D, and 5 units of human placental RNase inhibitor at 29 °C for 30 min. Then 50 µM ATP, 50 µM CTP, 5 µM GTP, and 0.185 Mbq of α-[32P]UTP were added and incubated for an additional 90 min. The RdRp activity with more than 0.2 mg/ml heparin was about 20% that without heparin and did not drop below 20% even when more heparin was added. Next, the time course with 0.2 mg/ml heparin was examined (Fig. 1B). Under these conditions, the accumulation of transcribed RNA

RESULTS

Single-round Transcription—First of all, the concentration of heparin for the single-round transcription was determined (Fig. 2A). HCV RdRp and cIRES template (10 pmol each) were treated with 3.1, 6.25, 12.5, 25, 50, 100, and 200 µg/ml of heparin after preincubation with 50 µM (G(−)) or 0.5 mM GTP (G+) in 20 mM Tris/HCl (pH 8.0), 100 mM KCl, 2.5 mM MnCl2, 25 µg/ml actinomycin D, and 5 units of human placental RNase inhibitor at 29 °C for 30 min. Then 50 µM ATP, 50 µM CTP, 5 µM GTP, and 0.185 Mbq of α-[32P]UTP were added and incubated for an additional 90 min. The RdRp activity with more than 0.2 mg/ml heparin was about 20% that without heparin and did not drop below 20% even when more heparin was added. Next, the time course with 0.2 mg/ml heparin was examined (Fig. 1B).

FIG. 1. Model RNA templates derived from the complementary strand of IRES. Structure and part of the sequence of model RNA templates derived from the complementary strand of IRES. The secondary structure of cSL1 and cSL2 is predicted by mFold. That of cSL3 and cSL4 is drawn as a mirror of that of IRES because mFold predicted 17 patterns for their secondary structure and it has yet to be determined (36, 37). The designation of the model templates indicates the name of the complementary sequences of the stem-loop structure of IRES. All the templates have UGGC (underlined) at their 3’ terminus. The 3’ termini of SL234–1D, SL234, SL34–S, SL34, SL4, and SL0 start from the position marked by arrowtails (A). In SL34–SS, 6 Gs (bold) are substituted by 6 As (bold) of SL34–S (B). Templates carrying only cSL1 and cSL2 (SL12, SL12–1S, and SL12–1LD) are designed as an internal deletion of cIRES (C, D, and E). The 3’ terminus of SL2 starts from the position marked by an arrowtail (C). In SL1234–1S and SL12–1S, the GC stem sequences are substituted with AU (D). In SL1234–1LD and SL12–1LD, the AAUC sequence of the loop structure is substituted with A (E).

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continued for 90 min and reached a plateau. Thus, 0.2 mg/ml heparin was used for the single-round transcription assay of HCV RdRp.

Effect of Stem-loop Structures on Transcription in Vitro—First, the activity of deletion mutant templates for stem-loop structures was measured by the single-round transcription assay because the initiation activity could be accurately measured with this assay (Figs. 1 and 3). Under de novo initiation by 0.5 mM GTP, the template activity of SL234–1D, SL234, SL34, SL4, and SL0 was 74.8, 88.2, 34.2, 27.9, and 9.7% that of cIRES, respectively (Fig. 3). There was no significant difference among cIRES, SL234–1D, and SL234. However, there was a big decrease in activity between SL234 and SL34. Without the initiation, far less RNA was synthesized de novo, and there was little difference among them.

Next, in order to analyze the effect of the stem-loop structures and sequences between SL234 and SL34 on de novo transcription more precisely, single-round transcriptions using SL34-S, SL1234–1S, SL1234–1LD, and SL34-SS were performed (Figs. 1 and 5). Again, the sequence UGGC was added to the 3’/H11032 terminus of each template. The amount of RNA synthesized from 10 pmol of cIRES, SL234–1D, SL234, SL34, SL4, SL0, SL34-S, SL1234–1S, SL1234–1LD, and SL34-SS was 8.06, 6.03, 7.11, 2.76, 2.25, 0.78, 3.90, 4.49, 9.22, and 5.16 fmol, respectively.

Several bands smaller than the template were found among the transcripts from cIRES, SL234–1D, SL234, and SL34 (Fig. 3, A and B). Additional transcripts were also found in SL34-S, SL1234–1S, SL1234–1LD, and SL34-SS (Fig. 5A). However, no additional bands were found in SL4 and SL0. From the pattern and size of the transcripts, we concluded that they were produced by early termination. From the size of the two major additional transcripts estimated from PAGE (Fig. 3, *1 and *2), transcription terminated in bulge *1 and *2 of cSL3, respectively (Fig. 1). Until this experiment, we only calculated the transcripts of template size, excluding those derived from early termination.

To compare the effect of cSL1 and cSL2, we designed templates without early termination as follows: SL12, SL2, SL12–1S, SL12–1LD, and SL0/H11001 SL1 (Fig. 1 and Fig. 5, B and C). From these templates few additional transcripts were obtained. The transcripts of SL2, SL12–1S, and SL12–1LD were 165.5, 258.4, and 91.7% the level of SL12 (Fig. 5B). When cSL1 was added to SL0 (SL0+SL1), the transcripts were 69.0% the level without cSL1 (SL0) (Fig. 1C).

Effect of Stem-loop Structures on Binding to HCV RNA-dependent RNA Polymerase—The same deletion mutant templates were examined for binding to HCV RdRp by gel shift assay (Fig. 4). The relative binding ratio was calculated after correcting for the number of UMP in the probes. The relative binding ratio with 0.5 mM GTP was 100, 74, 83, 27.9, and 9.7% that of cIRES, respectively (Fig. 3D). There was no significant difference among cIRES, SL234–1D, and SL234. However, there was a big decrease in activity between SL234 and SL34. Without the initiation, far less RNA was synthesized de novo, and there was little difference among them.

Next, in order to analyze the effect of the stem-loop structures and sequences between SL234 and SL34 on de novo transcription more precisely, single-round transcriptions using SL34-S, SL1234–1S, SL1234–1LD, and SL34-SS were performed (Figs. 1 and 5). Again, the sequence UGGC was added to the 3’/H11032 terminus of each template. The amount of RNA synthesized from 10 pmol of cIRES, SL234–1D, SL234, SL34, SL4, SL0, SL34-S, SL1234–1S, SL1234–1LD, and SL34-SS was 8.06, 6.03, 7.11, 2.76, 2.25, 0.78, 3.90, 4.49, 9.22, and 5.16 fmol, respectively.

Several bands smaller than the template were found among the transcripts from cIRES, SL234–1D, SL234, and SL34 (Fig. 3, A and B). Additional transcripts were also found in SL34-S, SL1234–1S, SL1234–1LD, and SL34-SS (Fig. 5A). However, no additional bands were found in SL4 and SL0. From the pattern and size of the transcripts, we concluded that they were produced by early termination. From the size of the two major additional transcripts estimated from PAGE (Fig. 3, *1 and *2), transcription terminated in bulge *1 and *2 of cSL3, respectively (Fig. 1). Until this experiment, we only calculated the transcripts of template size, excluding those derived from early termination.

To compare the effect of cSL1 and cSL2, we designed templates without early termination as follows: SL12, SL2, SL12–1S, SL12–1LD, and SL0+SL1 (Fig. 1 and Fig. 5, B and C). From these templates few additional transcripts were obtained. The transcripts of SL2, SL12–1S, and SL12–1LD were 165.5, 258.4, and 91.7% the level of SL12 (Fig. 5B). When cSL1 was added to SL0 (SL0+SL1), the transcripts were 69.0% the level without cSL1 (SL0) (Fig. 1C).
respectively. One pmol of cIRES and SL234–1D inhibited the binding of cIRES with HCV RdRp. One pmol of 3NTR almost inhibited the binding as well. Ten pmol of SL234, SL34, SL34-S, and XREG inhibited the gel shift with cIRES. From these results, cSL2 is concluded important for binding with HCV RdRp especially in 0.5 mM GTP.

**DISCUSSION**

The HCV RNA genome contains conserved 5’- and 3’-UTRs (8–12). As in the case of Flaviviridae family viruses, the 3’-terminal X-region is expected to play an important role in the synthesis of the minus strand, and the complementary strand of IRES is expected to serve as the origin for plus strand synthesis in genome replication (4). The complementary strand of IRES may also work as a promoter of transcription. Mutations in IRES also affected the replication of genomic RNA (35). The reason for this may be that the mutation in the 3’-terminus of the complementary strand (cIRES) affected the replication and transcription. Both termini of the viral genomic RNA have stem-loop secondary structures. The 3’-termini of both the plus and minus strands of genomic RNA are able to serve as templates for RdRp in vitro (16). The complementary sequences of IRES had the highest template activity for de novo RNA synthesis in vitro (26, 34). However, activity for the de novo synthesis of RNA in vitro by RdRp in the X region was very weak (26). Therefore, we determined the promoter/origin structure of

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**FIG. 2.** Determination of heparin concentration for single-round transcription. A, 10 pmol of cIRES was preincubated in 20 mM Tris/HCl (pH 8.0), 100 mM KCl, 2.5 mM MnCl2, 50 μM (G(+)) or 0.5 mM GTP (G(−)), 25 μg/ml actinomycin D, 5 units of human placental RNase inhibitor (Nacalai Tesque, Japan), 1 mM DTT, and 10 pmol NS5B at 29 °C for 30 min. Then heparin (0, 0.1, 3.1, 6.3, 12.5, 25, 50, 100, and 200 μg/ml), 50 μM ATP, 50 μM GTP, and 5 μM UTP, and 0.185 MBq [α-32P]UTP were added successively, and the reaction mixture was further incubated for 90 min. B, the time course of single-round transcription with 0.2 mg/ml heparin in TxG(+) was measured until 120 min. Inset, the autoradiogram of 6% PAGE, 7 M urea. The position of the template-sized transcribed products is indicated by the arrowhead.

**FIG. 3.** Analysis of deletion mutants of stem-loop structures. The template activity of cIRES, SL234–1D, SL234, SL34, SL4, and SLO was measured in 50 μl of the standard transcription buffer TxG(+), TxG(−) (A and B) or TxG(+) (C and D) under the multiround (A and C) and the single round (B and D) condition. After electrophoresis on a 6% PAGE containing 7 M urea, the radioactivity of each template-sized transcript was measured with a BAS image analyzer (A and B), and the amount of synthesized RNA was calculated (C and D). Average values with the S.D. (error bar) of the relative template activity were calculated from at least three independent measurements. The position of the template-sized transcribed products of cIRES (1), SL234–1D (2), SL234 (3), SL34 (4), SL4 (5) and SLO (6) is indicated at the right (A and B). Short additional transcripts from the first four templates are indicated by a star.

**FIG. 4.** RdRp binding activity of stem-loop structures of the complementary strand of IRES. A, RdRp binding activity. [32P]UMP-labeled (20,000 cpm) cIRES, SL234–1D, SL234, SL34, SL4, and SLO were incubated with or without 0.5 mM GTP. The relative binding ratio to that of cIRES with 0.5 mM GTP was calculated after correcting for the number of UMP in the probes and is indicated on the autoradiogram. B, competition assay. [32P]UMP-labeled cIRES (20,000 cpm) and HCV RdRp (3.2 pmol) were incubated with 1, 10, 100, and 1000 pmol of the indicated unlabeled competitor RNA (Fig. 1) (26). C, detection of HCV RdRp protein in the same 0.8% agarose gel by Coo massie Brilliant Blue R-250 (lane 2). The position of HCV RdRp is indicated by arrows.
the complementary strand of IRES.

In the multiround transcription system, the products from short templates were sometimes larger than those from long ones. To compare the initiation activity, we established a de novo single-round transcription system using 0.2 mg/ml heparin to treat HCV RdRp and templates followed by preincubation with 0.5 mM GTP (Fig. 2). Because HCV RdRp prefers a cytidine at the 3' terminus and interacts with GTP (28, 30, 32, 33), all the templates are designed to have UGGC at the 3' terminus (Figs. 1 and 5). We have temporally used the secondary structure of cIRES as a mirror image of that of IRES in Fig. 1 until it is determined experimentally (36, 37).

Because the transcription activity following the treatment with 0.5 mM GTP decreased markedly when cSL2 was deleted, cSL2 was important for de novo initiation (Fig. 3D). However, the activity of SL1234–1S was also half of that of cIRES. Because the T_m of the UA stem was 12 °C, a stem structure might not form in the reaction at 29 °C. A comparison of the activity of cIRES, SL1234–1D, SL1234–1S, and SL1234–1LD indicated the complicated secondary structure of the template, although cSL2 and the stem of cSL1 may affect the structure of the promoter/origin. The sequence between cSL2 and cSL3 could also affect the activity. The seven Gs, which exist between cSL2 and cSL3, did not affect the activity. From a comparison of the activity of SL32–S, SL34–SS, and SL34, the length of the single stranded sequence at the 3' terminus was confirmed important as reported previously (26).

In this series of experiments, we calculated the product amount from only the template-sized bands. Additional products were transcribed from the templates containing cSL3 (Figs. 2 and 5). We measured the size of the products of template size and smaller (Fig. 3, *1 and *2). By comparing their size with that of the templates, the products *1 and *2 were identified as early termination products from the templates. The positions of possible termination sites are mapped in the bulges of cSL3 (Fig. 1). There is a triple helical structure in IRES corresponding to bulge *1, and a complex stem-loop in the stem structure of stem-loop 3 corresponding to bulge *2. We predict a strong secondary structure for these sequences in cIRES. The results and the prediction of secondary structure by mFold (bioinfo.math.rpi.edu/~zukerm/) (38,
39), which predicted too many to be shown here, suggest a complicated secondary structure of ciRES. The results obtained with deletion mutants of stem-loop structures of ciRES were difficult to interpret. The cRES sequence may make complicated stem-loop structures which interact with each other. Therefore, we decided to design a simpler template.

Because we could not predict the secondary structure of cRES but wanted to elucidate the role of cSL1 and cSL2 in the template activity, we constructed templates carrying only cSL1 and cSL2 (SL2) to exclude early termination (Fig. 5B). In this experiment, the activity of SL12–1S was more than twice that of SL12 but that of SL12–ILD was similar to the activity of SL2. Because the Tm of the UA stem in Fig. 1D was 12°C, a stem structure might not form in the reaction at 29°C. The stem structure of cSL1 could inhibit the activity. The sequence of the cSL1 loop did not affect the activity. Because the results from the templates carrying mutant sequences of cSL1 were not conclusive, we made additional templates carrying simple structures. When cSL1 was added to SL0 (SL0 + SL1), the amount of product decreased, confirming that cSL1 was a negative element (Fig. 5C). cSL1 was always identified as shown in Fig. 1 when the secondary structure of the templates was predicted by mFold (data not shown). When cSL1 exists, the length of the 3′ single-stranded RNA is only four nucleotides, too short to initiate RNA synthesis, cSL2 would be a positive element of the promoter/origin of replication.

The RdRp binding activity of cSL2 was similar to that of the RdRp and NS3.

The results from the templates carrying mutant sequences of cSL1 were difficult to interpret. The cIRES sequence may have complicated secondary structure of cIRES. The results obtained, cSL2 would be a positive element of the promoter/origin structure because it binds specifically to RdRp with 0.5 mM GTP. 0.5 mM GTP may give specificity to the binding of RdRp to cSL2.

Although we did not show the results, copy-back products became apparent without 0.5 mM GTP preincubation even in single-round transcription. The mechanism of switching from de novo initiation with 0.5 mM GTP preincubation to copy-back initiation remains to be resolved.

Fig. 6 shows the proposed scheme of initiation from the 3′ termini of the complementary strand RNA as well as that of the positive strand RNA (14). Gel shift assay indicates that cSL2 is also important for the binding of HCV RdRp especially with 0.5 mM GTP (Fig. 4A). Without 0.5 mM GTP, the template–RdRp interaction was enhanced, and SL34 also bound to RdRp effectively. The RdRp binding activity of cSL2 was similar to that of the poly(U/C) tract in the 3′ termini of HCV genome RNA (Fig. 4B). RdRp could also bind to other stems or sequences although the binding was weaker than that of cIRES, SL234–1D, and SL234. Considering the results of transcription and RdRp binding, cSL2 would be a positive element of the promoter/origin structure because it binds specifically to RdRp with 0.5 mM GTP. 0.5 mM GTP may give specificity to the binding of RdRp to cSL2.