RNA-dependent RNA polymerase, NS5B protein, catalyzes replication of viral genomic RNA, which presumably initiates from the 3′-end. We have previously shown that NS5B can utilize the 3′-end 98-nucleotide (nt) X region of the hepatitis C virus (HCV) genome as a minimal authentic template. In this study, we used this RNA to characterize the mechanism of RNA synthesis by the recombinant NS5B. We first showed that NS5B forms a complex with the 3′-end of HCV RNA by binding to both the poly(U-U/C)-rich and X regions of the 3′-untranslated region as well as part of the NS5B-coding sequences. Within the X region, NS5B bound stem II and the single-stranded region connecting stem-loops I and II. Truncation of 40 nt or more from the 3′-end of the X region abolished its template activity, whereas X RNA lacking 35 nt or less from the 3′-end retained template activity, consistent with the NS5B-binding site mapped. Furthermore, NS5B initiated RNA synthesis from a specific site within the single-stranded loop I. All of the RNA templates that have a double-stranded stem at the 3′-end had the same RNA initiation site. However, the addition of single-stranded nucleotides to the 3′-end of X RNA or removal of double-stranded structure in stem I generated RNA products of template size. These results indicate that HCV NS5B initiates RNA synthesis from a single-stranded region closest to the 3′-end of the X region. These results have implications for the mechanism of HCV RNA replication and the nature of HCV RNA templates in the infected cells.

Hepatitis C virus (HCV)† is the etiological agent of non-A, non-B hepatitis, often causing liver diseases including chronic hepatitis, liver cirrhosis, and hepatocellular carcinoma (1–4). HCV has a positive-sense, single-stranded RNA genome of approximately 9700 nucleotides (nt) in length, which is terminated with a stretch (98 nt) of highly conserved sequence, termed the X region (5–11). The X region folds into a stable secondary structure consisting of three stem-loop domains (12, 13). Upstream of the X region is a stretch of poly(U-U/C)-rich sequences of variable length and highly variable sequences of about 30–40 nt (5–11). Infectivity assays showed that the X region and U-U/C-rich sequences are required for viral infectivity, but the variable sequences are not (14). As implicated by sequence conservation among all HCV genotypes, the structure and/or sequence of the X region of HCV is important for minus-strand RNA synthesis and translational regulation (15, 16). The replication of HCV RNA is mediated by NS5B, which is an RNA-dependent RNA polymerase (RdRp) (16–20).

The initial step of viral RNA replication is recognition of the 3′-end of RNA template by RdRp, which may occur directly or indirectly with the help of cellular proteins (21, 22). For example, Qβ bacteriophage replicase recognizes the replicable RNA templates with the help of cellular factors, including ribosomal protein S1 and translation elongation factor Tu (23–25), which are also important for template recognition in certain in vitro selected RNA templates (26–28). Qβ replicase contains two RNA-binding domains; one is the catalytic site, and the other is for sequence-specific recognition of template RNA. However, template specificity is conferred by the host factors. Encephalomyocarditis virus polymerase recognizes the 3′-untranslated region (UTR) of viral RNA only when it contains a poly(A) tail (29, 30). The influenza virus polymerase PB1 subunit also specifically binds, via three separate RNA-binding domains, to the 5′- and 3′-arms of either viral or complementary RNA panhandles (31). In contrast, poliovirus polymerase appears to bind the viral RNA genome through a nonspecific cooperative binding mechanism (32). HCV RdRp has an RNA binding activity and preferentially binds poly(U) and poly(G) over poly(C) and poly(A) homopolymeric RNA (18). However, no specific binding of HCV polymerase to the 3′-end of HCV viral RNA has been reported, although the X region is important for infectivity in vivo (14) and acts as a minimal RNA template in vitro (16).

The mechanism of initiation of RNA synthesis by RdRp for most RNA viruses is poorly understood. Brome mosaic virus RdRp appears to be able to recognize an internal promoter for subgenomic mRNA synthesis de novo (33, 34). However, it is not clear whether there is a direct interaction between the promoter and viral RdRp holoenzyme, since the purified RdRp complex contained two virus-encoded proteins, the polymerase and helicase, and a subunit of translation elongation factor eIF3 (35). Similarly, bovine diarrhea virus polymerase can also recognize the minimal 21-nt RNA template and synthesizes complementary RNA in a primer-independent manner and start RNA synthesis preferentially from a cytidylate at the most 3′-end of the template (36).

For HCV polymerase, RNA synthesis has been shown to depend on exogenous or snap-back RNA primers in vitro (17–20). However, we recently reported that an HCV NS5B expressed and purified from Escherichia coli is able to initiate RNA synthesis de novo using the full-length HCV genome or...
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the 3'-end of the HCV genome in both senses as templates without requirement of a primer (16). Furthermore, we demonstrated that NS5B can utilize the 98-nt, X region RNA at the 3'-end of plus-strand HCV genomic RNA as a minimal template. The upstream sequences including the variable sequence region and U/U/C-rich tract were found to enhance the efficiency of RNA synthesis. For the minus-strand template, a minimum of 239 nt at the 3'-end of minus-strand HCV RNA was required for efficient RNA synthesis in vitro. Thus, our recombinant HCV polymerase by itself appears to be able to recognize HCV RNA and utilize the X region as a minimal RNA template. We used this minimal RNA template to further elucidate the mechanism of HCV RNA synthesis. Our results show that HCV polymerase can bind to the X region directly, but its binding is enhanced by an upstream U/U/C-rich tract and part of the NS5B-coding region. Furthermore, we identified the RNA initiation site and determined the sequence requirement for initiation of RNA synthesis. These results shed further light on the mechanism of HCV RNA replication.

EXPERIMENTAL PROCEDURES

Purification of HCV RdRp—Recombinant HCV RdRp NS5B enzyme was expressed in E. coli BL21 transformed with plasmid pThNS5B and purified using a Ni2+–nitrilotriacetic acid (NTA)-agarose column (Qiagen) as described previously (16). The NS5B-containing fractions were collected and dialyzed against buffer A (50 mM Tris-HCl, pH 8.0, 50 mM NaCl, 5 mM MgCl2, 1 mM dithiothreitol, 10% glycerol) and applied to a heparin-Sepharose CL-4B column (Amersham Pharmacia Biotech) that had been equilibrated in the same buffer. The column was washed with buffer A and step-eluted with 100 mM to 1 M NaCl. The peak fractions containing pure NS5B were pooled, and the salt concentration of the pooled fractions was adjusted to 100 mM NaCl with buffer A. The adsorbed protein was then eluted with a 20-ml linear gradient of NaCl from 100 mM to 1 M. Fractions (1 ml) were collected, and small aliquots of the peak fractions were stored at −80 °C after dialyzing against buffer A containing 20% glycerol. Protein concentrations were determined by Bio-Rad protein assay, and the purity of protein was estimated by densitometric analysis of a silver-stained SDS-polyacrylamide gel.

Western Blot Analysis—NS5B proteins were subjected to a SDS-10% polyacrylamide gel electrophoresis and electroblotted onto a nitrocellulose membrane. The membrane was probed with rabbit anti-His6 antibody (Santa Cruz Biotechnology, Inc.), and proteins were detected by using goat anti-rabbit IgG conjugated with peroxidase (American Qualex) and enhanced chemiluminescence (ECL, Amersham Pharmacia Biotech).

RdRp Activity Assay—Polymerase activity assays were carried out as described previously (16). Briefly, 200 ng of RNA template was incubated with 2 pmol of NS5B enzyme in a 25-μl reaction containing 50 mM Tris-HCl, pH 8.0, 50 mM NaCl, 100 mM potassium glutamate, 5 mM MgCl2, 1 mM dithiothreitol, 20 μg/ml actinomycin D (Sigma), 20 units of RNase inhibitor (Promega), 10% glycerol, 0.5 μM each ATP, CTP, GTP, and 5 μM UTP with 10 μCi of [α-32P]UTP (3000 Ci/mmol; NEN Life Science Products) for 1 h at 25 °C. After reactions, the products were extracted with acidic phenol emulsion (phenol, chloroform, Ambion), 10% SDS, 0.5 mM EDTA (1:1:0:2:0.04), precipitated with 2.5 volumes of 95% ammonium acetate/isopropanol alcohol (1:5), denatured in a denaturing buffer containing 5% formamide with 10 mM EDTA and 0.025% each of xylene cyanol and bromphenol blue, and then resolved on an 8% urea, 6% polyacrylamide gel (14 × 17 cm). After electrophoresis, gels were stained with ethidium bromide to localize positions of template RNAs. For better resolution, products were also analyzed on a 5% denaturing sequencing gel (35 × 43 cm). The dried gels were exposed to x-ray film, and the amount of [32P] incorporated into the newly synthesized RNA was quantified using a PhosphorImager (Molecular Dynamics, Sunnyvale, CA).

RNA Preparation—Wild-type and mutant X region RNAs were synthesized in vitro transcription using polymerase chain reaction-amplified DNA templates fused to bacteriophage T7 RNA polymerase promoter as described previously (16). The full-length 3'-UTR of HCV containing a long U/U/C-rich tract (81 nt) (named HCV-3'-(+)) was amplified by polymerase chain reaction using the infectious genotype 1b HCV cDNA (7) as a template. The 3'-UTR of HCV containing a short U-rich sequence (13 nt) (named HCV-3'-X) was amplified from an HCV Korean isolate of 1b genotype (15, 16). The FCR RNA, which contains the HCV-3'-(+), and the neighboring NSS5B-coding region (nt 9300–9364 of the infectious genotype 1b HCV RNA) and the CR RNA, which contains C-terminal portion of NSS5B-coding region only (nt 9300–9364), was amplified in a same way. The gel-purified reverse transcription primer chain reaction-amplified DNA templates were used directly for in vitro transcription using T7 RNA polymerase. After transcription with T7 RNA polymerase, DNA templates were digested by RQ1 DNase (Promega) for 15 min at 37 °C. Then in vitro transcribed RNAs were purified using a Sephadex G-25 spin column, extracted with acidic phenol/ chloroform, and then precipitated with ethanol. RNA concentrations were estimated by measuring the absorbance at 260 nm.

5'-End Labeling of RNA—For 5'-end [32P]labeling of X region and FCR RNAs, in vitro transcripts were dephosphorylated with shrimp alkaline phosphatase (Roche Molecular Biochemicals) and phosphorylated with T4 polynucleotide kinase (New England Biolabs) and [γ-32P]ATP (6000 Ci/mmol; NEN Life Science Products). After heat inactivation of the enzyme, free nucleotides were removed using a Sephadex G-25 spin column, added with T4 RNA ligase, and purified by electrophoresis on a 6% polyacrylamide gel containing 8 μM 1x in 1x TBE buffer (90 mM Tris base, 90 mM boric acid, 1 mM EDTA, pH 8.2). The labeled RNAs were eluted in 2 ml of a buffer consisting of 0.5 mM ammonium acetate, 1 mM EDTA (pH 7.5), and 0.5% SDS. The 5'-end-labeled RNAs were then precipitated with ethanol and resuspended in double distilled H2O to a final concentration of 1.2 μg (specific activity: 4.4 × 106 cpm/pmol for X RNA and 3.7 × 106 cpm/pmol for FCR RNA).

Alkaline Hydrolysis of End-Labeled RNA—[32P]-Labeled RNA was incubated with 20 μl of Na2CO3/NaHCO3 buffer (pH 10) for 20 min at 90 °C. The partially hydrolyzed RNA was mixed with an equal volume of a denaturing buffer containing 5% formamide with 10 mM EDTA and 0.025% each of xylene cyanol and bromphenol blue and loaded onto a denaturing polyacrylamide gel.

Cross-linking of [32P]-Labeled NS5B and Purified NS5B—In vitro transcription using polymerase chain reaction-amplified HCV-3'-UTR RNA was synthesized by in vitro transcription using the HCV-3'-UTR RNA as a template. The labeled RNAs were then precipitated with ethanol and resuspended in double distilled H2O to a final concentration of 1.2 μg (specific activity: 4.4 × 106 cpm/pmol for X RNA and 3.7 × 106 cpm/pmol for FCR RNA) and applied to a heparin-Sepharose CL-4B column (Amersham Pharmacia Biotech) equilibrated in the same buffer. The column was washed with buffer A and step-eluted with 100 mM to 1 M NaCl. The peak fractions containing pure NS5B were pooled, and the salt concentration of the pooled fractions was adjusted to 100 mM NaCl with buffer A. The adsorbed protein was then eluted with a 20-ml linear gradient of NaCl from 100 mM to 1 M. Fractions (1 ml) were collected, and small aliquots of the peak fractions were stored at −80 °C after dialyzing against buffer A containing 20% glycerol. Protein concentrations were determined by Bio-Rad protein assay, and the purity of protein was estimated by densitometric analysis of a silver-stained SDS-polyacrylamide gel.

Electrophoretic Mobility Shift Assay—The purified NS5B diluted to appropriate concentrations in buffer A were mixed with the radiolabeled RNA in the same buffer and incubated for 15 min at 25 °C. RNA-protein complexes were formed in a 10-μl reaction mixture in the same buffer as that for the RdRp assay, with the exception that rNTPs and actinomycin D were omitted. The 5'-end [32P]-labeled RNA (50 fmol) was incubated with 2.5 pmol of NS5B, unless otherwise specified, or increasing amounts of NS5B (0.5, 0.7, 0.9, 1, 2, 3, 4, and 5 pmol) for 15 min on ice and then for an additional 15 min at 25 °C. After reactions, 2 μl of loading buffer (50% glycerol, 0.01% each xylene cyanol and bromphenol blue) was added, and the samples were loaded directly onto a 1-mm-thick nondenaturing 4% polyacrylamide gel (5:1 acrylamide/bisacrylamide) gel. Gels were prerun at 5 V/cm for 30 min and run at room temperature in 0.5X TBE. For competition assays, increasing amounts of the unlabeled RNAs were preincubated with NS5B for 15 min on ice, and incubation was continued for 15 min at 25 °C with the labeled RNA. The unlabeled competitor HCV RNAs or homopolymeric RNAs were used in the competition assay.

Footprinting Assay for Probing the NSS5B-binding Site on the X Region—The 5'-end [32P]-labeled X RNA (50 fmol) was preincubated with 3 pmol of NS5B in buffer A on ice for 15 min and then at 25 °C for 15 min. After reactions, RNAs were digested with RNase T1 (Ambion; 0.1 units/reaction) for 15 min at 25 °C in 10 μl of RdRp assay buffer (50 mM Tris-HCl, pH 8.0, 50 mM NaCl, 100 mM potassium glutamate, 5 mM MgCl2, 1 mM dithiothreitol, 10% glycerol). Samples were mixed with an
Expression and Purification of Recombinant NS5B—We have previously expressed and purified an enzymatically active, full-length recombinant HCV NS5B by Ni²⁺-NTA chromatography (16). This preparation contained trace amounts of E. coli proteins of 75 and 110 kDa, which were detectable by silver staining but not by Coomassie Blue staining (see Ref. 16; data not shown). To study RNA binding and enzymatic properties of NS5B, we first performed further purification of NS5B. The pooled NS5B fractions eluted with 250–350 mM imidazole from Ni²⁺-NTA column were loaded on a heparin-Sepharose CL-4B column. The bound NS5B was eluted as a broad peak by NaCl gradient (Fig. 1A). The bound NS5B was eluted with a buffer containing 500–700 mM NaCl. The eluate was adjusted the NaCl concentration to 100 mM. The NS5B was detected by anti-His6 antibody in Western blotting (Fig. 1A). A 65-kDa protein was labeled. To assess the specificity of this cross-linking, increasing amounts of various unlabeled RNAs were used for competition studies. We found that the unlabeled homologous 3′-UTR RNA competed very effectively with the labeled RNA for binding (lanes 2–5). We next used homopolymeric RNAs for competition to assess the nucleotide preference for NS5B binding. Among the homopolymeric RNAs used (Fig. 2B), poly(U) competed most efficiently (lanes 5–7), followed by poly(G) (lanes 11–13). In contrast, poly(C) and poly(A) were poor competitors (lanes 2–4 and lanes 8–10, respectively). This order of competition agrees well with the previous direct filter binding assays of NS5B using homopolymeric RNAs (17). However, the 98-nt X region alone did not significantly compete for binding; only in the presence of a 500-fold molar excess of unlabeled RNA was some inhibition of NS5B binding observed (Fig. 2C, lane 5). In direct UV cross-linking studies using 32P-labeled 98-nt X RNA, a very weak NS5B band was detected only after a very long exposure (data not shown). These results indicate that NS5B binds weakly to the X region. We have also tested the 3′-UTR (HCV-3′(+)) Full) from an infectious HCV genotype 1b RNA (14), which contains a longer (81-nt U/U-C) pyrimidine-rich tract; comparable binding activity was detected (data not shown). These results indicate that NS5B binds to the HCV 3′-UTR mainly through interaction with the U-rich sequence. It also weakly interacts with the X region.

Since UV cross-linking studies showed a weak but still detectable interaction between NS5B and the X region (data not shown), we took another approach, electrophoretic mobility shift assay (EMSA), to characterize the interaction. We first estimated the binding affinity of NS5B to X RNA. The NS5B proteins at various concentrations were incubated with a fixed amount of radiolabeled X RNA, and the RNA-protein complex was resolved on a nondenaturing polyacrylamide gel. As shown in Fig. 3A, an RNA-NS5B complex was retarded at the loading wells when increasing amounts of NS5B were used. The percentage of probe bound to NS5B was quantified using a PhosphorImager and graphically illustrated in Fig. 3B. The appar-
ent dissociation constant was estimated to be about 170 nM. The binding affinity of HCV NS5B to this RNA is lower than those of influenza virus polymerase subunit PB1 to the viral complementary RNA (70 nM) (31) or Qβ replicase to in vitro selected RNA templates (20–30 nM) (26–28), but it is slightly higher than that of Qβ replicase to unselected RNA ligand pools (>200 nM). Competition assays indicated that the NS5B-X RNA interaction can be disrupted efficiently with a 5–10-fold excess of unlabeled X RNA (Fig. 4A) and also very efficiently inhibited by poly(U) (Fig. 4B). Other homopolymers were less effective in competition, with the following order of binding efficiency: poly(U) > poly(C) > poly(G) > poly(A). These results were similar to the competition data observed with the full-length 3′-UTR by UV cross-linking, except that poly(C) was a slightly better competitor than poly(G) for the X RNA probe in EMSA. To further confirm that the binding of NS5B to X RNA was specific, we used several X RNA mutants that contain various degrees of deletion of stem I and/or II for competition assays. These mutants have deletions of 40, 46, and 57 nt from the 3′-end (−40X, −46X, and −57X, respectively), and none of them can serve as RNA templates (see Ref. 16; see also Fig. 7B, left panel, lanes 9–11). The results showed that all of them competed poorly as compared with the 98-nt X RNA for binding to NS5B (Fig. 4C). These results suggest that NS5B binding to the X region is necessary for polymerase activity. Nevertheless, −40X RNA competed slightly better than the −46X and −57X RNAs at 10-fold molar excess, suggesting that −40X RNA contains part of the NS5B-binding sequence.

A previous report suggested that NS5B binds mainly to the 3′-end of the NS5B-coding region but not to the 3′-UTR of HCV RNA (37). To reconcile this finding with our results, we examined the NS5B-binding capacity of an RNA (FCR) that contains the 3′-terminus of the NS5B-coding region linked to the full-length 3′-UTR of HCV. EMSA analysis showed that this RNA formed an NS5B-RNA complex (Fig. 5A, lane 2), which could be specifically competed by a 5-fold molar excess of unlabeled FCR RNA (lanes 3–5). But the corresponding amounts of the RNA (CR) that consists of only the NS5B-coding region competed less efficiently (Fig. 5A, compare lane 3 with lane 6). In contrast, poly(U), but not poly(A), abolished the complex formation as efficiently as the full-length FCR (Fig. 5B). This result suggests that NS5B binds more strongly to the poly(U) sequence than the NS5B-coding region, in contrast to the published report (37).

These data together indicate that NS5B does bind weakly but specifically to the X RNA, which is the minimal cis-acting RNA element at the 3′-end of HCV genome for RNA synthesis by NS5B (16). In addition, NS5B binds to the U-U/C-tract of the 3′-UTR and the 3′-end of the NS5B-coding region, with the former having a stronger binding activity.

**Footprinting Mapping of NS5B-binding Site on X RNA—**We further characterized the NS5B-binding site on the X region by footprinting assays using RNase T1 (which specifically cleaves G-residue on single-stranded RNA). The 5′-end-labeled X RNA probe was incubated with the purified NS5B, and the RNA-protein complex was treated with RNase T1 under limited digestion conditions. As previously shown (13), most G residues (nt 22, 32, 35, 37, 50, and 73, counting from the 5′-end of the X region) in the predicted single-stranded regions of the X RNA were digested with RNase T1 in the absence of NS5B (Fig. 6A, lane 2). In addition, three G residues in the predicted double-stranded regions were weakly digested; these include the G residue at nt −53, which can potentially base pair with the terminal U-residue at nt −98, and G at −41 and −42 on stem II neighboring loop II (Fig. 6A, lane 2). These residues and nt 50 were protected from RNase T1 digestion in the presence of NS5B (Fig. 6A, lanes 3 and 4, and Fig. 6B, Gs in open circles). In contrast, Gs on loops I and II (G at −32, −35, −37, and −73) were not protected. These results indicate that NS5B binds stem II and the hinge region between stems I and II. The footprinting result was consistent with the EMSA competition assays using X deletion mutants, which suggest that NS5B binds part of stem II and stem I (Fig. 4C). These results together suggest that NS5B binds stem II, part of stem I (shown in Fig. 5B with the shaded bar), and the hinge region between the two stems. This footprinting analysis further confirms the direct binding of NS5B to the X region.

**RNA Synthesis on X RNA Template Is Initiated Selectively from the Unpaired Nucleotide U78 on the Internal Single-stranded Region of Loop I—**We previously found that the major HCV NS5B-synthesized RNA products using X RNA (98 nt) migrated slightly faster than the template RNA on denaturing polyacrylamide gels (16). To characterize this product, we first determined exact size of the RNA product by resolving it on a denaturing polyacrylamide sequencing gel. A series of RNA size markers prepared by partial digestion of the 5′-end-labeled 98-nt X RNA with RNase T1 or alkaline hydrolysis was run in parallel. The results showed that the size of the major product from X RNA template is 78 nt long, smaller than the template RNA (98 nt) (Fig. 7A, indicated by an arrowhead). To determine whether this RNA product was initiated internally or initiated from the 3′-end of the template and terminated prematurely, we tested template activity of serial 3′-end truncation mutants derived from X RNA. All of those truncation mutants that retain at least part of the stem I structure (up to a 15-nt deletion) from the 3′-end could serve as templates for NS5B and generated the same 78-nt product (Fig. 7B, left panel, lanes 2–4). The exact sizes of some of these RNA products (X, −5X, −10X, and −15X) were also analyzed on a denaturing sequencing gel (Fig. 7B, right panel), which showed that the major RNA products of these templates were exactly 78 nt in length. These results together suggest that the 78-nt product was not derived from the 3′-end initiation; instead, it might be the result of an internal initiation from loop I at nt 78. A 20-nt deletion, which completely deleted stem I, generated two RNA products of different sizes (lane 5). Deletion of 25 or 30 nt generated products nearly equivalent to the respective template sizes (lanes 6 and 7). Deletion of 35 nt generated an RNA of exact template size (lane 8). These results suggest that RNA synthesis by HCV NS5B may be initiated from the single-stranded sequence closest to the 3′-end of HCV X RNA template, but the precise point of initiation may vary slightly depending on the sequence or
structure of templates. Most of these deletion mutant X RNAs were better substrates than X RNA (compare lane 1 with lanes 2–8); in particular, the 10- and 35-nt deletion mutants generated 5.3- and 3.4-fold, respectively, more products than the wild-type X RNA. The 40-, 46-, and 57-nt deletion mutants (−40X, −46X, and −57X) were very poor templates; nevertheless, small amounts of products appeared to be of template size. The loss of template activity of these truncated X mutants corresponded to their inability to bind NS5B efficiently, as shown by competition assay in EMSA (Fig. 4C). Some deletion X mutants also yielded an additional faint band of high molecular weight products, which might represent RNA synthesis using folded back RNA templates.

To confirm the site of internal initiation, we made several mutant X templates containing a single base change at nt 78 (78U→A, C, or G) and a deletion mutant at nt 77 (del77X). As expected, one-nucleotide deletion at nt 77 (C) resulted in the synthesis of a product that is 1 nt shorter than the product of the wild-type X RNA (Fig. 8A, lanes 3 and 4). Substitutions of U78 with A, C, or G affected the efficiency of RNA synthesis (Fig. 8B), with preference for a pyrimidine ribonucleotide as the first nucleotide of RNA synthesis. These data together indicate that the HCV polymerase indeed initiated RNA synthesis from the single-stranded unpaired U residue at nt 78 preferentially.

To assess the potential impact of the upstream HCV RNA sequences on the initiation site of RNA synthesis by NS5B, we used two full-length 3′-UTR HCV RNAs, which consist of the variable sequence, U-U/C-rich tract of different lengths, and X region as templates. One of them, HCV-3′(+)-Full (total size 225 nt), is derived from an infectious HCV 1b strain (7) and contains 81 nt of U-U/C sequences. The other, HCV-3′(X) (total size 152 nt), is derived from a Korean isolate of HCV (15) and contains 13 U residues. RNA products were analyzed on a denaturing sequencing gel. When HCV-3′(X) RNA was used as template, two major RNA products were generated, both of which range between 125 and 135 nt in length (Fig. 9, lanes 3 and 6, indicated by open arrowheads) and are smaller than the template (152 nt) by approximately 20 nt. This result is consistent with the initiation site within loop I. Similar to the X RNA template, the HCV-3′(X) RNA did not yield any template-sized products. When HCV-3′(+)-Full RNA, which has a long stretch of U-U/C-rich sequence, was used as a template, the
major products were heterogeneous RNA ladders in the range of 80–140 nt. These RNAs most likely represent the polymerase stuttering and abortive synthesis within the U-U/C-rich sequences (lanes 2 and 5). This stuttering was not apparent with HCV-39(X) RNA, which has only 13 U residues (lane 6). It is significant that the RNA ladder started almost right after the end of the X sequence. In addition, the HCV-39(1) Full RNA also yielded a product of approximately 200 nt, which is smaller than the template (225 nt) (indicated by an arrowhead in Fig. 6, lane 2), consistent with its initiation within loop I. The 3'-ends of truncated X mutants are presented in closed boxes. SL I–III, stem-loops I–III (13).

Effects of Single-stranded Nucleotide Extension at the 3’-End of the X Template on Transcription Initiation—The results described above suggested that NS5B can only initiate from single-stranded RNA regions. To test this idea, we examined whether HCV NS5B could initiate RNA synthesis from the precise 3’-end of an RNA template if it has single-stranded sequences at the end of stem I. We constructed a series of X RNA derivatives with a mutation in loop I. A, wild-type X RNA (X) and a deletion X mutant (d77X) containing a 1-nt deletion at nt 77 (C) were used for RdRp activity assays. Products synthesized were resolved on a sequencing gel with the RNA size markers (lanes 1 and 2) as described in Fig. 6. B, X mutants with a substitution of T78 with A (78A), C (78C), or G (78G) were used for RdRp activity assays. Products were analyzed as in A. The arrowhead indicates nt 78.
The nucleotide sequences added to the 3'-schematic diagram of RNA templates used for RdRp activity assays. The nucleotide sequences added to the 3'-end of X RNA are presented in underlined italic type. B, an autoradiogram showing the products resolved on an 8% urea, 5% polyacrylamide sequencing gel. White dots denote the positions of the RNA templates. The 78-nt RNA product is indicated by an arrowhead. C, an autoradiogram showing the products from X, -15XM (containing an unpaired 3'-end), and d77X (Fig. 8). The major RNA product of the -15XM RNA (6 nt longer than the 78-nt product derived from the X RNA) is indicated by an arrowhead. The templates used for the assays are indicated above the autoradiograms.

FIG. 10. Effect of single-stranded nucleotide extension at the 3'-end of X RNA on the initiation site of RNA synthesis. A, schematic diagram of RNA templates used for RdRp activity assays. B, an autoradiogram showing the products resolved on an 8% urea, 5% polyacrylamide sequencing gel. White dots denote the positions of the RNA templates. The 78-nt RNA product is indicated by an arrowhead. C, an autoradiogram showing the products from X, -15XM (containing an unpaired 3'-end), and d77X (Fig. 8). The major RNA product of the -15XM RNA (6 nt longer than the 78-nt product derived from the X RNA) is indicated by an arrowhead. The templates used for the assays are indicated above the autoradiograms.

Initiation of RNA synthesis is a crucial step in the replication of HCV genome, yet it has been poorly understood because of the lack of a suitable RdRp assay system. We have recently reported the expression and characterization of a recombinant HCV RNA polymerase, which is capable of carrying out de novo RNA synthesis and can utilize the 3'-UTR of the HCV genome as a minimal template (16). These findings allowed us to study the mechanism of initiation of HCV RNA synthesis in vitro.

We have demonstrated in this study that HCV NS5B binds weakly but specifically to the X region at the 3'-end of HCV RNA. The interaction was detected biochemically by UV cross-linking, footprinting, and EMSA. By footprinting analysis, we mapped the NS5S-binding site to stem II and the single-stranded hinge between stems I and II of the X region. Correspondingly, the RdRp assay showed that this region is necessary for template activity of the X RNA. The relatively low binding affinity of NS5B to the X region provides a mechanism for NS5B polymerase to bind to the promoter to initiate RNA synthesis and yet escape from the promoter to elongate RNA synthesis. Indeed, it has previously been shown that the template activity of homopolymeric RNAs for HCV NS5B-inversely correlated with the binding activities of these RNAs to NS5B (18). Nevertheless, the NS5B binding activity appears to be required for an RNA to serve as a template, since -40X, -46X, and -57X mutants, which have lost most of the NS5B-binding activity (Fig. 4C), also lost most of their template activity for NS5B (Fig. 7B). This conclusion is consistent with the previous reports that the template activity of RNAs selected based on their ability to bind Q8 replicase correlated with their binding affinity (26–28). Our studies further showed that NS5B binds the pyrimidine-rich region and part of the NS5S-coding region at a higher affinity than the X region. This binding probably contributes to the selectivity of NS5B for the 3'-end of HCV RNA. It should be noted that a recent report showed that NS5B interacts mainly with the NS5S-coding sequences (37) but not the 3'-UTR region, whereas our studies showed that NS5B binds the U-U/C-rich region more strongly than the NS5S-coding sequence. This discrepancy may be partially due to the fact that the NS5B used in the previous study (37) was enzymatically inactive and thus may not reflect its native conformation. Our results clearly indicate that HCV NS5B alone is capable of interacting with the 3'-end of HCV genome in the pyrimidine-rich region and the X domain, which are the cis-acting elements important for the initiation of RNA synthesis. Also, NS5B alone appears to be sufficient to initiate RNA synthesis using the X or longer RNA templates. However, it is possible that other viral proteins or cellular proteins, such as polypyrimidine tract-binding protein (13), can alter the binding affinity or polymerase activity of NS5B, particularly on a longer RNA template, such as the full-length viral genome.

Promoter elements required for initiation of HCV RNA synthesis had been difficult to study in the past, because the previously reported recombinant NS5S could only be HCV viral RNA only in a primer-dependent manner and without template specificity (17–20). Our recombinant HCV polymerase can carry out primer-independent de novo RNA synthesis and thus allows us to partially map cis-acting elements at the 3'-ends of minus-strands of HCV genome (16). The importance of the X region at the 3'-end of the HCV genome as a promoter for the initiation of RNA synthesis has been illustrated further in this study by experiments that showed that the full-length X and its deletion mutants lacking up to 35 nt from the 3'-end were functional templates, whereas the X mutants with a deletion of 40 nt or more from the 3'-end were inactive. Our previous results also showed that deletion of stem III abolished the template activity of X RNA (16). These results combined suggest that stem-loops II, III, and part of stem I constitute the minimal cis-acting element required for NS5B to initiate RNA synthesis in vitro.

Our studies also revealed an interesting and somewhat surprising finding that RNA synthesis mediated by NS5B initiated from an internal 3'-most single-stranded nucleotide (U78) of loop I. It is notable that the 3'-end of the X region is in a perfect
double-stranded form (Stem I). Thus, U₇₈ is the 3'-most single-stranded nucleotide of the entire X RNA. RNA structure prediction based on computer modeling showed that X RNA mutants with a deletion of 15 nt or less from the 3'-end preserved the 3'-end double-stranded structure and that U₇₈ remains as the 3'-most single-stranded nucleotide in these RNAs (Fig. 7 and data not shown). Significantly, all of these deletion mutants initiate RNA synthesis from the same U₇₈. When additional nucleotides are deleted (20 nt or more) from the 3'-end, stem I is destroyed, and the RNA is predicted to have a single-stranded 3'-tail attached to stem II; significantly, all of these templates generated RNA products of template size. These results suggest that RNA synthesis initiates from the 3'-most single-stranded nucleotide of the X RNA template. This conclusion is supported by the finding that the addition of 2 nt or more to the 3'-end of stem I resulted in an RNA product of template size; the internal initiation became less prominent (Fig. 10B). Furthermore, for −15XM RNA, which contains a single-stranded 3'-end because of the disruption of the 5-nt double-stranded stem in the −15X RNA, RNA synthesis initiated from the very 3'-end of RNA (Fig. 10C), in contrast to the −15X RNA, for which RNA synthesis initiated internally. The efficiency of RNA synthesis varied greatly depending on the nature of the initiating nucleotide and the length of the single-stranded tail (Fig. 10, B and C). These findings suggest that once NSSB binds to stems II and I of the X region, the catalytic center of the enzyme can only interact with the single-stranded tail of the RNA to initiate RNA synthesis.

The internal initiation of RNA synthesis by NSSB using the authentic 3'-end RNA was confirmed for both the X region only and the full-length 3'-U3R. The pattern of RNA synthesis from the latter template was more complex because of the presence of the polypyrimidine tract, which appears to allow NSSB to stutter and abort prematurely. This mechanism may explain the extreme heterogeneity in the length of polypyrimidine tract among various RNAs within the same HCV isolate (10, 11). Nevertheless, it is clear that the full-length RNA products made from the full-length 3'-U3R of HCV RNA represented internal initiation rather than initiation from the very 3'-end.

If this in vitro mechanism of RNA synthesis applies to HCV RNA synthesis in vivo, how then does the NSSB copy the full-length viral RNA faithfully without losing genetic information as a result of internal initiation? First, other viral proteins or cellular proteins may alter the specificity of initiation of RNA synthesis. For example, under some conditions, the stable duplex of stem I may be unwound spontaneously or with the help of HCV helicase, NS3 protein (38, 39), thus allowing NSSB to initiate RNA synthesis from the 3'-end. Alternatively, the presence of other RNA-binding proteins (e.g. polypyrimidine tract-binding protein) in the replication complex may affect the conformation of the polymerase and/or cis-acting RNA elements and thus alter the initiation site. A third possibility is that the 3'-end of genomic RNA may be extended with single-stranded nontemplated nucleotides by cellular terminal transferases and then used as the template for initiation from the 3'-end. Indeed, a cDNA clone containing two extra nucleotides (UU) at the 3'-end has been obtained from an HCV isolate (11).

Finally, another possibility is that HCV polymerase may be able to repair 3'-end genetic information lost during the synthesis of minus-strand RNA, i.e. during plus-strand RNA synthesis, the 3'-end of the nascent RNA molecules copied from the negative-stranded RNA template may fold back to recover the 3'-end sequences, since stem I has internally complementary sequences. The precise mechanism of the initiation of RNA synthesis in vivo is still an open question. Nevertheless, our studies have provided new insights into the properties of HCV RNA polymerase and the mechanism of HCV RNA synthesis.

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