making an elbow-like interpentameric contact. The glycine- and proline-rich sequence, which allows the elbow-like connection in papilloma virus, is not found in the BKV VP1 protein, nor is a similar contact fitting with the structural data available for the BKV VLPs. As a common feature, the variability in the geometry of the contacts in both the polyoma and papilloma virus is entirely due to the inherent flexibility of the C-terminal domains, while the structure of the monomer core (the pentameric ring) seems rather stable and does not change significantly between the $T=1$ and $T=7$ structures. Differing from the papilloma virus case, the $T=1$ and $T=7$ structures of the polyomavirus seem to have one interpentameric interaction in common.

We can here conclude that both Ca^{2+} and disulfide links are important for the stability of the recombinant BKV $T=7$ capsid. However, when the ionic strength of the buffer solution is increased, hydrophobic effects are more influential and keep the capsid intact also in a reducing environment, lacking calcium ions. Chen et al. (6) showed that the reducing and chelating agents worked in a specific order. The reducing agent had to be introduced in advance of the chelating agent in order for dissociation of the VLPs to occur. Thus, the disulfide bond appear to protect bound calcium ions from contact with the chelating agent. Our reassembly experiments also showed that the formation of small or large VLPs does not require the formation of disulfide bonds in vitro. This may suggest that the disulfide interaction is not necessary for the actual reassembly process but that the bond is formed after the particle has been reassembled, allowing for an increase in the stability of the VLP.

Recently, a similar study was done with SV40 VP1, where free pentamers were found to reassemble preferably into the $T=1$ particle in the absence of calcium (17). Contrary to our observations and to those of Salunke et al. (29), Kanesashi et al. (17) did not observe any reassembly into VLPs with buffers having physiological pH (pH 7.2) and salt concentrations (150 mM NaCl). This could be due to the use of a low, 60-μg/ml, protein concentration, compared to the 0.5- to 1-mg/ml concentration used in our and Salunke's experiments.

Both Kanesashi's and our study show that small $T=1$ polyoma VLPs can form without calcium, while the larger $T=7$ structure requires the calcium ion for assembly. Two calcium-binding sites were found in five of the six unique monomers (α, α′, α″, β, and β′), while only one was found in the twofold monomer (γ) in the SV40 structure (34). One amino acid in the C-terminal arm was thought to be involved in each calcium-binding site. Even though the structure in the C-terminal arm was different among the six unique monomers, the interpentameric interactions were quite similar. The difference in structure allowed the same amino acids within the six unique monomers to have similar interpentameric interactions (hydrogen bonds, hydrophobic interactions, or salt bridges) at the three unique interpentameric contacts; at the local threefold axis, around the icosahedral threefold axis, and at the icosahedral twofold axis. However, only in the interpentameric connections at the local threefold and at the icosahedral twofold could the possible calcium-binding amino acids in the C-terminal arm form salt bridges with the adjacent pentamer. The negatively charged amino acids holding the calcium ion would repel each other in its absence. A partial explanation as to why the $T=1$ structure could form without calcium, whereas the $T=7$ structure could not, would then be that only the interpentameric contacts around the $T=7$, icosahedral threefold axis requires calcium for stabilization.

In conclusion, our structural study of the two BKV VLPs has shown that the triple-helix bundle is preserved in both structures and can form without Ca^{2+}. The "flatter" contacts around the threefold axis in the $T=7$ particle is probably both stabilized and enforced by Ca^{2+}, since salt bridges would not substitute in a similar conformation. The calcium concentration will in this way be the critical factor controlling the assembly of the VLPs into different sizes.

Neither of the two assembly models discussed in the literature, namely the assembly nucleus of a "five-around-one" pentamer (34) or a dimer of pentamers (4), can be deduced in our case. All the three unique contacts identified in the BKV VLPs elaborate on the formation of the J strand in the invaded pentamer. As a strong interaction, considerable energy would be needed to replace any type of unique contacts with one another. The primary contact between soluble pentamers would rely on the formation of looser contacts. These are most likely provided by the C-terminal arm at a location before the strand J forming sequence. While further investigations are needed to reveal the steps in which the polyoma VP1 pentamer assembles into particles and virions, our recent data suggests that the position of the C-terminal helix and its following tail could be stabilized through interaction with internal DNA, resulting in a more stable native-like capsid (Nilsson et al., manuscript in preparation).

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HEPATOLOGY

Outbreak of hepatitis C virus infection in an outpatient clinic

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Abstract

Background: From January through September 2001, seven patients were admitted to Fukaya Red Cross Hospital with typical clinical manifestations of acute hepatitis. Six were outpatients of the clinic, which is located near the hospital. An extensive survey of clinic outpatients conducted by the local health department revealed six more new acute hepatitis cases during this period.

Methods: A case control study was carried out to identify potential risk factors for infection. In total, 1946 outpatients with clinic records were scheduled to undergo hepatitis C virus (HCV)-antibody testing. For the HCV-Ab positive patients, HCV-RNA was subtyped and quantified, and sequences of HCV hypervariable region 1 were determined.

Results: Ultimately, 12 patients with acute hepatitis and two asymptomatic subjects were found to be a part of this outbreak. HCV isolates were divided into three major groups using phylogenetic tree analysis. Only a past history of visiting the clinic was significantly associated with acute hepatitis. The timing of the parenteral medical procedure at the clinic and the onset of acute hepatitis strongly suggested association of the two events.

Conclusions: Our findings suggest that nosocomial HCV infection can occur in an outpatient clinic, even in countries where post-transfusion hepatitis has been almost entirely eliminated.

Key words: hepatitis C virus, nosocomial infection, outbreak, outpatient clinic.

INTRODUCTION

Hepatitis C virus (HCV) has infected 170 million people worldwide and the number of deaths annually in the USA from HCV-associated liver disease and cancer may have almost overtaken deaths caused by AIDS.[1–3] HCV causes persistent infection in approximately 80% of infected adults. Severe liver disease and hepatocellular carcinoma develop in an estimated 70% of those who cannot eliminate the virus. The development of a blood screening test in 1990 has virtually eliminated the spread of HCV through transfusions in industrial countries. As a result, the Center for Disease Control (CDC) estimates that new USA HCV infections dropped from approximately 230 000 a year in the 1980s to fewer than 36 000 in 1996. However, the CDC estimates that 1.8% of the USA population still harbors the virus. In Japan, after the introduction of second-generation HCV-Ab tests (February 1992), the risk of post-transfusion HCV infection became essentially negligible.[4,5] The incidence of HCV infection in Japan has decreased to 1.8–3.5/100 000 person-years,[6] which is comparable to that of the USA.[7] Furthermore, in 2000, nationwide nucleic acid amplification testing (NAT) for hepatitis B virus (HBV), HCV and human immunodeficiency virus type 1 (HIV-1) was implemented by the Japanese Red

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Cross Blood Transfusion Services for screening of voluntarily donated blood, such that post-transfusion hepatitis C is now virtually nearly zero in Japan.[8]

Nonetheless, nosocomial HCV infections can occur rarely. One way of contracting HCV may be transmission from infected medical personnel to susceptible patients during medical care. Spread from a cardiothoracic surgeon[9] and from an anesthesiology assistant to patients has been reported.[10] Patient-to-patient transmissions were reported in a hematology ward,[11] a pediatric oncology ward,[12] during colonoscopy in a gastrointestinal disease unit,[13] and in a hemodialysis unit.[14,15] Recently, an outbreak of acute hepatitis C among healthy volunteers participating in pharmacokinetics studies was reported.[16] However, nosocomial HCV infection in an outpatient clinic, which is rarely reported for HIV infection,[17,18] has been regarded as extremely rare in industrial countries.

We report here an outbreak of HCV infection at an outpatient clinic in a suburban city near Tokyo, Japan.

METHODS

Description of outbreak

From April through September 2001, seven patients were admitted to Fukaya Red Cross Hospital with typical clinical manifestations of acute hepatitis, including general malaise. HCV antibodies were detected in all seven patients. All seven were infected with HCV genotype 1b. Three patients (F1, F2, F4) were referred by an outpatient clinic (clinic A) located near Fukaya Red Cross Hospital. Three other patients (F3, F5, and F6) with acute hepatitis were from other clinics, but interviews with these patients revealed that they were outpatients of clinic A (Table 1). Only one patient was not an outpatient of clinic A (the serum level of HCV-RNA was negative in this patient when the survey was carried out in May 2001). Sequencing analysis of the serum (convalescent sera stored in November 2001) from the three patients (F 2, F3, F6) revealed sequence similarities among these three patients, which lead the local health department to survey the outpatients of clinic A.

Identification and clinical description of cases

For surveillance purposes, patients were defined as any outpatients who had visited clinic A between July 2000 and May 2002 with symptoms suggestive of acute hepatitis and with serum aspartate aminotransferase (AST) or alanine aminotransferase (ALT) elevation (more than 400 IU/L).

In May 2002, 1946 individuals had outpatient records at clinic A. All 1946 subjects were requested to be tested for HCV-antibody (HCV-Ab). For the HCV-Ab positive patients, confirmation was made by immunoblot assay (RIBA; recombinant immunoblot assay: Ortho-Clinical-Diagnostics, Raritan, NJ, USA), HCV-RNAs were subtyped, and quantified.

Laboratory methods

Serum samples from the patients were collected in May 2002 and frozen at −80°C. The presence of HCV-Ab was determined by the gelatin particle agglutination test (Ortho-Clinical-Diagnostics). Reactivity was confirmed by immunoblot assay (RIBA). HCV RNA was detected qualitatively and was also quantified with reverse transcription-polymerase chain reaction (RT-PCR) kits (HCV Amplicore v2.0; Roche Diagnostics, Tokyo, Japan). HCV isolates were genotyped, and HCV hypervariable region 1 (HVR1: nucleotides 1491–1572, numbered as reported by Choo *et al.*[19]) was amplified as described elsewhere.[20] Products of the second PCR were purified from the agarose gel and cloned into a plasmid vector (TOPO TA cloning kit; Invitrogen, Carlsbad, CA, USA). Four to six clones from each subject were sequenced in both directions with CEQ2000 (Beckman Coulter, Fullerton, CA, USA). The direct

Table 1 Clinical characteristics of patients with acute hepatitis C

Case	Sex	Age	ALT (IU/L)	Peak T Bil (mg/dL)	Peak	Onset genotype
F1	Female	71	991	1.9	January 2001	1b
F2	Female	68	3645	14.1	April 2001	1b
F3	Female	24	2365	7.0	April 2001	1b
F4	Female	54	918	1.9	April 2001	1b
F5	Male	26	1077	1.7	July 2001	1b
F6	Male	76	1590	11.2	September 2001	1b
N1	Female	40	554	0.8	December 2000	1b
N2	Female	83	1950	15.9	April 2001	1b
N3	Male	73	1370	9.6	May 2001	1b
N4	Male	77	421	1.0	June 2001	1b
N5	Female	71	800	3.0	August 2001	1b
N6	Male	61	594	0.9	August 2001	1b

ALT, alanine aminotransferase; T Bil, total bilirubin.

sequencing procedures were performed with the primers used in the second PCRs employing a Dye Terminator Cycle Sequencing Kit (Perkin Elmer, NJ, USA) and analyzed on an ABI PRISM 377 sequencer. To prevent possible cross-contamination of the samples, stringent procedures were used for nucleic acid extraction and amplification, and the analyses were performed independently in two laboratories (Tokyo University and National Institute of Infectious Diseases) separately. Identical sequence results were obtained.

Pylogenetic analysis

The degree of divergence between the sequences was estimated by the neighbor-joining (NJ) method using Kimura's two-parameter distance.[21] The set of distance matrices was then analyzed by the UPGMA (Unweighted Pair Group Method with Arithmetic Mean) program of the Genetyx-Mac (version 10.1., Software Development, Tokyo, Japan).

Case control study and statistical analysis

We conducted two case control studies. In the first case control study, we enrolled seven patients and 11 matched controls from among the outpatients of Fukaya Red Cross Hospital to identify potential risk factors for infection. We used a detailed questionnaire to interview patients and controls. Information included past history of blood transfusion, surgical operation, tattooing, drug abuse, familial liver diseases, and parenteral procedures at clinic A. Parenteral procedures were confirmed by the records of patients at clinic A.

In the second case control study, we enrolled the first nine (F1-F6, N2-N4) patients and matched 18 controls from among outpatients of clinic A to identify potential risk factors for HCV outbreak. We used a detailed questionnaire to interview patients and controls. Collected information included past history of blood transfusion, surgical operation, tattooing, drug abuse, familial liver diseases, and parenteral treatment at clinic A. The parenteral treatments included blood aspiration, percutaneous injection, intravenous pyelography, intravenous cholangiography, endoscopy, intravenous injection, and drip infusion.

In the case control study, odds ratios and 95% confidence intervals were calculated with the use of Statview Version 5.0 (SAS Institute, Cary, NC, USA).

RESULTS

Survey of outpatients

An active survey revealed six more patients (N1-N6) with acute hepatitis C among outpatients of clinic A (Table 1). Three (N2, N3, N4) had received injection therapy and had been followed up in the clinic. The other three (N1, N5, N6) patients had visited other clinics with typical manifestations of acute hepatitis. A nurse (N1) who had worked at clinic A (through December 2000) had been diagnosed with acute hepatitis in December 2000. Thus, the total number of patients with acute hepatitis C virus infection in this outbreak was 12 (Fig. 1). The clinical characteristics of these patients are summarized in Table 1.

To define the extent of the outbreak, we notified all patients who had visited clinic A and had received any form of parenteral therapy from July 2001 through to May 2002. Those patients were contacted by local health department personnel, and a brief, standardized questionnaire was administered.

Altogether, 1831 patients (94.1%) out of 1946 with outpatient records of clinic A were surveyed from May through December 2002. In total, 1776 of the 1831 tested patients had age data, while for the other 51 patients no age data were available. Those 51 outpatients were HCV-Ab negative. Age related HCV-Ab positivity was as follows: 0–9 years old; 0/197, 10–19 years old; 0/294, 20–29 years old; 0/228, 30–39 years old; 1/187, 40–49 years old; 8/203, 50–59 years old; 2/298, 60–69 years old; 10/209, 70–79 years old; 12/122, and over 80 years old; 3/42. Thirty-six (including six new cases: N1-N6 with acute hepatitis) patients (2.0%) were HCV-Ab positive. Of these 36 HCV-Ab positive patients, 20 had genotype 1b, six had genotype 2a, two had genotype 2b, and eight had the virus undetectable according to the HCV quantification analysis.

In May 2002, the HCV-RNA level remained high in all 12 patients with acute hepatitis and ALT was slightly elevated. None of these patients in this outbreak had cleared the virus in at least 10 months after the infection. All staff members (two doctors and three nurses), except the nurse (N1) who had acute hepatitis, were negative for HCV-Ab on third-generation assays.

Molecular comparison

The HCV isolates of the 12 patients (F1-F6, N1-N6) with acute hepatitis and the 14 (out of 20 excluding six new patients with acute hepatitis) HCV-RNA positive carriers with genotype 1b were determined by HVR1

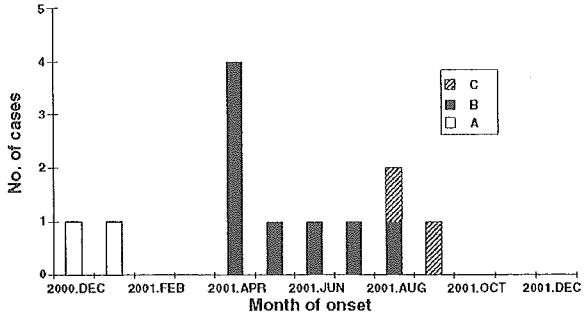

Figure 1 Onsets of hepatitis C virus (HCV) infection during the outbreak, according to the date of diagnosis. a-c shows the three major groups of HCV isolates in the outbreak that is described in Figure 2.

sequences. The HCV isolates of the patients with acute hepatitis were divided into three groups. The HVR1 sequences of two patients (AS1 and AS2) of the 14 asymptomatic patients were similar to those of patients with acute hepatitis, while those of the other 12 were not. Figure 2a shows the amino acid sequences of HVR1 for each of the isolates. The evolutionary distances in this outbreak were similar to those previously reported for HCV infections caused by needle-stick injuries.[22] HCV isolates were divided into three major groups using phylogenetic tree analysis. The first group (group A) consists of two patients, F1 and N1. HCV isolates from these two patients diverged from other isolates, but F1 and N1 had amino acid mutations in common.

The second group (group B) represented the major HCV type in this outbreak. F2 and F4 showed identical isolates and phylogenetic tree analysis strongly suggested that these patients were infected with the same HCV (Fig. 2b). Two asymptomatic carriers (AS1 and AS2) belonged to this group. It is difficult to determine whether these two patients were infected in this outbreak or were possible sources of this HCV infection. The third group (group C) consists of F6 and N5, who had completely identical sequences. These isolates diverged from group A, but had common amino acid substitutions with those of group B.

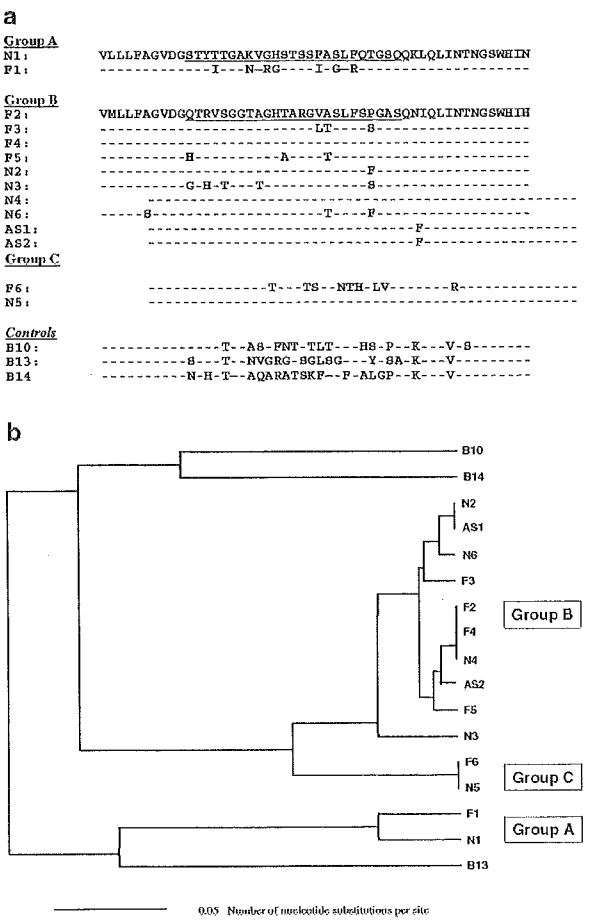

Figure 2 (a) Alignment of the amino acid sequences of hepatitis C virus (HCV) hypervariable region 1 from the patients. HCV isolates were divided into three major groups (groups A, B and C). AS1 and AS 2 are asymptomatic patients. The dashes denote amino acids identical to N1 in group A, and to F2 in group B, C, and control groups. Polymerase chain reaction (PCR) products of HCV isolates obtained from chronic hepatitis C patients in other areas were used as controls. (b) Phylogenetic tree analysis comparing coding sequences for HCV hypervariable region 1 from 17 isolates. For phylogenetic analysis, HVR1 sequences obtained from 14 HCV-RNA positive patients involved in this outbreak and three controls were compared.

Evaluation of risk factors for hepatitis C virus infection

In the first case control study, only a past history of visiting clinic A was significantly associated with acute hepatitis (OR, 42.6; 95% CI, 2.28–177.3). In the second case control study, intravenous pyelography (OR, 2.50; 95% CI, 0.24–25.68), intravenous cholangiography (OR, 2.50; 95% CI, 0.47–13.27), and drip infusion (OR, 2.14; 95% CI, 0.25–18.50) showed relatively high OR, but were not significantly associated with acute hepatitis. It is difficult to identify potential risk factors because controls had also received several parenteral treatments. Almost all patients and controls had undergone at least one of the parenteral procedures at this clinic (Table 2).

To determine the source of outbreak, we reviewed individual patient records. Each patient with acute hepatitis had received at least one of the following parenteral medical procedures; blood aspiration, percutaneous injection, intravenous pyelography, intravenous cholangiography, endoscopy, intravenous injection, or drip infusion. Figure 3 shows the relationship between

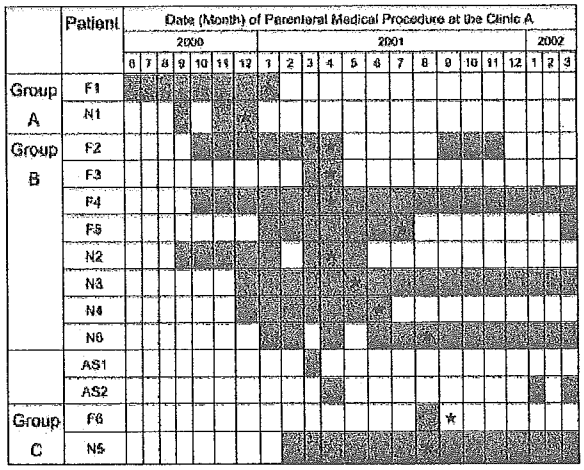

Figure 3 Relationship between a parenteral medical procedure and the onset of acute hepatitis. (★) show the onset of acute hepatitis.

Table 2 Risk factors possibly associated with parenteral medical procedures

Risk factor	Cases		Controls		OR	95% CI
	+	−	+	−		
Blood aspiration	9	0	17	1	1.11	0.09–13.84
Percutaneous injection	9	0	17	1	1.11	0.09–13.84
Intravenous pyelography	9	0	15	3	2.50	0.24–25.68
Cholangiography	6	3	8	10	2.50	0.47–13.27
Endoscopy	1	8	2	15	0.94	0.07–12.00
Intravenous injection	0	9	0	17	1.80	0.10–31.99
Drip infusion	2	7	2	15	2.14	0.25–18.50

Intravenous pyelography, intravenous cholangiography, and drip infusion showed relatively high odds ratio (OR). It is difficult to identify potential risk factors because controls had also received several parenteral treatments. CI, confidence interval.

the parenteral procedure and the onset of acute hepatitis. This data strongly suggests an association between the onset of acute hepatitis and parenteral treatment at clinic A. Patient F3 had visited clinic A only once in March 2001, and had received a percutaneous injection and intravenous pyelography, and manifested acute hepatitis in April.

DISCUSSION

We reported an outbreak of HCV infection in an outpatient clinic. Most of the patients had typical manifestations of acute hepatitis. Acute HCV infection is usually asymptomatic, despite significant viremia and hepatic cytolysis. Only one-third of patients develop jaundice or symptoms.[23] One of the characteristics of this outbreak was that most of the patients had accompanying symptoms of general malaise or jaundice. Although all patients recovered, the symptoms in the acute phase were severe and peak ALT levels were relatively high. The serum level of HCV-RNA had persisted in all patients at least 10 months after the infection. Only two asymptomatic carriers were identified in the retrospective survey of outpatients, suggesting that a high percentage of patients were symptomatic in this outbreak. Although drug abuse, high-risk sexual behavior, and poverty are considered to be risk factors for HCV infection,[24] these risk factors were not identified in the present outbreak. No surgical exposure prone procedures were found in this clinic.

Transmission risk is determined by the infectivity of the body fluids and tissues to which an individual is exposed. In this sense, HCV RNA concentrations in the inocula were assumed to be high. Alternatively, the HCV isolates in this outbreak may have been exceptionally infectious. We cannot rule out either of these possibilities.

The mean incubation period to onset of symptoms is about 7 weeks (range: 3–20 weeks)[23] in acute hepatitis C. All patients in this outbreak had at least one parenteral treatment at clinic A 7–8 weeks before the onset of symptoms (Fig. 3), suggesting that the infection was contracted when these procedures were carried out. The survey of the clinic by the local health department showed that glass syringes had been used after sterilization with an autoclave until 2001. Previously, an iatrogenic HCV infection with glass syringes was suggested in parenteral antischistomal therapy in Egypt[25] and in a rural area of Japan.[26] Furthermore, it was found that bottles had been shared among several patients receiving parenteral treatments, particularly percutaneous injection, intravenous pyelography, intravenous cholangiography, and drip infusion. It is probable that the needles were exchanged, but the injection equipment, including the bottles, was used for several patients. This may have been the source of the infection.

Fukaya city is a suburb of Tokyo, with a population of approximately 100 000. The incidence of HCV infection in Japan has fallen to less than 1.8–3.5/100 000 person-years,[6] which is comparable to that of the USA. In Japan and Italy,[27] the proportions of HCV positive patients are higher among the elderly (particularly those in their sixties and older in contrast to the USA where the incidence is highest in the fourth and fifth decades [3.0–3.9%]).[28] We must keep in mind that some percentage of the general population will have HCV infection, and can become a reservoir for an acute HCV outbreak even in general practice. Minor violations in safety procedures may result in the spread of blood borne agents even in the countries where the incidence of HCV infection is low.

Characteristic features of this outbreak are its high percentage of chronicity. None of the patients with acute hepatitis had eliminated HCV before May 2002. Symptomatic acute hepatitis C infection has been described to have a better outcome.[29,30] Lately, in the study of the natural course of acute symptomatic hepatitis C, spontaneous clearance was observed in 52% of the patients, usually within 12 weeks after the onset of symptoms.[31] Treatment of acute hepatitis C with interferon alfa-2b was reported to be very effective, if the patients started to receive interferon within the preceding 4 months after the known or suspected exposure.[32] In this outbreak, at least 10 months had passed after the suspected exposure, so for some of the patients under 60 years of age, we started ribavirin plus interferon therapy.[33] However, for those over 70 we are

conducting periodic follow-up, because in studies with 10–20 years of follow-up, cirrhosis develops in less than 20–30% of patients.[30]

Epidemiological and phylogenetic analyses disclosed horizontal nosocomial HCV infection. We were not able to pinpoint the precise mechanisms leading to these infections. Nonetheless, this study underlies the importance of constant surveillance of infectious diseases. Our findings suggest that nosocomial HCV infection can occur in an outpatient clinic, even in countries where post-transfusion hepatitis is apparently well controlled.

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Exploiting *cis*-Acting Replication Elements To Direct Hepatitis C Virus-Dependent Transgene Expression

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We describe here a novel targeting gene therapy strategy to direct gene expression responsive to hepatitis C virus (HCV). The goal was approached by engineering a construct containing the antisense sequence of the transgene and internal ribosome entry site of encephalomyocarditis virus flanked by 5′- and 3′-end sequences of HCV cDNA that contain *cis*-acting replication elements. Thus, expression of the transgene is only promoted when the minus-strand RNA has been synthesized by the functional replication machinery present in infected cells. Reporter assay and strand-specific reverse transcription-PCR showed selective transgene expression in Huh-7 cells harboring an autonomously replicating HCV subgenome but remaining silent in uninfected cells. Furthermore, using the cytosine deaminase suicide gene as a transgene coupled with recombinant adenovirus delivery, we demonstrated that cytosine deaminase was specifically expressed in replicon cells, resulting in marked chemosensitization of replicon cells to the cytotoxic effects of flucytosine. This new targeting strategy could be extended to other single-stranded RNA viruses encoding the unique RNA-dependent RNA polymerase that has no parallel in mammalian cells.

Chronic hepatitis C virus (HCV) infection, which frequently leads to liver cirrhosis and hepatocellular carcinoma (3, 34), remains a major public health problem worldwide. It has been estimated that more than 3% of the world population is infected with HCV. The plus-strand HCV RNA genome is approximately 9,600 nucleotides in length and encodes a polyprotein precursor of about 3,010 amino acids, which is cleaved co- and posttranslationally by cellular and viral proteases to produce structural and nonstructural (NS) proteins (8, 13, 15). One of the NS proteins, NS5B, is an RNA-dependent RNA polymerase (RdRp) that catalyzes the replication of HCV (5, 30).

Current treatment modalities available for HCV infection, including alpha interferon, has limited effectiveness. Only 20 to 30% of alpha interferon-treated patients develop a sustained remission, and increased or prolonged systemic administration is often associated with severe side effects or viral resistance. The combination of ribavirin and interferon is known to be significantly more effective than interferon monotherapy in naïve and relapser patients, but it induces a sustained response only in 41% of patients and in less than 30% of patients infected with genotype 1 (18). The therapeutic potential of small inhibitory molecules that target serine protease, helicase, or RdRp has proved to be promising (4, 6, 10), however, one unavoidable problem of this approach is selection of resistant mutants conferred by single or multiple mutations due to the error-prone nature of RdRp (24, 26, 29). Thus, an alternative approach for treating HCV patients that results in the death of infected cells, thereby limiting or eliminating virus production, while leaving uninfected cells unharmed would have an advantage over the HCV therapies now available.

Here we describe a targeting gene therapy approach that harnesses the viral replication machinery by constructing an HCV-like minigenome that consisted of the antisense sequence of cytosine deaminase (CD) suicide gene and internal ribosome entry site (IRES) element from encephalomyocarditis virus (EMCV) flanked by the 5′- and 3′-end regions of HCV, so that expression of CD is initiated only when the minus-strand RNA has been synthesized by the functional replication components present in infected cells. Using recombinant adenovirus delivery, we demonstrate that CD is specifically expressed in infected cells, resulting in marked chemosensitization of infected cells to the cytotoxic effects of flucytosine (5-FC).

MATERIALS AND METHODS

Cells. The cell lines Huh-7, HepG2, A549, and 293 were 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 the HCV subgenomic replicon was grown in DMEM containing 300 μg/ml G418 (Geneticin, Invitrogen).

Plasmids. For construction of the reporter vectors pT7cRLs, a fragment containing HCV cDNA (1 to 377) with the T7 promoter directly coupled at the 5′ end was amplified by PCR with primers 5′-tataagcttTAATACGACTCACTATAGCCAGCCCCCGATTGGGGGC-3′ and 5′-tgctctagaTTTGGTTTTTCTTTGAGGTT-3′ (capital letters indicate the sequences originally contained in the target sequence, and lowercase letters indicate the attached sequences which were introduced for the convenience of molecular manipulation, such as restriction sites). The EMCV IRES sequence was amplified from pEMCVRL (42) with primers 5′-cgcggatccAACTAACTAACTAAGCTAGC-3′ and 5′-ctctctagaGTATTATCGTGTTTTTCAAA-3′. The PCR products were digested with HindIII and XbaI or BamHI and XbaI, respectively, and cloned into HindIII/BamHI-digested pUC18 to generate pTCE. The 3′ part of the NS5B coding region connected 3′ untranslated region (UTR) of HCV was amplified by PCR using primers 5′-cgcggatccGGAAACTTGGGGTCCCACCC-3′ and 5′-ataggcgc

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cagcgaggaggctgggaccatgccggccACATGATCTGCAGAGAGGCC-3′, digested with BamHI and NarI and cloned, along with the annealed oligonucleotides containing the partial sequence of the HDV ribozyme, sense 5′-CGCCGGCTG GGCAACATTCCGAGGGGACCGTCCCCTCGGTAATGGCGAATGGGA CCg-3′ and antisense 5′-aattcGGTCCCATTCGCCATTACCGAGGGGACGG TCCCCTCGGAATGTTGCCCAGCCGG-3′, into BamHI/EcoRI-cut pTCE, generating pTCECD. The *Renilla* luciferase gene was amplified from pRL-TK (Promega) using primers 5′-ctctctagaATGACTTCGAAAGTTTATGA-3′ and 5′-ctctctagaTTATTGTTCATTTTTGAGAA-3′, digested with XbaI, and inserted into XbaI-digested pTCECD to generate pT7cRLNS5B1 or pT7RLNS5B1. pT7cRLNS5B2 or pT7cRLUTR, which contain the NS5B coding region from nucleotides 9307 to 9371 plus the 3′-UTR or 3′-UTR alone, was constructed similarly.

To construct pmCMVcRLmpA, the HCV minigenome was amplified from pTcRLNS5B1 by primers 5′-atagagctcTCTGGCTAACTGCCAGCCCCCGAT TGGGGGC-3′ and 5′-ctcactagtACATGATCTGCAGAGAGGCC-3′, digested with SacI and SpeI and cloned, together with the annealed minimal poly(A) oligonucleotides, sense 5′-CTAGAACTAGTAATAAAGGATCCTTTATTTTC ATTGGATCCGTGTGTTGGTTTTTTGTGTGCGGCCGCG-3′ and antisense 5′-AATTCGCGGCCGCACACAAAAAACCAACACACGGATCCAATGAA AATAAAGGATCCTTTATTACTAGTT-3′ into SacI/EcoRI-digested pShuttle (Clontech).

For construction of pmCMVcRLHD, a PCR product amplified from pT7cRLNS5B1 with primers 5′-ATGACTTCGAAAGTTTATGA-3′ and 5′-ata cttaagGGTCCCATTCGCCATTACC-3′ was digested with BstBI and AflII and cloned with the SacI-BstBI fragment from pmCMVcRLmpA into SacI/AflII-digested pShuttle. Using pmCMVcRLmpA as a template DNA, the 5′ hammerhead ribozyme sequence was fused to the 5′ UTR by PCR using the primers 5′-tatactagtGGGCTGGCCTGATGAGTCCGTGAGGACGAAACATGCATC TCCATGCATGTCGCCAGCCCCCGATTGGGGGC-3′ and 5′-TTTCTCCGC ACCCGACATAG-3′, after digestion with SpeI and EcoRI, the fragment was cloned into NheI/EcoRI-digested pShuttle generating pCMVHHcRLmpA. The CD gene was amplified by PCR using primers 5′-gtgtctagaAGGCTAACAAT GTCGAATAA−3′ and 5′-atatctagaAGACAGCCGCTGCGAAGGCA−3′, digested with XbaI, and ligated with the XbaI-digested pmCMVcRLmpA to generate pmCMVcCDmpA. The sequences of these constructs were confirmed by nucleotide sequencing.

Adenovirus. The expression cassette in pmCMVcCDmpA, which is flanked by I-CeuI and PI-SceI sites, was digested with these two restriction enzyme, and ligated to the E1- and E3-deleted Adeno-X viral DNA (I-CeuI and PI-SceI digested) (Adeno-X Expression System, Clontech). The resultant adenoviral DNA (AdmCMVcCDmpA) was digested with PacI and then transfected into low-passage 293 cells. Seven days following transfection, crude virus was prepared from the transfected cells by three cycles of freeze-thawing, and further amplified in 293 cells by several rounds of infection. The purified virus was aliquoted and stored at −80°C before use. The authenticity of recombinant adenoviral DNA was verified before preparing high-titer viral stocks. AdsiNS5B was constructed as described previously (42), the sense-strand sequence of short hairpin RNA is 5′-GAAGGTCACCTTTGACAGA-3′.

In vitro transcription. Plasmids were linearized at SalI site located immediately downstream of the HDV ribozyme, and these fragments were used as templates for runoff RNA synthesis with T7 RNA polymerase according to the protocol supplied by the manufacturer (Roche). For capped-RNA synthesis, T7 Cap-Scribe (Roche) was used. After transcription, 10 units of RQ DNaseI (Promega) were added to the reaction mixture to digest DNA templates. The mixture was extracted with phenol-chloroform and RNA was precipitated with ethanol-7.5 M ammonium acetate.

Transfection. Cells were seeded onto 35-mm-diameter tissue culture dishes 24 h before transfection. One microgram of each reporter vector, 1 μg of pAM8-1 (when expression from the T7 promoter was desired), and 0.1 μg of pGL3-Control vector were cotransfected into cells with TransFast Transfection Reagent (Promega). For RNA transfection, 1 μg of each reporter RNA and 0.5 μg of capped firefly luciferase RNA were cotransfected into cells with Lipofectin reagent (Invitrogen). The cells were harvested after 48 h, 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 were 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).

Strand-specific RT-PCR. RNAs were isolated from transfected cells with Trizol reagent (Invitrogen) and treated with RNase-free DNase (Promega). The DNA-free RNA was extracted with phenol-chloroform and precipitated with ethanol. The absence of DNA in the RNA templates was confirmed by a control PCR without reverse transcriptase. For reverse transcription (RT), 1 μg of RNA was denatured at 65°C for 2 min, and cDNA synthesis was performed in 20-μl reaction volume with Superscript II reverse transcriptase (Invitrogen) at 42°C for 1 h using a primer complementary to *Renilla* luciferase gene, 5′-CTTATCTTG ATGCTCATAGC-3′. The resulting cDNA was amplified for 35 cycles using primer 5′-ATGACTTCGAAAGTTTATGA-3′ and the primer used in the RT reaction.

Northern blot analysis. Total RNAs were isolated and purified as described above, separated by denaturing agarose gel electrophoresis, and analyzed by Northern blot using Digoxigenin-labeled sense and antisense *Renilla* luciferase sequence to detect plus- and minus-strand transcripts.

Real-time RT-PCR. One microgram of DNase-treated total RNA was reverse-transcribed as described for strand-specific RT-PCR using a primer complementary to NS5B 5′-ACGGAGCGGATGTGGTTGAC-3′. After an incubation at 95°C for 5 min, the resulting cDNA was quantified with SYBR Green according to the protocol supplied by the manufacturer (Takara). PCR was performed with a primer 5′-TGGTCTACGCCACAACATCC-3′ and the primer used in the RT reaction. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA level in each sample was simultaneously quantified to normalize the value of HCV replicon RNA.

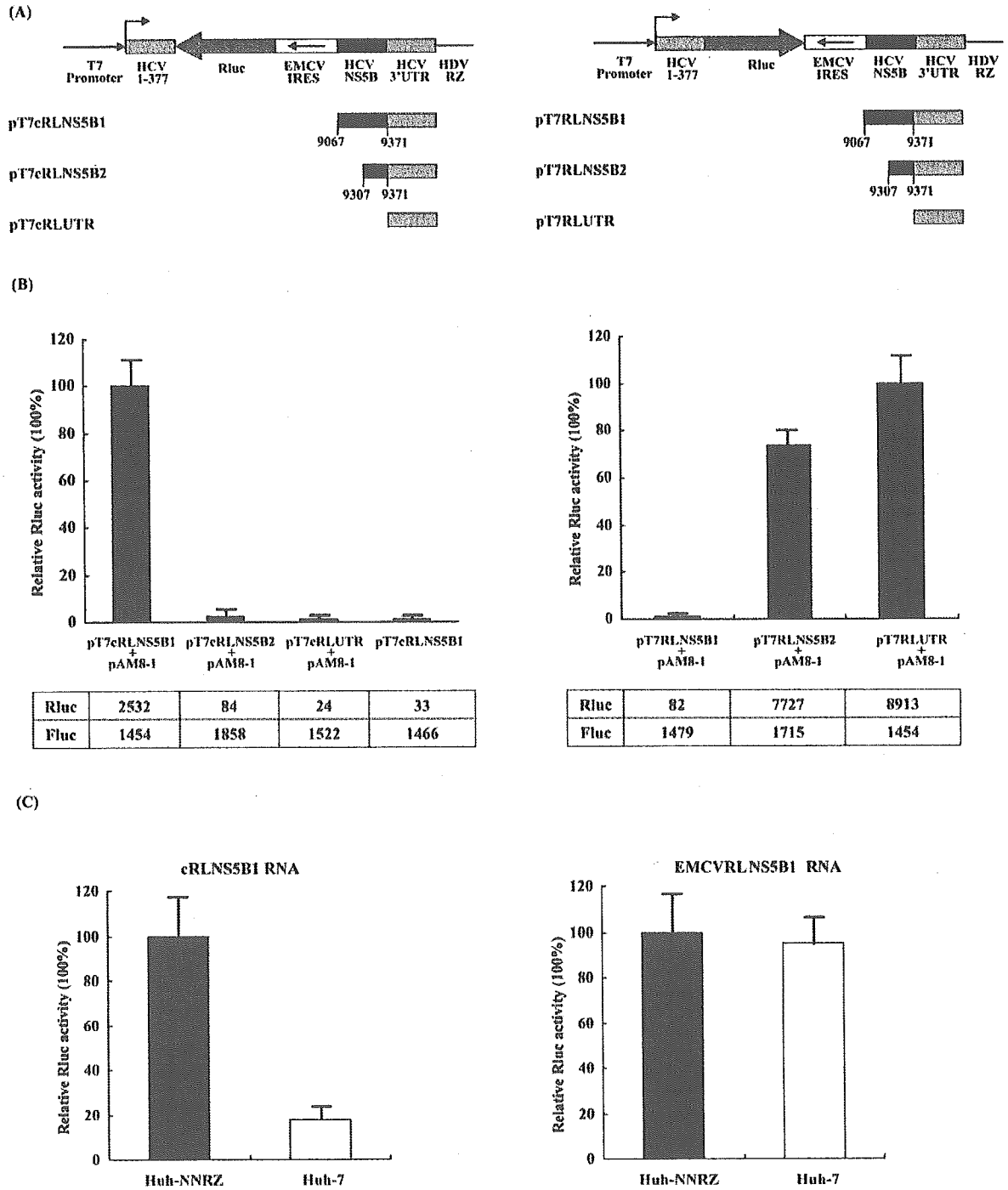
CD enzymatic assay. Huh-NNRZ or Huh-7 cells (1×10^6) were infected with AdmCMVcCDmpA at a multiplicity of infection (MOI) of 0, 10, 20, 50, 100, and 200. After 48 h, cells were harvested, washed once with phosphate-buffered saline, and resuspended in 0.5 ml 10 mM Tris-HCl. The cell suspension were sonicated, briefly centrifuged, and 100 μl supernatant was mixed with 170 μl PBS and 30 μl of 30 mM 5-FC solution. After incubation at 37°C for 24 h, 50 μl of reaction mixture was taken and quenched in 0.95 ml of 0.1 N HCl, the optical densities (ODs) were measured on a UV spectrophotometer at 255 and 290 nM. The amounts of 5-FC and 5-fluorouracil (5-FU) in the reaction mixture were calculated using the equations $(0.1191 \times OD_{290} - 0.02485 \times OD_{255}) \times 20$ = mM 5-FC and $(0.1849 \times OD_{255} - 0.04907 \times OD_{290}) \times 20$ = mM 5-FU; the conversion of 5-FC to 5-FU was then calculated as [mM 5-FC/(mM 5-FC + mM 5-FU)] × 100% (23).

Cytotoxic assay. Cells (Huh-NNRZ or Huh-7) were mock infected or infected with AdmCMVcCDmpA (MOI 80). Twenty-four hours later, the medium was changed with fresh DMEM containing 0 or 0.5 mM 5-FC. After an additional 4-day culture, cell viability was measured with cell proliferation reagent WST-1 (Roche) according to the manufacturer's instructions.

RESULTS

Determining *cis*-acting elements in the NS5B coding region essential for viral minus-strand RNA synthesis. Synthesis of HCV minus-strand RNA is initiated by recognition of the 3′ end of RNA template by RdRp, which requires a membrane-associated replication complex of viral and cellular proteins and a viral RNA template containing *cis*-active replication elements. It was known that conserved sequences and structures in the 5′ and 3′ untranslated region (UTR) in HCV RNA function as *cis*-acting elements essential for viral replication (12, 21, 38). However, the precise mechanism of the initiation of RNA synthesis is not fully understood. Although Oh et al. (31) previously showed that RdRp can utilize the 3′ UTR of HCV RNA as a minimal template in vitro, increasing evidence has supported that the 3′ UTR may not be a good template by itself for efficient RdRp binding and subsequent RNA synthesis, and the 3′ part of NS5B coding region with conserved stem-loop structure may also harbor functional *cis*-acting element required for viral replication (7, 39).

To further define the sequence of the NS5B coding region essential for HCV minus-strand RNA synthesis, we constructed HCV minigenome reporter vectors by inserting the antisense sequence of the *Renilla* luciferase gene and EMCV IRES between the 5′ end (nucleotides 1 to 377) and differently truncated NS5B coding region-connected 3′ UTR or 3′ UTR alone (Fig. 1A). In view of the consideration that functional



Rluc	2532	84	24	33
Fluc	1454	1858	1522	1466

Rluc	82	7727	8913
Fluc	1479	1715	1454

FIG. 1. Determination of *cis*-acting replication elements in the NS5B coding region. (A) Schematic display of T7-based HCV minigenome reporter constructs. HCV minigenome containing the antisense (left) or sense (right) sequence of the *Renilla* luciferase gene and the antisense sequence of the EMCV IRES flanked by the 5′ end (1 to 377) and differently truncated NS5B coding region-connected 3′ UTR or 3′ UTR alone was juxtaposed precisely at the T7 transcription start site and followed by the HDV ribozyme gene. (B) The indicated reporter vectors were transfected into Huh-NNRZ cells with or without pAM8-1 expressing T7 RNA polymerase. Relative *Renilla* luciferase activities in the lysates were determined at 48 h posttransfection. The columns and bars represent the means and standard deviations of three independent triplicate transfections. (C) Huh-NNRZ cells were transfected with in vitro transcribed cRLNS5B1 RNA (left) or EMCVRLNS5B1 RNA (right) together with capped firefly luciferase RNA as an internal control. Relative *Renilla* luciferase activities in the lysates were determined at 24 h posttransfection. Absolute values of *Renilla* and firefly luciferase activity are listed below the corresponding bars.

HCV minigenome may require authentic 5′ and 3′ ends without overhang, the cassette was juxtaposed precisely at the T7 transcription start site and was followed by self-cleaving HDV ribozyme (36). Huh-NNRZ cells, a Huh-7-derived cell line stably replicating the HCV subgenomic replicon (22, 25, 42), were transfected with each reporter vector, pAM8-1 plasmid expressing T7 RNA polymerase (40) and pGL3-Control vector. The cell lysates were collected and *Renilla* luciferase activities were measured 48 h after transfection. The firefly luciferase (Fluc) activity from cotransfected pGL3-Control vector was simultaneously measured to normalize the transfection efficiency. As shown in Fig. 1B, the expression of *Renilla* luciferase, which directly reflects the level of minus-strand RNA synthesized, was only detected in cells transfected with pT7cRLNS5B1, which contains the NS5B coding region from nucleotides 9067 to 9371 upstream of 3′ UTR. Neither of the cells transfected with pT7cRLNS5B2 and pT7cRLUTR, which respectively contain the NS5B coding region from nucleotides 9307 to 9371 plus 3′-UTR and 3′-UTR alone, expressed *Renilla* luciferase. This indicates that the 3′ part of NS5B coding region from nucleotides 9067 to 9306 contains one or more *cis*-acting elements, which are absolutely required for HCV minus-strand RNA synthesis. Importantly, the *Renilla* luciferase activity in Huh-NNRZ cells transfected with pT7cRLNS5B1 alone (not cotransfection with pAM8-1) was almost negligible, providing a evidence that minus-strand RNA detected here does not result from cryptic transcription but from a HCV-dependent trans replication using minigenome transcript as a template (see below). On the basis of this finding, pT7cRLNS5B1 was used for subsequent experiments. For comparison, reporter assay with pT7RLNS5B1, pT7RLNS5B2 and pT7RLUTR, which different from their counterpart in that the *Renilla* luciferase gene was inserted in the opposite direction (Fig. 1A right), was also conducted. Interestingly, inclusion of the same NS5B coding sequence (9067 ~ 9306) dramatically inhibited the HCV IRES-directed *Renilla* luciferase expression in pT7RLNS5B1, suggesting that the 3′-partial NS5B coding region may contain bifunctional element(s) participating in both repression of HCV IRES-dependent translation and initiation of minus-strand RNA synthesis to coordinate these two processes.

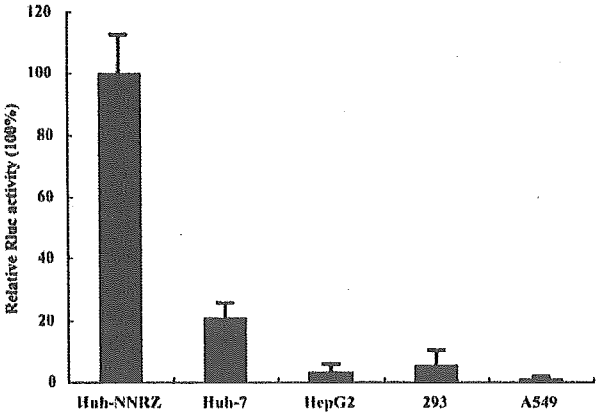
To further rule out the possibility that the minus-strand RNAs which function as mRNAs for *Renilla* luciferase expression were transcription products by a cryptic promoter element in the 3′ part of NS5B coding region, we monitored the *Renilla* luciferase activity after transfection with RNAs in vitro transcribed from pT7cRLNS5B1. As a control, transcripts in vitro transcribed from pT7EMCVRLNS5B1, which is identical to pT7cRLNS5B1 except for the sense (not antisense) sequence of EMCV IRES and *Renilla* luciferase gene inserted between 5′- and 3′-end sequences of HCV, was also included. Capped firefly luciferase RNA was cotransfected as an internal control to normalize the transfection efficiency. As shown in Fig. 1C, *Renilla* luciferase activity was also detected in Huh-NNRZ cells transfected with cRLNS5B1 RNA, which was significantly higher than that in transfected Huh-7 cells. In contrast, after transfection with EMCVRLNS5B1 RNA, both Huh-NNRZ and Huh-7 cells expressed comparable *Renilla* luciferase, indicating that the higher level of *Renilla* luciferase expression in Huh-NNRZ cells was not due to the differences in mRNA stability and/or translation between Huh-NNRZ and Huh-7 cells. In view of this result together with the observation that the *Renilla* luciferase activity in pT7cRLNS5B1-transfected Huh-NNRZ cells was almost negligible in the absence of T7 RNA polymerase (Fig. 1B), we conclude that the minus-strand RNA was synthesized by HCV-dependent trans replication rather than transcription by a cryptic promoter element in pT7cRLNS5B1.

Specific gene expression in HCV replicon cell line. Our targeting strategy in this study is devised by inserting the antisense sequence of transgene and EMCV IRES between 5′- and 3′-end of HCV cDNA, and thus in principle, the foreign gene should be specifically expressed in HCV-infected cells containing functional replication apparatus. To verify this, the reporter vector pT7cRLNS5B1 and pAM8-1 were transfected into Huh-NNRZ, Huh-7, HepG2, 293 and A549 cells, and *Renilla* luciferase activities in the lysates were determined as described above. Figure 2A shows the only cell line with significant *Renilla* luciferase activity was Huh-NNRZ. Other cell lines, including both hepatic- and nonhepatic cells, expressed only low or undetectable levels of *Renilla* luciferase.

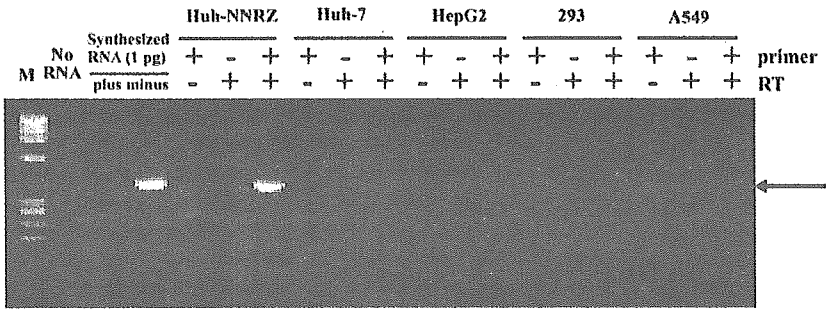
To further confirm that the transgene expression in pT7cRLNS5B1 is HCV-responsive, we performed strand-specific RT-PCR for minus-strand sequence using the RNA extracted from the transfectants described above. Three different controls (no RNA, no reverse transcriptase, and no primer in the reverse transcription) were included as specificity control and were negative. No minus-strand RNA was detected in the RT-PCR using 1 pg of in vitro transcribed plus-strand control RNA, confirming the strand specificity of the RT-PCR assay (Fig. 2B lane 3). Fully consistent with the results from reporter assay, minus-strand RNA was only detected in Huh-NNRZ cells cotransfected with pT7cRLNS5B1 and pAM8-1, while other transfected cells were negative. Simultaneously, the extracted RNA was subjected to Northern blot analysis using digoxigenin-labeled sense and antisense *Renilla* luciferase probes to detect plus- and minus-strand transcripts. Similar to the result from strand-specific RT-PCR, minus-strand RNA transcripts of the expected size (2.46 kb in length) were specifically detected in Huh-NNRZ cells transfected with pT7cRLNS5B1 and pAM8-1, although the plus-strand transcripts detected were comparable among different cell lines

FIG. 2. Specific gene expression in HCV replicon cell line. (A) The indicated cell lines were cotransfected with the pT7cRLNS5B1 and pAM8-1 vectors. Relative *Renilla* luciferase activities in the lysates were determined as described for Fig. 1. The columns and bars represent the means and standard deviations of two separate triplicate experiments. (B) Total RNAs were prepared from each transfectant. Strand-specific RT-PCR for minus-strand RNA was performed using 1 μg of extracted RNA. The arrow indicates the expected 408-bp PCR products. (C) Northern blot was performed on 5 μg of extracted RNA using digoxigenin-labeled sense and antisense *Renilla* luciferase RNA probes to detect plus- (upper panel) and minus-strand (middle panel) transcripts. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) served as a loading control. RNA size markers are shown on the left, and the bands corresponding to plus- and minus-strand RNA are indicated on the right.

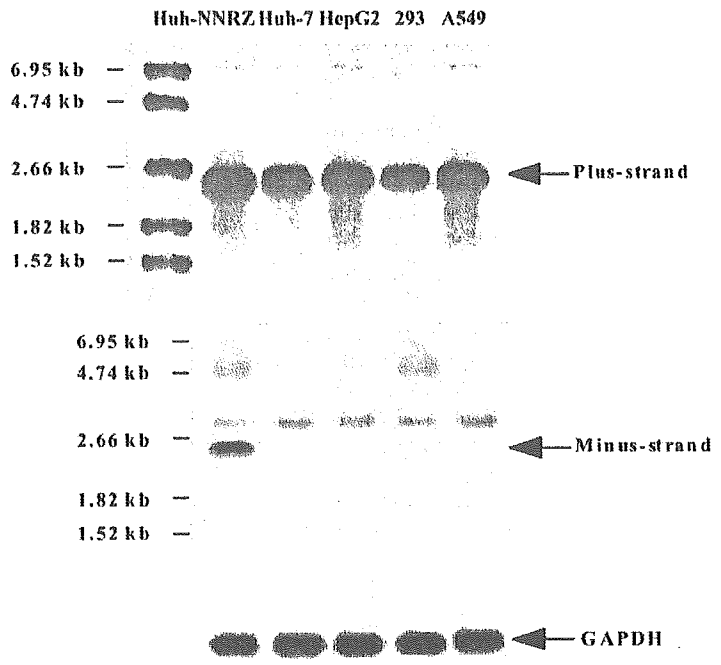
(A)


120
100
80
60
40
20
0
Relative Rluc activity (100%)
Huh-NNRZ
Huh-7
HepG2
293
A549

(B)


Huh-NNRZ
Huh-7
HepG2
293
A549
Synthesized RNA (1 pg)
No RNA
M
plus minus
primer
RT

(C)


Huh-NNRZ Huh-7 HepG2 293 A549
6.95 kb
4.74 kb
2.66 kb
1.82 kb
1.52 kb
Plus-strand
6.95 kb
4.74 kb
2.66 kb
1.82 kb
1.52 kb
Minus-strand
GAPDH

(Fig. 2C). Taken together, these experiments demonstrate that our approach can selectively direct gene expression in HCV replicon cells containing functional replication components.

Inhibition of transgene expression by short interfering RNA directed against HCV NS5B. To determine if the transgene expression in pT7cRLNS5B1 was dependent on HCV replicase in the replicon cell, Huh-NNRZ cells were infected with AdsiNS5B at MOIs of 80, 30, and 10 and then cotransfected with pT7cRLNS5B1 and pAM8-1. The cell lysates were assayed for *Renilla* luciferase expression and total RNAs were subjected to real-time RT-PCR for quantification of HCV replicon RNA levels. As shown in Fig. 3, transduction with AdsiNS5B resulted in a substantial and dose-dependent reduction in the replicon RNA level, whereas the HCV RNA level in cells transduced with irrelevant AdsiGFP, expressing short interfering RNA against green fluorescent protein, even at an MOI of 80, was comparable to that in mock-infected cells. More importantly, *Renilla* luciferase expression in AdsiNS5B-transduced Huh-NNRZ cells was also dose-dependently inhibited, fully parallel to the HCV RNA levels quantified. Likewise, in an experiment investigating the effect of a nonnucleoside inhibitor of the HCV NS5B polymerase on transgene expression, it was found that *Renilla* luciferase activity and minus-strand RNA production in replicon cells were dose-dependently reduced after incubation with the NS5B inhibitor (data not shown). These observations provide further support that the transgene expression is strictly dependent on the presence of HCV replicase.

HCV-dependent gene expression from polymerase II-derived transcripts. Application of our targeting strategy to gene therapy would require expression from polymerase II RNA polymerase rather than T7-directed transcription. It has been reported for other positive-strand RNA viruses that full-length transcripts produced in the nucleus by polymerase II are delivered in a functional form into the cytoplasm (1, 20). Artificially synthesized RNA transcripts from the cDNA of positive-strand RNA viruses require that both 5′ and 3′ ends accurately reflect those found in the viral genome in order to maximize infectivity (14).

To ensure that the polymerase II-derived transcripts contain a minimal or no overhang at the 5′ end, we adapted two distinct approaches: one by positioning the viral cDNA immediately adjacent to the transcription start site of the cytomegalovirus (CMV) promoter (Fig. 4A, pmCMVcRLmpA and pmCMVcRLHD), and another by inserting a *cis*-acting hammerhead ribozyme (17) that cleaves to produce authentic 5′ end (pCMVHHcRLmpA). Similarly, to minimize the overhang at the 3′ end of polymerase II-derived transcripts, we used a synthetic, minimal poly(A) signal (37) (pmCMVcRLmpA and pCMVHHcRLmpA) or inserted the self-cleaving HDV ribozyme upstream of the unmodified bovine growth hormone poly(A) signal (pmCMVcRLHD).

To ascertain whether the polymerase II-derived transcript could act as a functional template for minus-strand RNA synthesis, Huh-NNRZ, Huh-7 and HepG2 cells were transfected with these constructs. Compared with the transfected Huh-7 or HepG2 cells, *Renilla* luciferase activities in transfected Huh-NNRZ cells were consistently higher, the order being pmCMVcRLmpA > pmCMVcRLHD > pCMVHHcRLmpA (Fig. 4B). This indicates that the polymerase II-derived transcripts, especially that from pmCMVcRLmpA, were competent as templates for minus-strand RNA synthesis, suggesting that 5′ cap structure may not impair the template ability of the polymerase II-derived transcript to replicate. The lower *Renilla* luciferase activity in cells transfected with pmCMVcRLHD or pCMVHHRZcRLmpA compared with that in the pmCMVcRLmpA transfectant may be attributable to inefficient cleavage by the ribozyme.

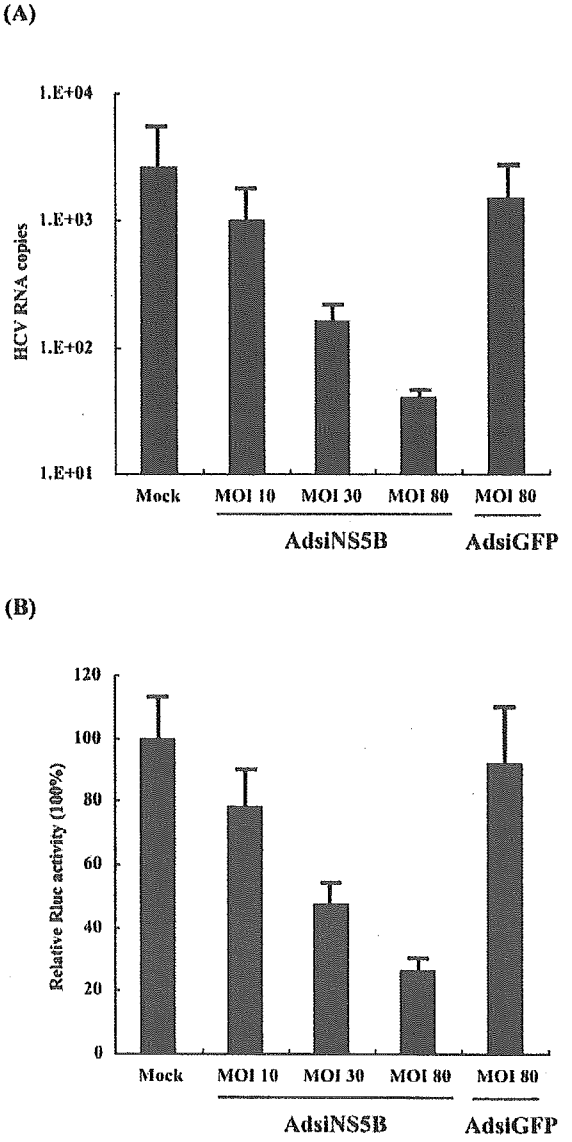


FIG. 3. Inhibition of transgene expression by silencing of HCV NS5B. Huh-NNRZ cells were infected with AdsiNS5B at an MOI of 80, 30, and 10 and then transfected with pT7cRLNS5B1 and pAM8-1. The transduced cells were harvested at day 2 posttransfection. HCV replicon RNAs were quantified with real-time RT-PCR (A), and relative *Renilla* luciferase activities were determined as described for Fig. 1 (B). The columns and bars represent the means and standard deviations of two independent experiments.

Chemosensitizing effect of CD expressed from adenovirus-delivered HCV-like minigenome. To further investigate the therapeutic feasibility of our targeting approach, we next sub-

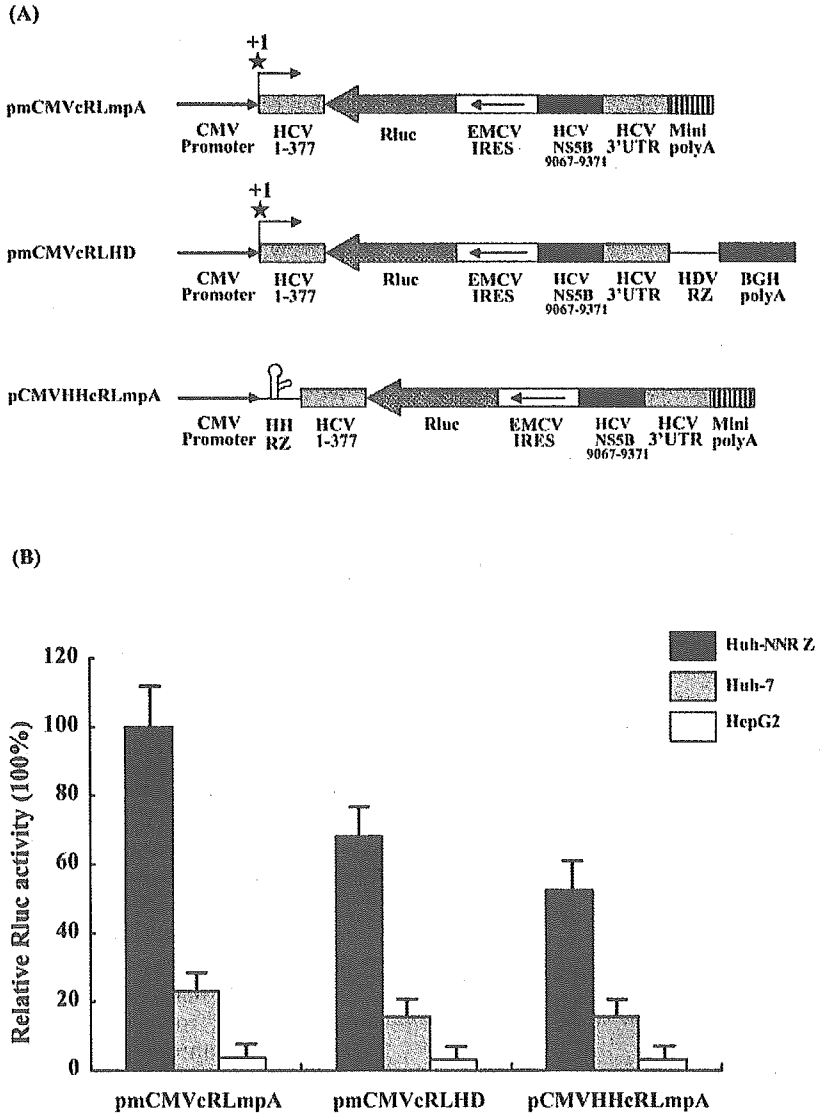


FIG. 4. Polymerase II-derived transcripts are functional templates for minus-strand RNA synthesis. (A) Schematic diagram of polymerase II-directed HCV minigenome reporter constructs. The HCV minigenome containing the antisense sequence of *Renilla* luciferase and the EMCV IRES flanked by the 5′ end (1 to 377) and 3′ partial NS5B coding region (9067 to 9371)-connected 3′ UTR was placed immediately next to the cytomegalovirus transcription start site or combined with a hammerhead ribozyme and was followed by a minimal poly(A) or HDV ribozyme upstream of the unmodified bovine growth hormone poly(A). (B) Huh-NNRZ, Huh-7, and HepG2 cells were transfected with the indicated reporter vectors. Relative *Renilla* luciferase activities in the lysates were determined as described for Fig. 1. The columns and bars represent the means and standard deviations of two separate triplicate transfections.

cloned the expression cassette from pmCMVcCDmpA, which contains the antisense sequence of the CD suicide gene instead of the *Renilla* luciferase in pmCMVcRLmpA, into recombinant adenovirus to generate AdmCMVcCDmpA. CD is a prokaryotic enzyme capable of converting the nontoxic prodrug 5-FC to the chemotherapeutic agent 5-fluorouracil (5-FU), which has been widely used in anticancer gene therapy (9, 16). Huh-NNRZ and Huh-7 cells were infected with AdmCMVcCDmpA at an MOI of 0, 10, 20, 50, 100, and 200. After 48 h, cells were harvested and CD enzymatic activity was determined by measuring the conversion of 5-FC to 5-FU. There was a dose-dependent increase in CD activity in Huh-NNRZ cells infected with AdmCMVcCDmpA, while infected Huh-7 cells showed only basal levels of CD activity, demonstrating an HCV-specific CD expression from AdmCMVcCDmpA (Fig. 5A).

To further verify the data from the enzymatic assay, we next investigated whether Huh-NNRZ cells are sensitized to the cytotoxic effect of 5-FC by transduction of AdmCMVcCDmpA. Huh-NNRZ and Huh-7 cells were infected with AdmCMVcCDmpA at an MOI of 80, followed by the addition of 5-FC (0.5 mM). Controls included AdmCMVcCDmpA alone, 5-FC alone, and no treatment. Four days later, the cytotoxic effects were evaluated with cell proliferation reagent WST-1.

Following a 4-day exposure to 5-FC, the viable cell count

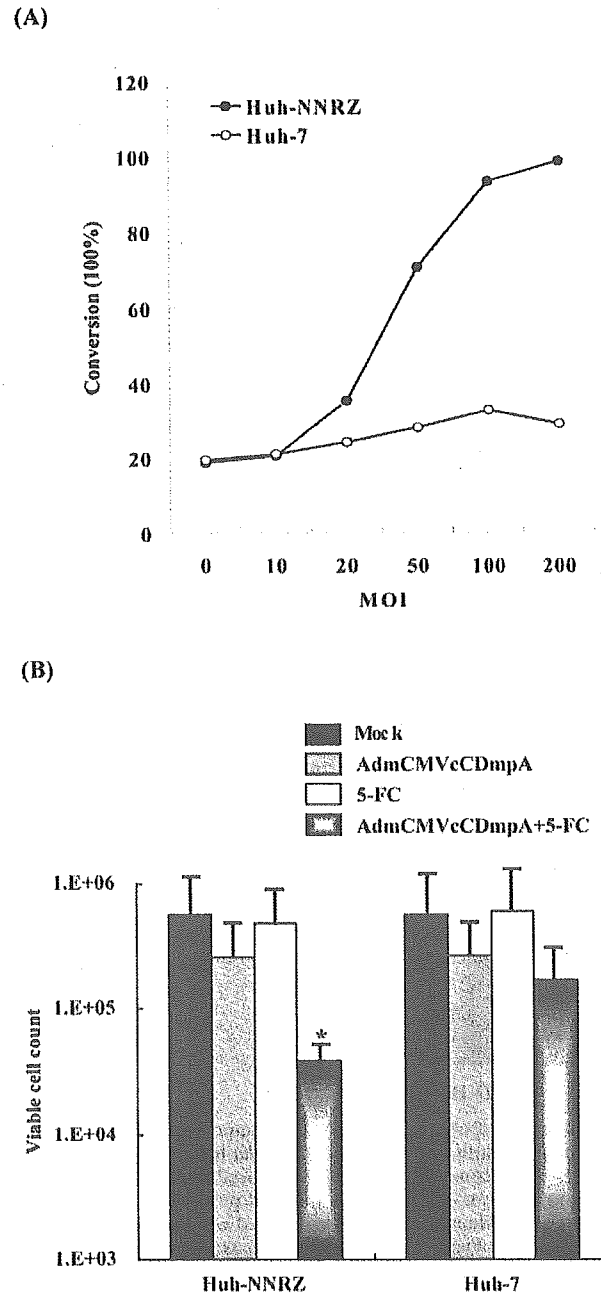


FIG. 5. Adenovirus-delivered HCV-responsive CD expression. (A) Huh-NNRZ and Huh-7 cells were infected with AdmCMVcCDmpA (MOI, 0 to 200). After 48 h, cells were harvested and CD enzymatic activity was determined by measuring the conversion of 5-FC to 5-FU. (B) Huh-NNRZ and Huh-7 cells were mock infected or infected with AdmCMVcCDmpA at an MOI of 80 and maintained in the absence or presence of 5-FC. Viable cells were quantified after 4 days. Representative data are from three separate experiments. *, $P < 0.05$ compared with the control.

of Huh-NNRZ cells transduced with AdmCMVcCDmpA was reduced by 14–fold (compared to the control, $P < 0.05$), whereas the viability of Huh-NNRZ cells infected with AdmCMVcCDmpA in the absence of 5-FC or treated with 5-FC alone was comparable to that of the control with no treatment (Fig. 5B). In contrast, transduction with AdmCMVcCDmpA did not significantly confer sensitivity to 5-FC on parental Huh-7 cells, indicating that the deaminating reaction converting 5-FC to 5-FU did not occur in uninfected Huh-7 cells due to lack of CD expression. These results further demonstrate that our strategy, by exploiting *cis*-acting replication elements, renders transgene (CD) expression responsive to the presence of HCV, thereby approaching the goal of selectively killing HCV-infected cells in combination with 5-FC while keeping uninfected cells unharmed.

DISCUSSION

We demonstrate here a proof-of-concept for a new targeting strategy to direct gene expression responsive to HCV by using a construct containing the antisense sequence of transgene and EMCV IRES flanked by the 5′- and 3′-end regions of viral cDNA. Thus, expression of the transgene is initiated only when the minus-strand RNA has been synthesized by the functional replication components present in HCV-infected cells. By using this strategy in combination with recombinant adenovirus delivery, our data showed that expression of the therapeutic gene (CD) depends strictly on the presence of functional HCV replication machinery in replicon cell, resulting in marked chemosensitization of replicon cells to the cytotoxic effects of 5-FC, while having little effect on noninfected cells. In addition to the CD suicide gene, other therapeutic genes such as apoptosis-inducing genes may also be employed in this strategy for eradication of HCV-infected cells. These experiments also show that viral replication components, including RdRp, can act in *trans* to synthesize minus-strand RNA using the transcript from the engineered construct as a template.

Using HCV minigenome reporter vectors with differently truncated NS5B coding sequences fused upstream of the 3′ UTR, we found that the 3′ part of NS5B coding region from nucleotides 9067 to 9371 is required for HCV minus-strand RNA synthesis, although it is not excluded that this *cis*-acting sequence could be further reduced by deletion mutagenesis. This result is in agreement with that reported by You et al. (39), who identified a stem-loop (5BSL3.2) at nucleotides 9262 to 9311 and found that both the structure and primary sequence of 5BSL3.2 are essential for HCV RNA replication. Although expression of the transgene from our engineered construct was dependent on the presence of functional HCV replication machinery in replicon cell, an over background level of *Renilla* luciferase activity was consistently observed in naïve Huh-7 cells, which was higher than in other hepatic and nonhepatic control cells (Fig. 2A). The reason for this "leaky" expression in naïve Huh-7 cells is currently unknown; one possible interpretation is antisense transcription mediated by the T7 polymerase. Further clarification of this HCV-independent expression in naïve Huh-7 cells is under way to determine whether it truly limits the possibility of using cytotoxic gene to selectively kill HCV-infected cells. If this is the case, other "mild" therapeutic genes such as interferons may be alternatively employed in this targeting strategy; directing HCV-responsive interferon expression should be advantageous to avoid toxic side effects.

While a low but detectable *Renilla* luciferase activity was observed, *Renilla* luciferase RNA was not detected in Huh-7

cells by Northern blot analysis (Fig. 2C). This discordance may be attributable to a lower sensitivity of the Northern blot compared to the luciferase assay. Similarly, because using RT-PCR for detection of minus-strand RNA is likely to create false positive due to self-priming or random priming of the wrong strand during the RT step, a compromise between sensitivity and strand specificity is required despite efforts to reduce such nonspecific cDNA synthesis. For this reason, to ensure high strand specificity, the sensitivity of the strand-specific RT-PCR procedure used here was limited, which may account for the failure to detect low-level *Renilla* luciferase coding transcripts in Huh-7 cells. Further experimentation is in progress to elucidate whether the discrepancy in the results of the reporter assay and Northern blot or strand-specific RT-PCR is simply due to the variance in the sensitivity of different assays.

Consistent with those reported previously (27), our study demonstrated that polymerase II-derived transcripts are competent as templates for minus-strand RNA synthesis by the HCV replication complex. Compared with the T7 expression system, polymerase II expressed lower levels of the *Renilla* luciferase reporter gene, probably due to the lower levels of polymerase II-derived transcript present in transfected cells. Additionally, while rapid amplification of cDNA end analysis of the transcript from pmCMVcRLmpA, which was constructed by trial and error to position the 5′ UTR 4 nucleotides downstream from the putative start site, confirmed that the transcription start site coincided with the first nucleotide of the 5′ UTR, it was shown that this RNA transcript terminated 14 nucleotides downstream of the AAUAA poly(A) signal. Thus, the transcript from pmCMVcRLmpA still contained an overhang of 32 nucleotides at the 3′ end, although it was much shortened by using a minimal poly(A) signal. Further efforts to minimize the overhang at the 3′ end of the polymerase II-derived transcript may be necessary to improve its template activity for minus-strand synthesis.

In an experiment to investigate the duration of reporter gene (*Renilla* luciferase) expression using pT7cRLNS5B1 (data not shown), we found that *Renilla* luciferase activity decreased considerably at day 4 and was insignificant at day 6 posttransfection, suggesting that the newly synthesized minus-strand RNA may be incompetent as a template for subsequent plus-strand production. The reason for this is currently unknown, but one possibility is that plus-strand RNA synthesis may require another *cis*-acting element(s) in addition to those included in our construct. Studies are under way to define this, as well as to identify the minimal *trans*-acting viral components essential for HCV replication, which will shed light on the development of HCV element-based gene delivery system.

Synthetic minigenomes have been described in a number of minus-stranded RNA viruses of several different families (28, 32) and plus-stranded RNA viruses belonging to the *Coronaviridae* family (19). Coupled with infectious helper viruses or plasmid-encoded proteins, minigenomes have contributed greatly to the analysis of *cis*-acting sequences and *trans*-acting proteins required for viral replication, maturation, and packaging. In addition, coronavirus-derived minigenomes have been used to express transgene in tissue culture and in animals (2). Different from the coronavirus minigenomes with the transgene in the sense orientation, which directs tissue-specific transgene expression by virtue of viral tropism, the HCV minigenome described here contains the transgene and IRES element in the antisense orientation, and the minus-strand RNA synthesized by the replication machinery in HCV-infected cells functions as the mRNA for transgene expression, rendering transgene expression responsive to the presence of HCV. However, when the antisense sequence of the reporter gene was inserted in the minigenome derived from transmissible gastroenteritis virus, the transgene was not expressed (19). Whether the discrepancy between the finding in transmissible gastroenteritis virus and that presented here is simply due to the lack of the IRES element for translation initiation in the former minigenome or due to the intrinsic difference between transmissible gastroenteritis virus and HCV remains to be investigated.

Because the HCV genome RNA and the therapeutic minigenome share the same replication machinery in infected cells, it is possible that replication of the HCV genome may be competitively inhibited when the replication machinery is being actively recruited by the therapeutic HCV minigenome. If this is the case, an additive antiviral effect may be expected. Additionally, given that the HCV-responsive expression construct described here contains the 5′ (nucleotides 1 to 377) region of the HCV genome, which was reported to interact with HCV nucleocapsid (core) protein and play an important role in viral encapsidation (11, 33, 35, 41), it is tempting to speculate that the HCV minigenome may also be packaged inside the virion and budded from infected cells and, if so, it could direct secondary transgene expression by sequential infection, ultimately augmenting its anti-HCV effect.

In summary, we have developed a novel targeting therapeutic approach directed against cells containing the HCV replication apparatus, which may be useful as a prophylactic in the early stage of hepatitis, during limited infection of the liver, or for ex vivo therapy of hepatocytes. It may also reduce virus loads in chronically infected patients and, in combination with interferon and ribavirin therapy, might eradicate HCV from the infected host. In addition to its therapeutic application, the HCV minigenome reported in this study also offers a useful tool for studies aimed at investigation of the molecular mechanisms involved in replication and expression of the HCV genome.

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Role of Cyclophilin B in Activation of Interferon Regulatory Factor-3*

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IRF-3 is a member of the interferon regulatory factors (IRFs) and plays a principal role in the induction of interferon-β (IFN-β) by virus infection. Virus infection results in the phosphorylation of IRF-3 by IκB kinase ε and TANK-binding kinase 1, leading to its dimerization and association with the coactivators CREB-binding protein/p300. The IRF-3 holocomplex translocates to the nucleus, where it induces IFN-β. In the present study, we examined the molecular mechanism of IRF-3 activation. Using bacterial two-hybrid screening, we isolated molecules that interact with IRF-3. One of these was cyclophilin B, a member of the immunophilins with a *cis-trans* peptidyl-prolyl isomerase activity. A GST pull-down assay suggested that one of the autoinhibition domains of IRF-3 and the peptidyl-prolyl isomerase domain of cyclophilin B are required for the binding. A knockdown of cyclophilin B expression by RNA interference resulted in the suppression of virus-induced IRF-3 phosphorylation, leading to the inhibition of the subsequent dimerization, association with CREB-binding protein, binding to the target DNA element, and induction of IFN-β. These findings indicate that cyclophilin B plays a critical role in IRF-3 activation.

Interferon regulatory factors (IRF(s))[1] are a family of transcription factors that regulate a variety of biological events, including innate immunity. Once activated by the invasion of a pathogen, such as viruses and bacteria, IRFs regulate the expression of various genes encoding immunomodulatory cytokines and chemokines and limit the spread of infection. Among these factors, interferons (IFNs) play important roles in host defense, cell growth regulation, and immune activation (1, 2). IFNs include the type I IFN-αs and IFN-β and the type II IFN-γ. Type I IFNs are immediately induced in response to various viral infections, and IRF-3 and IRF-7 play an important role in their induction (3, 4). The mode of IRF-3 involvement against virus infection has been analyzed by using Newcastle disease virus (NDV) and Sendai virus (5–9). IRF-3 is expressed in the cytoplasm as a latent, inactive form, and its C-terminal serine/threonine residues are phosphorylated by IκB kinase ε and TANK-binding kinase 1 (10, 11). Virus-induced C-terminal phosphorylation of IRF-3 represents an important posttranslational modification, leading to dimerization (6, 7), translocation from the cytoplasm to the nucleus, association with CBP/p300 coactivators (6, 9), stimulation of DNA binding to the IFN-stimulated response elements (ISREs), and activation of the corresponding genes (5, 8, 9).

IRF-3 consists of an N-terminal DNA-binding domain that specifically binds to an ISRE motif, and a C-terminal IRF association domain (IAD) that mediates protein-protein interactions. IRF-3 uses the IAD for both intramolecular autoinhibition interactions and intermolecular dimerizations (6, 12). Furthermore, IRF-3 possesses a transactivation domain (amino acids 134–394) and two autoinhibition domains found within the proline-rich sequence (amino acids 134–197) and at the C-terminal end (amino acids 407–414). The two autoinhibition domains are thought to interact with each other to generate a closed conformation that masks the C-terminal IAD, the DNA-binding domain, and the nuclear localization signal of IRF-3, which prevents homodimerization and DNA binding in uninfected cells. The C-terminal phosphorylation of IRF-3 might open the conformation, leading to dimer formation and exposure of the nuclear localization signal and the DNA-binding domain (6, 13, 14). However, the molecular events associated with such a drastic conformational change remain unknown. In the present study, we demonstrate the interaction of IRF-3 with cyclophilin B (CypB), an immunophilin with *cis-trans* peptidyl-prolyl isomerase and chaperone-like activities (15). The knockdown of CypB by RNA interference prevented the NDV-induced IRF-3 phosphorylation, dimerization, association with CBP, binding to the ISRE, and induction of IFN-β.

EXPERIMENTAL PROCEDURES

Bacterial Two-hybrid Screening—A BacterioMatch™ two-hybrid SystemXR Plasmid cDNA library (Stratagene, La Jolla, CA) was used to screen IRF-3 interacting proteins according to the manufacturer's protocol. For the construction of the bait plasmid, the GST fusion expression plasmid pGEX-IRF-3 was digested at the NcoI site, which overlapped with the initiation codon of IRF-3, filled in with the Klenow fragment of DNA polymerase I, and then digested by XhoI. The plasmid pBT was digested by BamHI, filled in with the Klenow fragment of DNA polymerase I, and then digested by XhoI. The NcoI/Klenow-XhoI fragment containing the IRF-3 coding region was ligated with the pBT fragment. The junction of the cloning site of the resultant plasmid pBT-IRF-3 was verified by sequencing. Competent cells of the BacterioMatch two-hybrid system *Escherichia coli* reporter strain were transformed with pBT-IRF-3, together with the pTRG-cDNA library (Human HeLa cell plasmid cDNA library, number 982208, Stratagene). As every

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[1] The abbreviations used are: IRF, interferon regulatory factor; IFN, interferon; CBP, cAMP-response element-binding protein-binding protein; ISRE, interferon-stimulated response element; NDV, Newcastle disease virus; ISG, interferon-stimulated gene; GST, glutathione *S*-transferase; CypB, cyclophilin B; PPIase, peptidyl-prolyl isomerase; siRNA, small interfering RNA.