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Helper virus-independent *trans*-replication of hepatitis C virus-derived minigenome

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Abstract

We have previously described a synthetic T7-driven cDNA minigenome containing the antisense sequence of luciferase gene and internal ribosome entry site of encephalomyocarditis virus flanked by 5′- and 3′-end sequences of hepatitis C virus (HCV) that contain *cis*-acting replication elements. Synthesis of minus-strand RNA from the artificial minigenome was determined by using Huh-7 cells harboring autonomously replicating HCV subgenome as a helper for provision of functional replication components. To further confirm and extend these studies, we investigated here whether the minigenome replication system could be reconstituted by transfection of naïve Huh-7 cells with plasmid expressing nonstructural (NS) proteins. Reporter assay and Northern blot analysis revealed that *trans*-expression of NS proteins from 3 to 5 resulted in high level of luciferase activity and synthesized minus-strand RNA. The analogous result was also obtained with the minigenome derived from HCV 2a, and both HCV 1b- and 2a-derived NS protein were able to support the chimeric minigenomes whose 5′- or 3′-end was replaced by the respective region of the heterologous virus. These results provide a basis for establishing the reverse genetic system that is helpful to study *cis*- and *trans*-acting factors involved in HCV RNA replication.

Keywords: Hepatitis C virus; Minigenome; *trans*-Replication

Hepatitis C virus (HCV) is an important human pathogen with an estimated 170 million chronic carriers throughout the world, and many of them are at a high risk for developing liver cirrhosis and hepatocellular carcinoma [1]. HCV is a member of the *Flaviviridae* family with a positive-sense RNA genome of ~9600 nucleotides in length. The genome is flanked by highly structured nontranslated regions (NTRs) important for both RNA translation and replication. The viral genome encodes a polyprotein precursor of approximately 3010 amino acids, which is processed by viral and cellular protease to produce the structural proteins (core, E1, and E2) and nonstructural (NS) proteins (p7 and NS2 to NS5B).

Like other plus-stranded RNA viruses, HCV genomic RNA is first transcribed into a minus-strand intermediate, which in turn serves as the template for production of progeny plus-strand RNA. Although the basic steps in replication have been well established, little is known about the detail of these processes. Studies of HCV replication have been hampered by the lack of an efficient tissue culture system. Although the development of subgenomic replicon has facilitated the investigation of viral RNA replication in cell culture [2], culture-adaptive mutations within the NS proteins are required for efficient replication [3,4], and full-length genomes carrying such mutations do not produce infectious virus particles [5,6]. More recently, it was reported that genotype 2a JFH1 genome replicates efficiently independent of the culture-adaptive mutations and supports production of viral particles [7]. This *in vitro* system, together with the later-developed JFH1-based

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chimeras [8,9], are an important progress in HCV research, allowing the study of unknown aspects of HCV life cycle. However, a comparison study showed that JFH1 differs from the earlier-generated HCV 1b replicon in independence of the cellular cofactor (cyclophilin B) for the replication and less sensitivity to antiviral reagent [10], suggesting that the strain- or genotype-specific properties may exist and the observation obtained with JFH1 cannot be simply extrapolated to other isolates.

Synthetic minigenomes have been described in a number of minus- and plus-stranded RNA viruses, which has contributed greatly to the analysis of *cis*-acting sequences and *trans*-acting proteins required for viral replication, maturation, and packaging [11,12]. We previously established a helper virus-dependent expression system utilizing HCV-derived minigenome, and Huh-7 cells harboring autonomously replicating HCV subgenome [13]. In this study, we further investigated whether the minigenome replication system could be reconstituted by transfection of naïve Huh-7 cells with plasmid expressing NS proteins. It was shown that synthesis of minus-strand RNA from HCV minigenome can be supported by *trans*-expressed polyprotein NS3 to NS5B, and the NS proteins were able to replicate not only the homologous minigenome but the heterologous and chimeric minigenome as well.

Materials and methods

Plasmids. HCV 1b-derived minigenome p1b-1b was previously referred to as pT7cRLNS5B1 [13]. For construction of chimeric minigenome p2a-1b, the first 376 nucelotides of HCV 2a cDNA with the T7 promoter directly coupled at the 5′-end were amplified by PCR with primers 5′-tataa gcttTAATACGACTCACTATAACCTGCCCCTAATAGGGGC-3′ and 5′-tgcgcatgcTTTGGTTTTTCTTTGAGGTT-3′. The *Renilla* luciferase gene was amplified from pRL-TK (Promega) using primers 5′-ctctctagaA TGACTTCGAAAGTTTATGA-3′ and 5′- tgcgcatgcTTATTGTTCATT TTTGAGAA-3′. The resulting PCR products were digested with *Hin*dIII–*Sph*I and *Sph*I–*Xba*I, respectively, and inserted into the *Hin*dIII/*Xba*I sites of p1b-1b. The 3′- part of the NS5B coding region fused 3′ UTR of HCV 2a cDNA was amplified by PCR using primers 5′-ataggatccCCTCAGAA AACTTGGGG-3′ and 5′-ataggcgccagcgaggaggctgggaccatgccggccACAT GATCTGCAGAGAGACC-3′, digested with *Bam*HI and *Nar*I, and cloned, along with the annealed oligonucleotides containing partial sequence of the HDV ribozyme [13], into *Bam*HI/*Eco*RI-cut p1b-1b or p2a-1b, creating p1b-2a and p2a-2a, respectively.

To construct plasmid pNS3-51b expressing polyprotein from HCV 1b, a cDNA containing the ORF of NS3 to NS5B was amplified with primers 5′-atatctagaATGGGCCCATCACGGCTTA-3′ and 5′-ataggcgcgccTCA CCGGTTGGGGAGCAGG-3′, digested with *Xba*I and *Asc*I, and cloned, along with *Hin*dIII/*Xba*I-cut HCV sequence (1–341 nt), into pGEMEX-1 vector (Promega) which was modified by deletion of all of the T7 gene 10 and introduction of *Hin*dIII and *Asc*I sites between T7 promoter and terminator [14]. pNS3-52a, which expresses the polyprotein from HCV 2a, was constructed similarly except with the primers of corresponding sequence from genotype 2a. The sequences of these constructs were confirmed by nucleotide sequencing.

Cells. The cell line Huh-7 was purchased from the American Type Culture Collection (ATCC) and maintained in Dulbecco's modified Eagle's medium (DMEM, Invitrogen) supplemented with 10% fetal calf serum and 50 U/ml penicillin and streptomycin in a 5% CO_2 humidified atmosphere. A Huh-7-derived cell line (Huh–NNRZ) stably replicating HCV subgenomic replicon was grown in DMEM containing 300 μg/ml G418 (Geneticin, Invitrogen) [15,16].

Transfection. Huh-7 cells were seeded at 1×10^5 per well of 12-well plates. Twenty-four hours later, 0.5 μg *Eco*RI-linearized minigenome (p1b-1b, p1b-2a, p2a-1b, or p2a-2a), 0.5 μg pGEMEX-1, pNS3-51b, or pNS3-52a, 0.5 μg pAM8-1, and 0.1 μg pGL3-Control vector were cotransfected into cells with Fugene HD Transfection Reagent (Roche). The cells were harvested at the indicated time points, and cell lysates were assayed for luciferase activity as described below.

Luciferase assay. Cell lysates were prepared from transfected cells, centrifuged briefly, and 20 μl of the supernatants was used for luciferase assays with Dual-Luciferase Reporter Assay System (Promega) according to the manufacturer's instructions. Luciferase activities were measured using a TD-20/20 Luminometer (Promega).

Western blot analysis. Protein was electrophoresed on a sodium dodecyl sulfate–polyacrylamide gel, transferred to Hybond-P PVDV Membrane (Amersham). The blots were probed with Antiserum Product 2871 and 2881 (ViroStat) for detection of NS3 and NS4, rabbit polyclonal antibody (ab2594, Abcam Limited) for NS5A, and goat polyclonal antibody (sc-17532, Santa Cruz Biotechnology, Inc.) for NS5B. Signals were visualized with ECL Plus Western Blotting Detection Reagents (Amersham).

Northern blot analysis. RNAs were isolated from transfected cells with Trizol reagent (Invitrogen) and treated with RNase-free DNase (Promega). The purified RNAs were separated by denaturing agarose gel electrophoresis and analyzed by Northern blot using digoxigenin-labeled antisense *Renilla* luciferase sequence.

Results

Synthetic minigenome derived from HCV

The minigenome construct derived from HCV 1b consists of the antisense sequence of the *Renilla* luciferase gene and internal ribosome entry site (IRES) of encephalomyocarditis virus (EMCV) flanked upstream by 5′-end (nucleotides 1–377) and downstream by 3′-end sequence containing NS5B coding region from nucleotides 9067 to 9371 plus 3′-UTR of HCV cDNA. The cassette was positioned precisely at the T7 transcription start site followed by self-cleaving HDV ribozyme to ensure authentic 5′- and 3′-ends (Fig. 1A, p1b-1b). If the minigenome could be accepted as a template by the replication complex provided in *trans*, the luciferase gene, which is encoded by synthesized minus-strand RNA, would express in HCV-infected cells. Fully consistent with this hypothesis, luciferase activity was selectively detected in Huh-7 cells harboring an autonomously replicating HCV subgenome (Huh–NNRZ) [13].

Replication of HCV minigenome in Huh-7 cells expressing polyprotein NS3 to NS5B

The ability of the synthetic minigenome to replicate in replicon cells prompted us to investigate whether the replication of minigenome could be supported by HCV proteins expressed in *trans*. For this purpose, Huh-7 cells were transfected with the minigenomic construct p1b-1b, plasmid encoding a polyprotein encompassing NS3 to NS5B under the control of T7 RNA polymerase promoter, pAM8-1 plasmid expressing T7 RNA polymerase [14], and pGL3-Control vector. The cells were harvested at 3

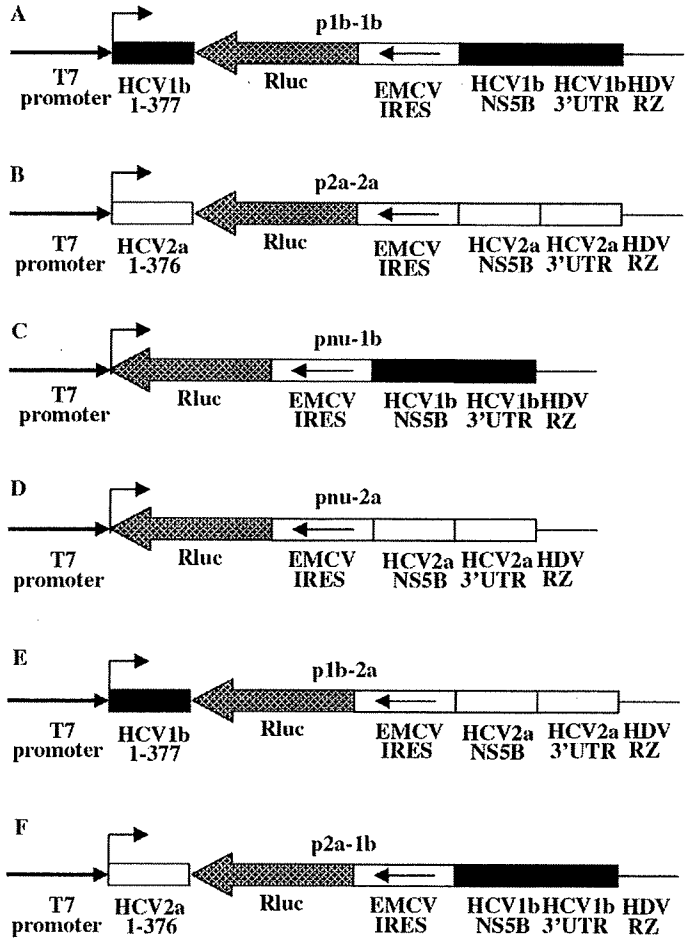

Fig. 1. Schematic diagrams of T7-based minigenomes derived from HCV 1b (A), HCV 2a (B), chimeric minigenomes p1b-2a consisting of 5′-end of HCV 1b and 3′-end of HCV 2a (E) and p2a-1b consisting of 5′-end of HCV 2a and 3′-end of HCV 1b (F). HCV minigenome containing the antisense sequence of the *Renilla* luciferase gene and EMCV IRES flanked by the 5′-end and 3′-partial NS5B coding sequence-connected 3′-UTR was juxtaposed precisely at the T7 transcription start site and followed by the HDV ribozyme sequence. pnu-1b (C) and pnu-2a (D) were identical to p1b-1b and p2a-2a except for the 5′-end sequences deleted.

days posttransfection, protein expression was verified by Western blot analysis (Fig. 2) and the replication of minigenome was determined by luciferase assay and Northern blot analysis. The firefly luciferase activity from cotransfected pGL3-Control vector was simultaneously measured to normalize the transfection efficiency. As shown in Fig. 3A, only background level of *Renilla* luciferase (Rluc) activity was detected in cells transfected with the empty vector. Cotransfection of the plasmid encoding the polyprotein NS3 to NS5B (pNS3-51b) resulted in significant *Renilla* luciferase expression. Omission of pAM8-1 in the transfection mixture completely abrogated *Renilla* luciferase activity, largely ruling out the possibility that the minus-strand RNA used here as the mRNA for reporter gene expression was synthesized as a consequence of the transcription by a cryptic promoter. Consistent with the results reported previously [13], *Renilla* luciferase activity was also detected in Huh–NNRZ cells stably replicating the HCV subgenomic replicon, although it was lower than that in cells *trans*-expressing the polyprotein. The fact that the replicase complex reconstituted by *trans*-expressed polyprotein could support more efficient replication of the minigenome may be attributable to the higher expression level of plasmid-encoded protein on a per-transfected-cell basis. Alternatively, the recruitment of the replication complex to the minigenome may be competed by the subgenomic replicon, because both of these share the replication machinery in replicon cells.

To further confirm the result of reporter assay, RNA was extracted from transfected cells and subjected to Northern blot analysis using digoxigenin-labeled antisense *Renilla* luciferase probes. Also, minus-strand RNA transcripts of the expected size were specifically detected in Huh-7 cells expressing NS 3–5 protein and Huh–NNRZ cells replicating HCV subgenomic replicon (Fig. 3B, lanes 2 and 4). These data demonstrate that *trans*-replication of HCV minigenome does not require replication of the helper viral RNA.

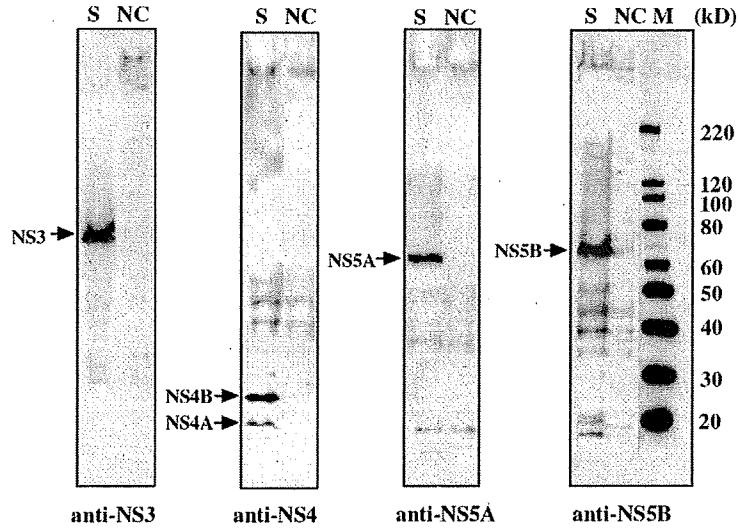

Fig. 2. Expression of NS proteins in Huh-7 cells transfected with plasmid encoding a polyprotein encompassing NS3 to NS5B. Huh-7 cells were transfected with pNS3-51b together with pAM8-1 plasmid expressing T7 RNA polymerase and harvested at day 3 posttransfection. Cell lysates of the transfected cells were analyzed by Western blot using antibodies against each NS protein. Protein standard is shown on the right, and the band corresponding to each NS protein is indicated by an arrowhead. Huh-7 cells transfected with the empty vector served as a negative control (NC).

To document that the reporter gene expression detected above was dependent on HCV replicase reconstituted by *trans*-expressed NS proteins, we employed inactive mutant pNS3-51b/dGDD (in which the GDD motif of NS5B was deleted) and AdsiNS5B expressing siRNA directed against NS5B [13] in the reporter assay. As shown in Fig. 3C, the deletion of GDD motif significantly attenuated the ability of NS proteins to support minigenome replication, and transduction with AdsiNS5B resulted in a substantial and dose-dependent reduction in luciferase expression. These results provide further evidence that the reporter gene was expressed as a result of replication of HCV minigenome by *trans*-supplied NS proteins.

Chimeric minigenomes as templates for HCV replication complex

Next, we were interested in investigating whether the replicase of HCV can recognize the heterologous signals for synthesis of minus-strand RNA. For this purpose, HCV minigenome from distantly related genotype 2a (Fig. 1B, p2a-2a), minigenomes with 5′-end deleted (Fig. 1C and D, pnu-1b and pnu-2a), and chimeric minigenomes whose 5′- or 3′-end was replaced by the respective region of the heterologous virus (Fig. 1E and F, p1b-2a and p2a-1b) were constructed. Huh-7 cells were transfected with these minigenomes together with the plasmid expressing HCV 1b- or 2a-derived NS proteins, pAM8-1, and *Renilla* luciferase activities were measured as fore-mentioned. Consistent with the results described above, replicase of HCV 1b and 2a accepted its respective minigenome as the template for synthesis of minus-strand RNA, and exchange of NS proteins between HCV1b- and 2a-derived minigenome systems also led to reporter gene expression (Fig. 4), implying that the replication complex is not strictly specific for the homologous RNA template. Deletion of the 5′-end region in the minigenome fully abrogated its replication, both NS proteins from HCV 1b and 2a, however, could support the replication of chimeric minigenomes, suggesting that both RNA–protein interaction between replicase and viral genome and long range RNA–RNA interaction between 5′- and 3′-terminal sequence involved in HCV minus-strand RNA synthesis are functionally conserved between genotype 1b and 2a. Additionally, in all tested minigenomes, the NS proteins originated from HCV 1b constantly yielded higher levels of luciferase expression than that from 2a, suggesting that intrinsic differences in the replication capabilities of the replicase complex from different strains may exist. More likely, the superior capability of pNS3-51b in supporting the minigenomes replication may be attributable to the fact that the coding sequence in pNS3-51b was amplified from the replicon which harbors the adaptive mutations due to long-term culture, whereas the coding region in pNS3-52a was directly amplified from HCV 2a-infected serum.

Discussion

Successful establishment of the minigenome system has been described in a number of minus-stranded RNA viruses from different families and plus-stranded RNA viruses belonging to the *Coronaviridae* family, which has contributed greatly to the analysis of *cis*-acting sequences and *trans*-acting proteins essential for viral replication [11,12]. The rescue of synthetic minigenomes was achieved either through helper virus infection of minigenome-transfected

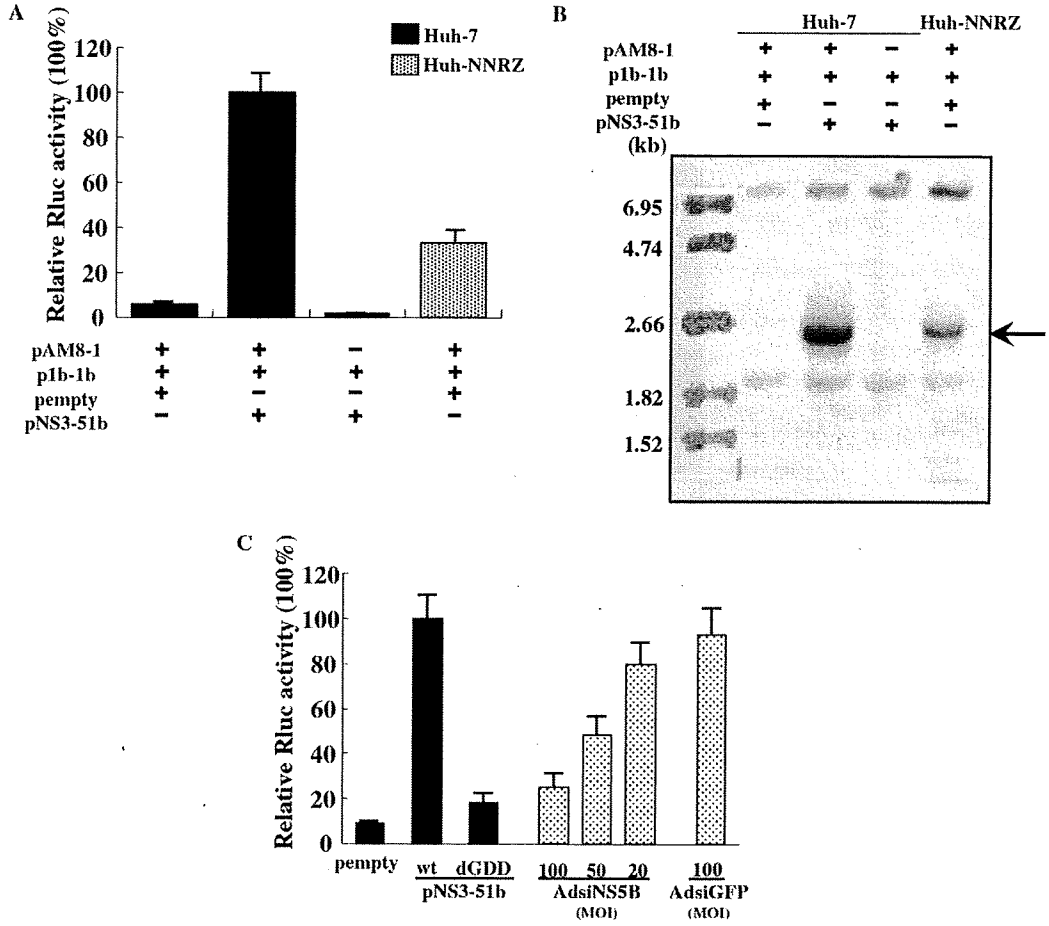

Fig. 3. Replication of HCV minigenome in Huh-7 cells expressing polyprotein NS3 to NS5B. (A) Huh-7 or Huh–NNRZ cells were transfected with p1b-1b, pNS3-51b expressing polyprotein NS3 to NS5B, together with or without pAM8-1. Relative *Renilla* luciferase activities in the lysates were determined at 72 h posttransfection. The columns and bars represent mean and standard deviation of four independent experiments. (B) Northern blot was performed on 8 μg of extracted RNA using digoxigenin-labeled antisense *Renilla* luciferase RNA probe to detect minus-strand transcripts. RNA size markers are shown on the left, and the bands corresponding to minus-strand RNA are indicated on the right. (C) Huh-7 cells were transfected with p1b-1b, pAM8-1, and pempty, pNS3-51b or pNS3-51b/dGDD (column 1–3), or infected with AdsiNS5B at an MOI of 100, 50, and 20 (column 4–6) before transfection, and relative *Renilla* luciferase activities in the lysates were determined as described above.

cells with virus particles or through co-transfection of plasmids expressing viral proteins. For viruses of *Flaviviridae* family, however, a little has been reported in the development of similar approach except an *in vitro* replication system which utilizes cytoplasmic extracts from viral-infected cells and exogenous RNA template containing 5′- and 3′-terminal regions was described for dengue virus [17]. Together with those reported previously [13], the data shown here represent the first example of minigenome system for HCV, indicating that both the replicase complex supplied from replicating subgenomic replicon and that reconstituted by plasmid-encoded NS proteins are capable of supporting the replication of HCV minigenome.

The data shown here further confirm that the viral 5′- and 3′-end sequence together with the 3′-partial NS5B coding region represent sufficient *cis*-acting signals for minus-strand RNA synthesis. These results, however, do not rule out the possibility for the existence of *cis*-acting elements in other coding region, which may act as regulatory elements (either enhancers or silencers) in RNA synthesis. The presence of noncontiguous *cis*-acting signals involved in viral RNA replication has been reported in the viral genome of the brome mosaic virus [18], tobacco mosaic virus [19], and the double-stranded RNA virus of yeast [20].

Similar to that found in dengue virus, it was shown that deletion of the 5′-end region in the minigenome fully abrogated its replication, but substitution of the 5′-end with the respective sequence from heterologous virus (p1b-2a or p2a-1b) did not significantly affect its template ability, suggesting that the long range RNA–RNA interaction between 5′- and 3′-ends essential for RNA replication is functionally conserved between HCV 1b and 2a. In addition to homologous minigenome, both HCV 1b- and 2a-derived replicase were able to accept the heterologous and chimeric minigenomes as the templates for synthesis of minus-strand

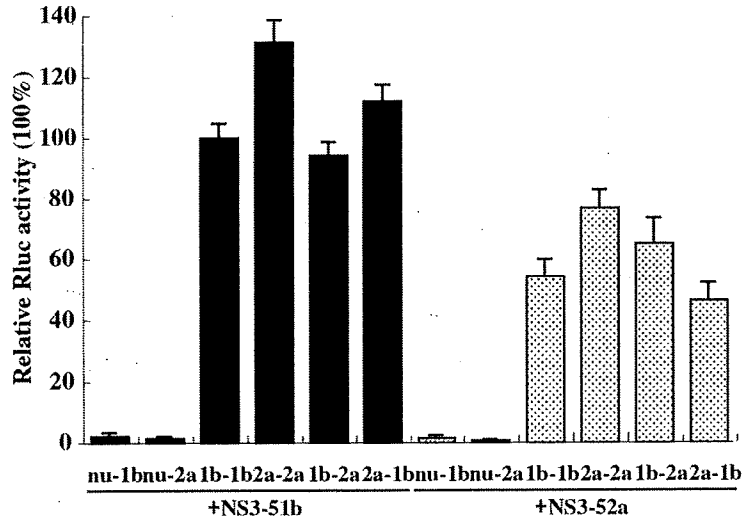

Fig. 4. Replication of chimeric HCV minigenomes. Huh-7 cells were transfected with each indicated minigenome, pAM8-1, and pNS3-51b or pNS3-52a expressing polyprotein NS3 to NS5B derived from genotype 1b or 2a. Relative *Renilla* luciferase activities in the lysates were determined as described for Fig. 3A. The columns and bars represent means and standard deviations of three independent transfections.

RNA, indicating that the replicase-catalyzed RNA synthesis is not strictly strain- or genotype-specific.

Using replicon system, Bartenschlager's group obtained the evidence showing that only mutations in NS5A, but not mutations in NS3, NS4B, and NS5B, could be rescued by *trans*-complementation [21]. Our data presented here indicate that replication of the minigenome can be supported by *trans*-expressed NS proteins. One scenario may make these two different findings compatible: the *cis*-expressed, lethally mutated NS proteins may exert dominant negative effect in reconstituting replication complex, and thus interfere with the incorporation of *trans*-supplied NS proteins into a functional replication complex, which may account for the failure of NS proteins (other than NS5A) to *trans*-complement HCV RNA replication; however, such a dominant negative effect does not exist in the minigenome system described here because there is no NS protein expressed in *cis*, and *trans*-expressed NS proteins might be able to reconstitute the functional replication complex to support minigenome replication. Further experiments are now in progress to substantiate this assumption.

It is generally believed that the HCV replication follows the pathway used by other plus-strand RNA viruses: the input RNA is first transcribed into a minus strand, which in turn serves as the template for production of progeny plus strand. The negative strand intermediates are postulated to exist as a dsRNA form. However, there is no direct evidence demonstrating this postulation in HCV, and whether there is free HCV-specific RNA of negative polarity in infected cells is still an issue to be elucidated. On the other hand, increasing evidence showed that the RNA in native replication intermediates of some positive strand RNA viruses is single-stranded. For example, in polio virus-infected cells, a careful electron microscope analysis using a membrane-permeable cross-linking reagent demonstrated that the native replication intermediate *in vivo* has a predominantly single-stranded backbone attached to several nascent RNA chains with few or no regions of extensive base-pairing, although deproteined (phenol-extracted) replication intermediate has a backbone mostly double-stranded [22]. More recently, Fujimura et al., reported that native replication intermediates of 20 S RNA virus have a single-stranded RNA backbone [23]. After completion of product-strand elongation, both the product and template strands are released from the replication complex as single-stranded RNA. The data presented here indicate that the minus strand RNA could serve as the mRNA for transgene expression, implying a similar scenario may also occur in HCV replication and minus strand RNA may be dissociated and present as a free single-strand form after RNA synthesis is completed.

One issue of concern in using minigenome to study the molecular mechanism of viral replication is whether the elements controlling viral replication in the context of minigenome could authentically reflect those that occurred in the context of full-length genome. Recently, differential effect of a point mutation in the replicase gene on genome and minigenome replication was reported in coronavirus, emphasizing the need to use full-length genome to validate the replication signals obtained from minigenome system [24]. Nonetheless, the HCV minigenome system described here represents a useful tool for identification of *cis*- and *trans*-acting factors involved in viral replication while eliminating biosafety constraints required for work with infectious systems. Additionally, it will be of interest to explore whether the HCV minigenome can be packaged by additional provision of the viral structural protein in *trans*, and its success will not only further broaden the

application of the HCV minigenome, but also facilitate the development of HCV-based gene delivery system.

We describe here a reverse genetic system for HCV that is based on T7-driven minigenome coupled with plasmid-encoded NS proteins. This system opens the possibility of manipulation of *cis*-acting signals and *trans*-acting factors involved in the control of HCV RNA synthesis, which may facilitate future studies aimed at investigation of the mechanisms involved in the replication of viral RNA.

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Genetic Analysis of Hepatitis C Virus with Defective Genome and Its Infectivity in Vitro▽

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Replication and infectivity of hepatitis C virus (HCV) with a defective genome is ambiguous. We molecularly cloned 38 HCV isolates with defective genomes from 18 patient sera. The structural regions were widely deleted, with the 5′ untranslated, core, and NS3-NS5B regions preserved. All of the deletions were in frame, indicating that they are translatable to the authentic terminus. Phylogenetic analyses showed self-replication of the defective genomes independent of full genomes. We generated a defective genome of chimeric HCV to mimic the defective isolate in the serum. By using this, we demonstrated for the first time that the defective genome, as it is circulating in the blood, can be encapsidated as an infectious particle by *trans* complementation of the structural proteins.

Viruses with a deletion mutation in their genome have been identified as defective interfering (DI) particles for many virus species (1, 3, 9, 16). Part of the DI virus genome is deleted, but regions indispensable for replication and packaging are preserved. Most DI viruses occur spontaneously in the course of cell culture infected with a high titer of wild-type viruses. Hepatitis C virus (HCV) with a defective genome has been found in liver and serum specimens of some HCV patients (4, 8, 15). HCV has a plus-strand RNA genome that encodes the viral core, E1, E2, and p7 structural proteins and NS2, NS3, NS4A, NS4B, NS5A, and NS5B nonstructural proteins (10). According to the reports, the deletions have been found mainly in the structural region and most of the deletions are in frame, but some deletions are out of frame (4), raising questions about whether the defective HCV genome is merely a by-product of a full genome or a self-replicating genome and whether it can be encapsidated into an infectious virus particle.

In the present study, we molecularly cloned 38 HCV isolates with defective genomes from HCV patient sera to address these questions by genetic analyses and infection experiments. As long as we explored, all of the deletions were in frame, indicating the potential to support translation from the authentic initiation codon to the termination codon, although the structural region was widely deleted, as reported previously. Phylogenetic analyses evidenced self-replication of the defective genomes independent of full genomes. We demonstrated for the first time, by *trans* complementation experiments, that the defective genome, as it is circulating in the blood, can be encapsidated as an infectious particle, designated HCV_{CCD}.

First, to amplify HCV cDNAs in 21 serum specimens from 18 HCV patients (genotype 1b), we performed three sets of long-distance reverse transcription (RT)-PCRs flanking (i) the 5′ untranslated region (UTR) to the 5′ part of the NS3 region, (ii) the remaining part of the NS3 region to the end of NS5B, and (iii) the 5′ UTR to the end of the NS5B region (Fig. 1A). The specimens were collected with informed consent. cDNA was synthesized with RNase H-deficient reverse transcriptase Superscript III (Invitrogen, Carlsbad, CA) at a higher temperature (55°C) to reduce template switching and mispriming. PCRs were performed in a (hemi)nested manner with high-fidelity polymerase KOD plus or KOD FX (Toyobo, Osaka, Japan) as described previously (5). For some target nucleotide positions, a mixture of two or three primers was used to reduce mismatches due to sequence heterogeneity (Table 1). Of the 21 specimens examined, representative results are shown in Fig. 1. An amplicon of the 5′ UTR-NS3 region of the predicted size (ca. 3.7 kb) was detected in all specimens (18/18), and representative results are shown in Fig. 1B. In addition, a shorter amplicon suggestive of a defective HCV genome was simultaneously present in four specimens from 1 (R4) of 12 cases of clinically mild hepatitis and from 3 (T5, K3, and K4-pre) of 6 cases of active hepatitis (clinical data not shown). Defective genomes were found in the patients with relatively higher copy numbers of HCV RNA (>8.1×10^5 copies/ml in the 5′ UTR, Table 2), suggesting that the coexistence of a defective genome is related to hepatitis severity. The authentic-size amplicon was poorly detected when coexisting with a defective HCV genome shorter than 2 kb (T5 and K3), presumably because of preferential amplification of the shorter amplicon. A shorter amplicon was not detected for the NS3-

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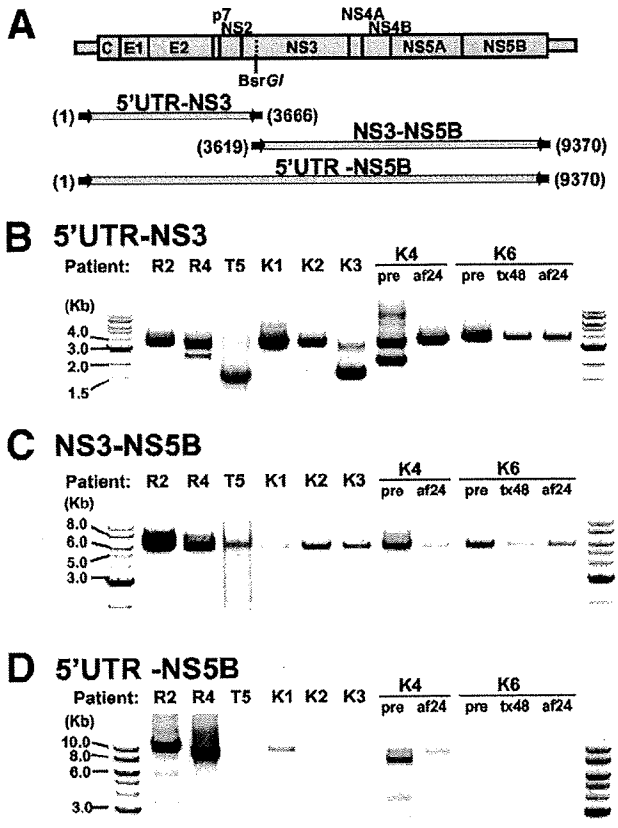

FIG. 1. Representative results of long-distance RT-PCRs for serum HCV. (A) The three sets of long-distance RT-PCR used: 5′ UTR-NS3 (5′ UTR to the 5′ part of the NS3 region), NS3-NS5B (the remaining part of the NS3 region to the end of NS5B), and 5′ UTR-NS5B (5′ UTR to the end of the NS5B region). The nucleotide positions of the 5′ and 3′ ends of each amplicon are indicated in parentheses. PCR products were electrophoresed and stained with ethidium bromide. Results of the representative 11 specimens (eight patients) are shown for 5′ UTR-NS3 (B), NS3-NS5B (C), and 5′ UTR-NS5B (D). Serum specimens were collected from patients K4 and K6 before interferon treatment (pre), at the end of the full 48-week treatment period (tx48), and 24 weeks after the full treatment period (af24). DNA molecular size markers are at both sides of panels B to D.

NS5B region, while the amplicon of the predicted size (ca. 5.6 kb) was detected in all of the specimens, albeit with various efficiencies (Fig. 1C). The 5′ UTR-NS5B region, which covers almost the whole genome, was then amplified, and an amplicon of the predicted size (9.4 kb) was detected in 10 specimens from nine patients (5 specimens in Fig. 1D). Of the successfully amplified specimens, two (R4 and K4-pre) also contained a shorter amplicon, in accordance with the results of the 5′ UTR-NS3 PCR. The NS3-NS5B region is essential for autonomous replication of HCV as an RNA replicon in vitro (5–7). It has been shown that NS5A is the only nonstructural protein that can *trans* complement HCV replication (13). They used a nonadaptive mutation of NS5A as a replication-incompetent NS5A protein instead of a deletion mutant protein. Thus, we speculate that deletion of the NS3-NS5B region cannot be complemented in *trans*. Intriguingly, the shorter amplicon was not detected after full-term interferon treatment in patient K4 (K4-af24), although it was detected prior to treatment (K4-pre) (Fig. 1B and D). The possible reasons for this are that (i) the defective genome disappeared naturally, (ii) packaging of the defective genome by the helper virus was impeded by an unknown mechanism of interferon, or (iii) replication of the defective genome is preferentially inhibited by the interferon pathway. Further studies are needed to reveal the effect of a defective HCV genome on the pathogenesis and treatment of HCV.

A total of 38 isolates with defective HCV genomes were molecularly cloned into plasmid vector pASGT (unpublished data) from the shorter amplicons of the 5′ UTR-NS3 PCR from four serum specimens (R4, T5, K3, and K4-pre in Fig. 1B) at the AscI and BsrGI restriction sites. The nucleotide sequences were determined with an autosequencer (3730 DNA analyzer; Applied Biosystems, Foster City, CA). Sequence analyses revealed that the structural region was widely deleted in all of the defective isolates and that the deletion ranges were quite diverse among the isolates (extending up to the NS2 region) (Fig. 2A). In contrast, the 5′ UTR and core regions were constantly preserved, suggesting that these regions, as well as the NS3-NS5B region, are indispensable for the production of HCV with a defective genome. Intriguingly, defective genomes with different deletion patterns coexisted in single specimens from two patients (three patterns in patient K3 and four patterns in patient K4-pre). Moreover, two deletions in a single genome were observed in five isolates from patient R4 (isolate R4S-5). As many as three deletions in a single genome were observed in the isolate from patient K3 (e.g., isolate K3S-15), in which two small deletions resulted in two tiny residual fragments. Such diversity in deletion ranges indicates flexibility of the remaining structural region for the replication of defective HCV genomes. Nevertheless, all of the deletions identified in the 38 isolates were in frame (Fig. 2B), implying that these defective HCV genomes have the potential for translation from the core to the authentic end of NS5B without a frameshift.

To determine the ratio of defective to full genomes, we performed quantitative PCRs targeting a relatively conserved E2 sequence, which is commonly deleted in the defective genomes, with primers listed in Table 1. Calculation of the 5′ UTR/E2 ratio, which must theoretically be 1 without the existence of the defective genome, showed higher values (1.7 to 2.45) in specimens containing the defective genomes (R4, K3, and K4pre in Table 2), indicating that the defective genome level in serum is 0.7 to 1.45 times the full genome level. However, to clarify the impact of defective genomes on pathogenesis and their effect on the treatment of HCV, accumulation of more data is needed.

The nucleotide sequence comparison of 38 defective HCV isolates showed sequence diversity. Such diversity was observed even among isolates obtained from the same specimen. Perhaps such diversity is a result of self-replication and the subsequent evolution of the defective HCV genome. To explore this possibility, phylogenetic analyses were performed on the nucleotide sequence data from patient K4. Sequences at the 5′ and 3′ maximum overlapping regions located outside the deletions were separately compared (Fig. 2A), and phylogenetic trees were created by the neighbor-joining method with GENETYX software (Genetyx Inc., Tokyo, Japan). As a re-

TABLE 1. Primers used for long-distance and quantitative RT-PCRs in this study

Test and region(s)	Direction	Primer(s)[a]	Sequence[b]	Position[c]
Long-distance PCR				
5′ UTR-NS3	RT	606R/712R	GTTTCCATAGACTC(A/G)ACGGG	3930–3949
5′ UTR-NS3, 5′ UTR-NS5B	1st forward	420	GGCGACACTCCACCATAGATCACTC	1–42
5′ UTR-NS3	1st reverse	605R/713R	ACCGGAATGACATCAGCATG(T/C)CTCGT	3741–3766
5′ UTR-NS3, 5′ UTR-NS5B	2nd forward	AscT7-420	ATCGTAGGCGCGCCTC*TAATACGACTCACTATA*GC CAGCCCCCGATTGGGGGCGACACTCCACCATAGATCACTC	1–42
5′ UTR-NS3	2nd reverse	604R/714R	CGAGGTCCTGGTCTACATT(G/A)GTGTACAT	3639–3666
NS3-NS5B, 5′ UTR-NS5B	RT	386R	AATGGCCTATTGGCCTGGAG	9390–9392
NS3-NS5B	1st forward	602/723	CCACCGCAACACAATCTTTCCT(G/A)GCGAC	3529–3556
NS3-NS5B, 5′ UTR-NS5B	1st reverse	719R/720R/721R	GAGTGTTTAGCTCCCCGTTCA(T/C/G)CGGTTGGG	9363–9392
NS3-NS5B	2nd forward	603/724	CAAAGGGTCCAATCACCCA(A/G)ATGTACAC	3619–3646
NS3-NS5B, 5′ UTR-NS5B	2nd reverse	607R/654R/722R	CGGTTGGGGAGCAGGTA(G/A/G)A(T/T/C)GCCTAC	9345–9370
Quantitative PCR				
5′ UTR	RT	738RH	ACTCGCAAGCACCCTATCAGGC	291–312
5′ UTR	Forward	736	AAGCGTCTAGCCATGGCGTTAGTA	73–96
5′ UTR	Reverse	737R	GGCAGTACCACAAGGCCTTTCG	272–293
5′ UTR	Probe	733FB	FAM-TCTGCGGAACCGGTGAGTACAC-BHQ1	147–168
E2	RT	743RH/744RH/753RH/753RH	CAACGCTCTCCTCG(A/A/G/G)GTCCA(A/G/A/G)TTGCA	2271–2296
E2	Forward[d]	751/752	GGCCTCCACATGGCAA(C/T)TGGTTCGG	1972–1993
E2	Forward[d]	739/740	CCGCCGCAAGGCAACTGGTT(C/T)GG	1974–1993
E2	Reverse	741R/742R	GCCTCGGGGTGCTTCCGGAAGCA(G/A)TCCGT	2088–2116
E2	Probe	734FB/735FB	FAM-TGGATGAA(T/C)AGCACTGGGTTCACCAAGAC-BHQ1	2001–2029

[a] Primers separated by slashes harbor a nucleotide substitution(s) (in parentheses) in the sequence in the same order.
[b] An underline and a double underline indicate recognition sequences for AscI and BsrGI, respectively, with which the PCR products were subcloned into plasmid vector pASGT5. Italics denote the T7 promoter, which was used to synthesize RNA in vitro from the T5S2 isolate (Fig. 4A).
[c] Nucleotide positions correspond to the HCV-JS sequence (12).
[d] Forward primers for E2 were mixed in the reaction mixture.

sult, isolates with the same deletion pattern formed genetic clusters that were distinct from each other, as well as from those of nondefective HCV isolates (Fig. 3A and B). Similar results were obtained for the other patients with defective HCV genomes (data not shown). These results suggest that a defective HCV genome is capable of replication to accumulate mutations and to evolve independently of the nondefective HCV genome.

TABLE 2. Quantitative PCRs for the 5′ UTR and E2 regions of HCV[a]

Region for quantification	No. of copies/ml		5′ UTR/E2 ratio
	5′ UTR	E2	
R2	2.0×10^6	1.7×10^6	1.17
R4	5.3×10^6	2.2×10^6	2.44
T5	ND[b]	ND[b]	
K1	8.3×10^5	8.4×10^5	0.99
K2	3.6×10^5	3.5×10^5	1.01
K3	8.6×10^5	5.1×10^5	1.7
K4pre	8.1×10^5	3.3×10^5	2.45
K4af24	4.5×10^5	4.4×10^5	1.01

[a] For quantification of the 5′ UTR and E2 regions, the TaqMan Fast PCR Universal mixture and the 7500 Fast Real-Time PCR system (Applied Biosystems) were used in a two-step method with the primers and probes shown in Table 1 according to the manufacturer's protocol. The copy number of HCV was determined by the standard-curve method with serial dilutions of the synthesized full-length HCV RNA.
[b] ND, not determined due to sample shortage.

Next, the ability of the defective HCV genome to be encapsidated and released from cells as HCV_{CCD} was examined. A genotype 1b replicon RNA lacking the structural region was synthesized by using defective isolate T5S-2 from patient T5 (Fig. 2 and 4A) as the template in an in vitro transcription system (MEGAscript T7 kit; Ambion, Inc., Austin, TX) under the control of the T7 promoter. Also, capped mRNA encoding the genotype 1b structural proteins from the same patient (designated C-NS2 in Fig. 4A) was synthesized in vitro with the mMessage mMachine T7 kit (Ambion). Both synthesized RNAs were cotransfected into Huh7.5 hepatoma cells. However, HCV_{CCD} was not obtained, presumably because of low replication or virus productivity of genotype 1b HCV per se. In fact, we transfected the defective RNA alone and observed the replication and protein expression of HCV, but with low efficiency (data not shown). Thus, to augment virus productivity, a JFH1-based chimeric HCV genome (genotype 1b/2a) and its deletion mutant were generated to mimic isolate T5S-2 (designated TNS2J1 and TNS2J1ΔS, respectively, Fig. 4A). JFH1 is genotype 2a HCV isolate that can produce high levels of infectious virus (14). To verify the virus productivity of TNS2J1, Huh7.5 cells (10-cm plate) were transfected with 10 μg of in vitro-synthesized RNA from TNS2J1 or JFH1 by lipofection with TransMessenger transfection reagent (Qiagen, Valencia, CA) according to the manufacturer's protocol. Two days later, the culture medium was concentrated 10-fold and inoculated into naïve Huh7.5 cells (four-well chamber

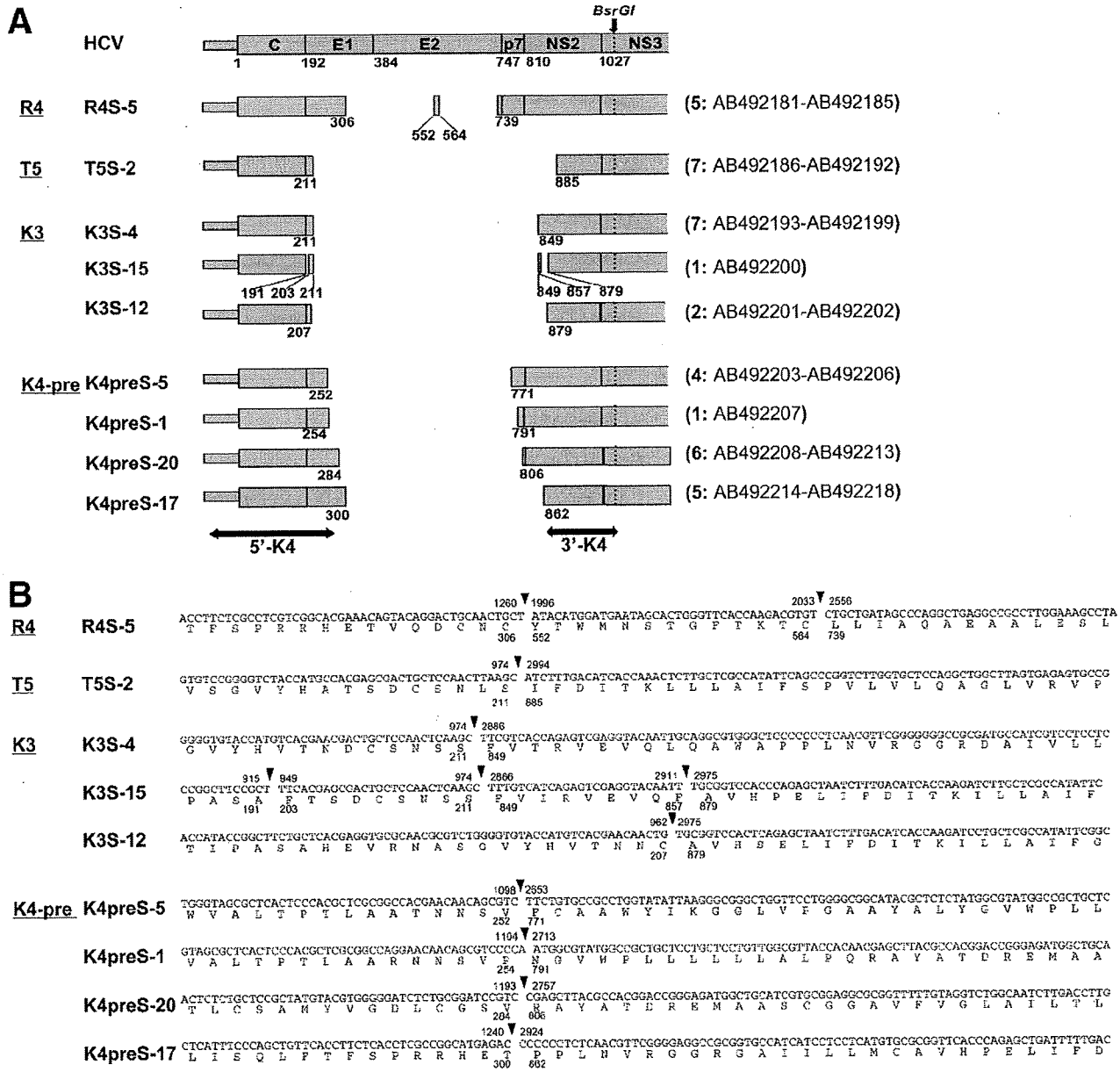

FIG. 2. Sequence analysis of the defective HCV genomes. A total of 38 isolates were molecularly cloned into a plasmid vector and sequenced. Data from representative isolates are presented. Nucleotide positions and deduced amino acid positions correspond to those of genotype 1b strain HCV-JS (12). (A) Defects located in the structural region were compared. The remaining regions are illustrated as shaded boxes. Below the boxes are numbers indicating amino acid positions at the end of each remaining region. At the top of the panel is the HCV genome with the amino acid position at the N terminus of each HCV protein below. The BsrGI restriction site that was used to clone the PCR products is shown as a dotted line. Each value in parentheses at the right is the number of isolates showing the same deletion pattern, followed by the GenBank accession number(s). The two-headed arrows indicate the 5′ and 3′ maximum overlapping regions among the defective HCV isolates in the K4-pre specimen that are compared in the following phylogenetic analyses (5′-K4 and 3′-K4; see Fig. 3). (B) Deletion breakpoints and their adjacent nucleotides and deduced amino acid sequences are indicated. Solid triangles denote breakpoints, and numbers indicate the nucleotide positions (above) and amino acid positions (below) at the junctions.

slide). Cells inoculated with the culture medium from TNS2J1 RNA-transfected cells markedly expressed HCV protein, as shown by immunofluorescent staining (Fig. 4B). The percentage of HCV-positive cells in chimera-infected cells, 40% (565/1,240), was greater than that of JFH1, 3% (37/1,210), demonstrating that the chimeric genome TNS2J1 can produce infectious HCV more robustly than JFH1 can ($P < 0.0001$).

Taking advantage of this chimeric genome, we conducted *trans* complementation experiments. To mimic the T5S-2 iso-

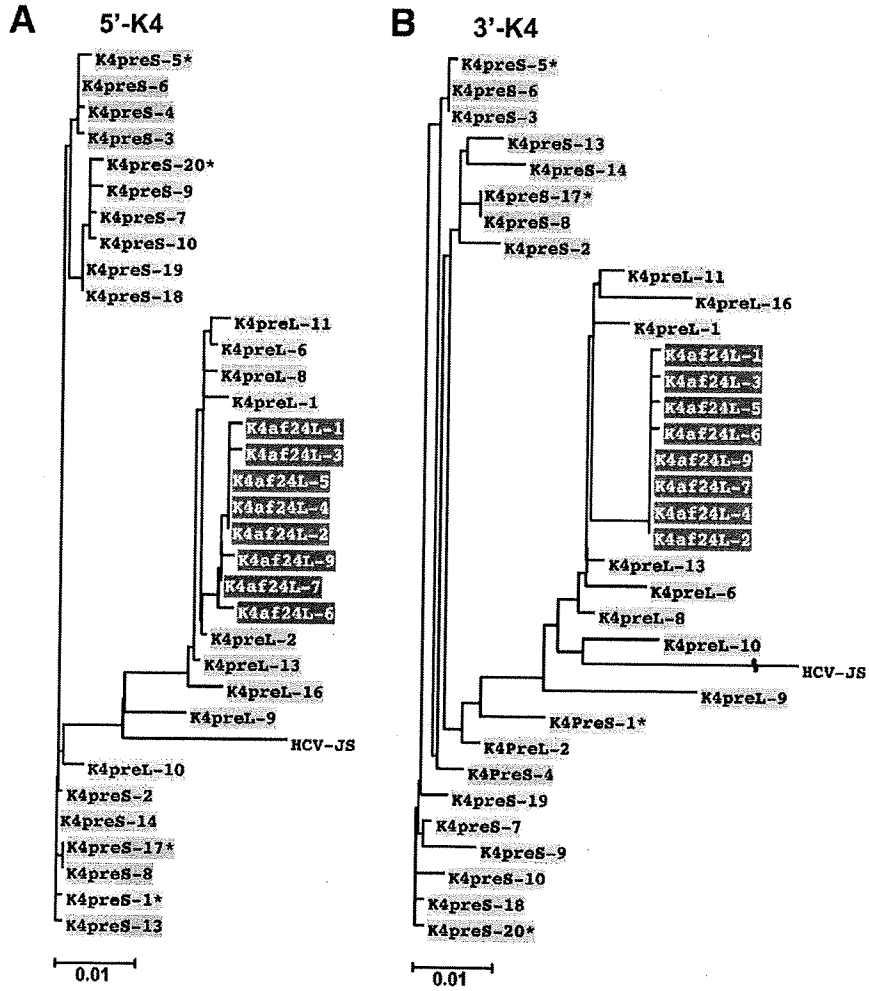

FIG. 3. Phylogenetic analyses of defective HCV genomes. Nucleotide sequence data from 33 isolates from patient K4 were used for phylogenetic analyses. The defective HCV genome (16 isolates) and the nondefective HCV genome coexisting before interferon treatment (9 isolates; GenBank accession no. AB492219 to AB492227) and those after treatment (8 isolates; GenBank accession no. AB492228 to AB492235) were compared in the 5′ and 3′ maximum overlapping regions separately (5′-K4 and 3′-K4 in Fig. 2A). Phylogenetic trees were created for the respective regions (A and B). In the isolate designations, pre and af24 stand for before and after interferon treatment and S and L stand for defective and nondefective HCV genomes, respectively. Isolates with the same deletion pattern (according to K4-pre in Fig. 2) are shaded in the same color. Asterisks denote the representative isolates illustrated in Fig. 2.

late, the region corresponding to the defect found in T5S-2 was identically deleted from the TNS2J1 genome (designated TN2J1ΔS, Fig. 4A). Ten micrograms of synthesized RNA of TN2J1ΔS was cotransfected into Huh7.5 cells (10-cm plate) together with 10 μg of synthesized capped mRNA encoding the structural region, including part of the nonstructural region of TNS2J1, designated C-NS2 or C-NS3P (Fig. 4A). Two days later, the culture medium was concentrated and inoculated into naïve Huh7.5 cells as previously described. HCV protein was expressed when cells were inoculated with the medium of cells cotransfected with TN2J1ΔS RNA and C-NS2 or C-NS3P mRNA, whereas no expression was observed in the case of TN2J1ΔS RNA alone (Fig. 4C). To stably provide the structural proteins in *trans*, packaging cell lines were established by retroviral transduction (2) of Huh7.5 cells with genes encoding the C-NS2 or C-NS3P region (Fig. 4A). These packaging cell lines were transfected with TN2J1ΔS RNA, and HCV protein was expressed in cells inoculated with the culture medium from the RNA-transfected packaging cells (Fig. 4D). Notably, the construct C-NS2 helped to produce HCV_{CCD} more efficiently than C-NS3P did (Fig. 4C). We observed less expression of the structural proteins with the C-NS3 construct than with the C-NS2 construct in a transient expression experiment (data not shown). One possible reason for this is that the C-NS3 construct needs one additional process, i.e., cleavage between NS2 and NS3, to produce NS2 and may affect the other proteins. Otherwise, it is simply because of the difference in the lengths of the constructs. These results indicate that a defective HCV genome lacking the structural region can be encapsidated by *trans* complementation of the structural proteins, thus conferring infectivity in vivo. Recently, a *trans*-packaging system consisting of an HCV subgenomic replicon and a reporter gene

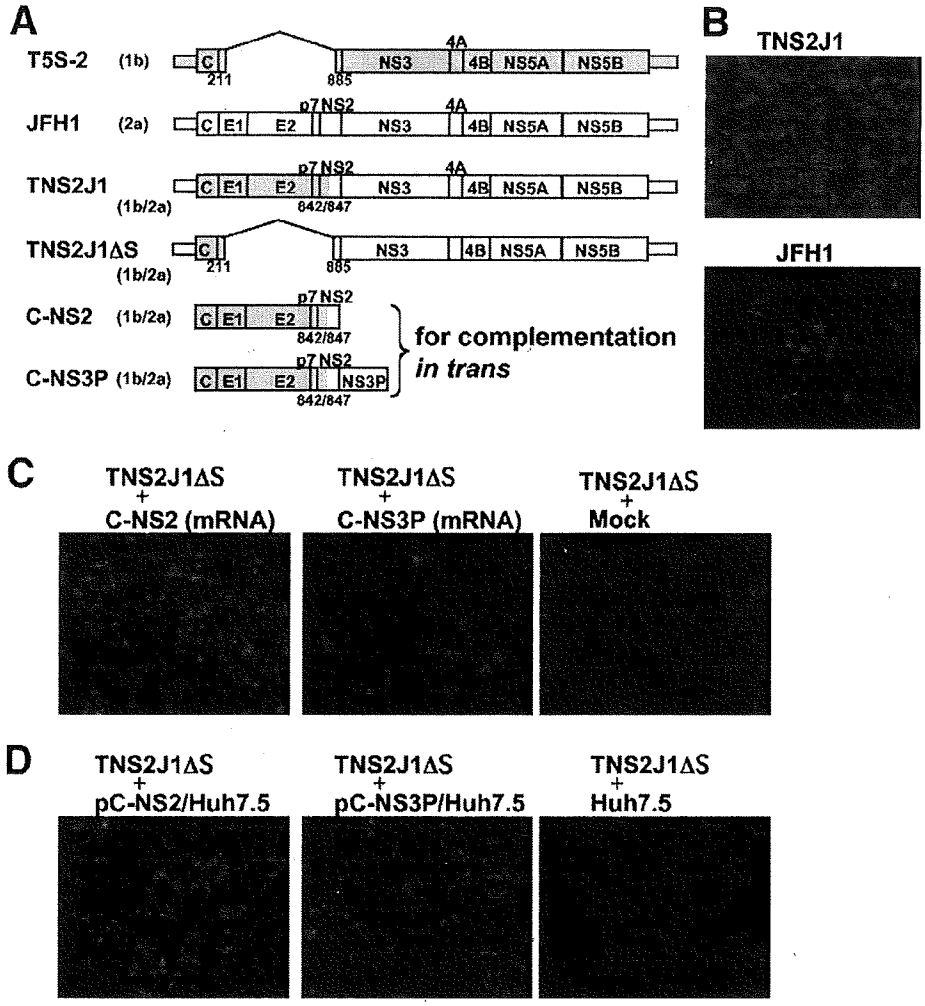

FIG. 4. In vitro infectivity of deletion mutant of chimeric HCV conferred by *trans* complementation of structural proteins. (A) Schematics of the following HCV genomic constructs: the defective HCV isolate (T5S-2), JFH1, chimeric virus of genotypes 1b and 2a (TNS2J1), and its deletion mutant (TNS2J1ΔS). C-NS2 and C-NS3 are fragments encoding the region from the core to the C terminus of the NS2 region and to the C terminus of the serine protease moiety in NS3, respectively. For the *trans* complementation experiments, the latter two constructs were inserted into pcDNA3.1 (Invitrogen) to synthesize capped mRNAs or into retroviral vector pCX4bsr (GenBank accession no. AB086384) to establish packaging cell lines stably expressing the proteins. Shaded and open boxes represent genotypes 1b (isolate from patient T5) and 2a (JFH1), respectively. The numbers below the boxes are amino acid positions at deletion breakpoints or at PCR-based recombination junctions. Naïve Huh7.5 cells were inoculated with the culture medium from cells transfected with JFH1 or TNS2J1 RNA (B), from cells cotransfected with TNS2J1ΔS RNA together with the structural region mRNA (C-NS2 or C-NS3P) or TNS2J1ΔS RNA alone (C), and from the packaging cell line (C-NS2/Huh7.5 or C-NS3P/Huh7.5) transfected with TNS2J1ΔS RNA and parental Huh7.5 cells transfected with TNS2J1ΔS RNA (D). HCV protein was detected by human HCV serum (1:500) by the indirect immunofluorescent method with Alexa Fluor 568 goat anti-human immunoglobulin G (1:200; red; Invitrogen). Nuclei were counterstained with 4′,6′-diamidino-2-phenylindole (DAPI; blue).

was also reported in which an intragenotypic chimera (2a/2a) was used as the most efficient packaging construct (11). Our packaging system used an efficient intergenotypic chimera (1b/2a) to encapsidate a genome mimicking a naturally occurring deletion (1b). Thus, although its efficiency may be different, our system could be a useful tool for the study of HCV_{CCD} of chimeric genome 1b/2a or genotype 1b.

Taken together, genetic analyses of the defective HCV genome showed the potential of its translation and self-replication. These defective genomes can be encapsidated into infectious virus-like particles by *trans* complementation of the structural proteins in vitro. The 5′ UTR and core regions, which are preserved in defective HCV genomes, are targets for the clinical quantification of HCV. Therefore, measured values may represent additive values for defective and nondefective HCVs and the method used for HCV quantification should be reevaluated.

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