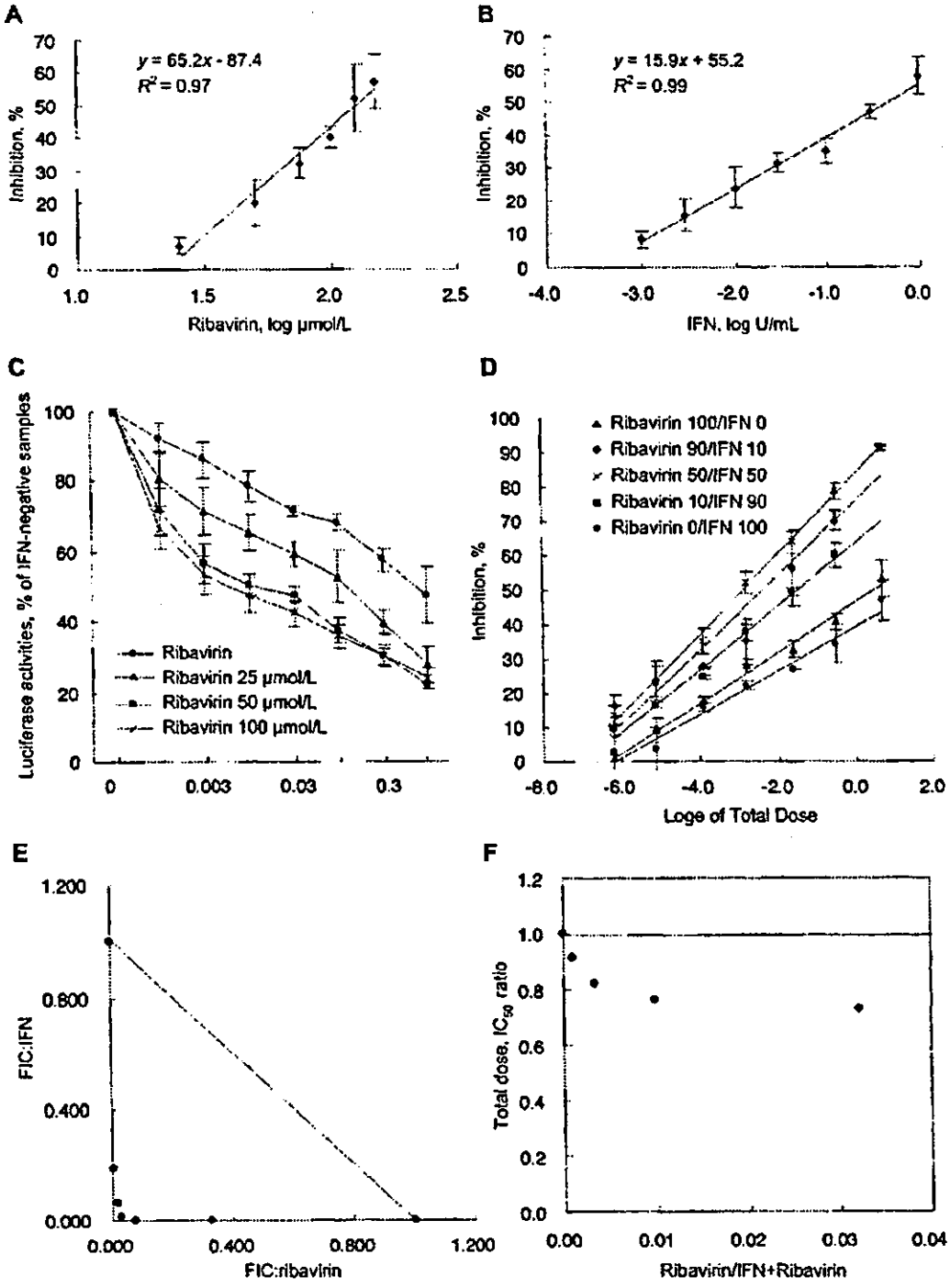

Figure 6. Effect of combination of interferon (IFN) and ribavirin on intracellular hepatitis C virus (HCV) replication. Huh7/Rep-Feo cells were cultured with various concentrations of ribavirin and IFN. Luciferase activities of the cell lysates were measured after 2 days of exposure. Error bars indicate mean ± 2 SD. *A*, Relative log dose-response curves of ribavirin. *B*, Relative log dose-response curves of IFN. *C*, Dose-inhibition curves of IFN combined with the concentrations of ribavirin indicated. The luciferase activities are displayed as percentages of the IFN-negative samples. *D*, Dose-effect curves of IFN and ribavirin combined in proportions indicated, adjusted by the IC_{50} of each drug. *E*, Graphic representation of the isobologram analysis. The fixed ratios adjusted by the IC_{50} (FICs) at 50% inhibition were calculated from the plots in panel *D*. FICs for IFN and ribavirin were plotted on the *X*-axis and *Y*-axis, respectively. A theoretical line of additivity is drawn between the FIC ratio of 1 for each drug that indicates additive effects. All of the FIC plots for the ribavirin-IFN combinations of 1:0, 100:1, 10:1, 1:1, 1:10, 1:100, and 0:1 fell below the line of unity, indicating strong synergy. *F*, The synergy of IFN and ribavirin within clinically relevant ranges of concentration were studied. The dose-inhibition analyses were conducted at 5 different IFN-ribavirin combinations of 1:0, 1000:1, 300:1, 100:1, and 30:1, and the IC_{50} values were calculated for each combination. For better graphic representation, the combination index (CI; see Materials and Methods for definition) was plotted against the proportions of ribavirin in the total combination. The horizontal bar at a level of 1.0 indicates additivity (CI, 1).

U/mL; figure 6*B*). The detailed mechanism by which IFN so effectively suppresses HCV replication is unknown, but a variety of IFN-stimulated genes (ISGs) could be involved—protein kinase PKR, which inhibits viral protein translation; 2′5′-oligoadenyl synthetase, which activates RNase L to degrade viral RNA; and other as-yet-uncharacterized genes [35]. In contrast, HCV proteins, E2 and NS5A in particular, have been shown to counteract the effects of IFN through interaction with the ISGs or with IFN signal transduction, which may contribute to establishment of continuous intracellular replication against IFN treatment, not only clinically but also for the replicon [36–38]. Thus, even slight increases in substitutions in these viral proteins induced by a suboptimal dose of ribavirin could drastically attenuate viral fitness to IFN and further enhance susceptibility of the HCV replicon to IFN, leading to the synergistic effect observed clinically, as well as experimentally, in the present study. Alternatively, ribavirin might directly enhance the expression or activity of the ISGs. In any case, the strong synergistic effects of IFN and ribavirin may explain the virological basis of improved clinical antiviral effects of combination therapy over monotherapy with IFN. However, even with combination therapy, approximately one-half of the patients treated were unable to eradicate HCV. Elucidation of the mechanism of the synergistic effect of ribavirin and IFN is needed urgently to improve the prognosis for HCV-infected patients.

Given the absence of singly effective, proven antiviral agents against HCV, other than IFN, combinations of IFN with agents that possess potential antiviral effects will continue to dominate therapy. The present study has shown strong synergistic effects of IFN and ribavirin on intracellular HCV replication and has demonstrated that the synergistic effects are attributable to direct and specific inhibition of viral replication. These results suggest that the antiviral effects of treatment with IFN may be improved by combination with other, ribavirin-derived nucleoside analogues. Continuation of the search for more-potent and less-toxic antiviral drugs based on ribavirin is mandatory for improving clinical anti-HCV chemotherapeutics.

References

1. Alter MJ. Epidemiology of hepatitis C. Hepatology 1997; 26:62S–5S.
2. Seeff LB, Buskell-Bales Z, Wright EC. Long-term mortality after transfusion-associated non-A, non-B hepatitis. N Engl J Med 1992; 327: 1906–11.
3. Tong MJ, El-Farra NS, Reikes AR, Co RL. Clinical outcomes after transfusion-associated hepatitis C. N Engl J Med 1995; 332:1463–6.
4. Hoofnagle JH, Di Bisceglie AM. The treatment of chronic viral hepatitis. N Engl J Med 1997; 336:347–56.
5. Poynard T, Bedossa P, Chevallier M. A comparison of three interferon alfa-2b regiments for the long-term treatment of chronic non-A, non-B hepatitis. N Engl J Med 1995; 332:1457–62.
6. Carithers RL, Emerson SS. Therapy of hepatitis C: meta-analysis of interferon alfa-2b trials. Hepatology 1997; 26:83S–8.
7. Davis GL, Esteban-Mur R, Rustgi V, et al. Interferon alfa-2b alone or in combination with ribavirin for the treatment of relapse of chronic hepatitis C. International Hepatitis Interventional Therapy Group. N Engl J Med 1998; 339:1493–9.
8. McHutchison JG, Gordon SC, Schiff ER, et al. Interferon alfa-2b alone or in combination with ribavirin as initial treatment for chronic hepatitis C. Hepatitis Interventional Therapy Group. N Engl J Med 1998; 339:1485–92.
9. Di Bisceglie AM, Conjeevaram HS, Fried MW, et al. Ribavirin as therapy for chronic hepatitis C: a randomized, double-blind, placebo-controlled trial. Ann Intern Med 1995; 123:897–903.
10. Patterson JL, Fernandez-Larsson R. Molecular mechanisms of action of ribavirin. Rev Infect Dis 1990; 12:1139–46.
11. Hultgren C, Milich DR, Weiland O, Sallberg M. The antiviral compound ribavirin modulates the T helper (Th) 1/Th2 subset balance in hepatitis B and C virus-specific immune responses. J Gen Virol 1998; 79: 2381–91.
12. Crotty S, Maag D, Arnold JJ, et al. The broad-spectrum antiviral ribonucleoside ribavirin is an RNA virus mutagen. Nat Med 2000; 6:1375–9.
13. Lohmann V, Koerner F, Koch J, Herian U, Theilmann L, Bartenschlager R. Replication of subgenomic hepatitis C virus RNAs in a hepatoma cell line. Science 1999; 285:110–3.
14. Guo JT, Bichko VV, Seeger C. Effect of alpha interferon on the hepatitis C virus replicon. J Virol 2001; 75:8516–23.
15. Yokota T, Sakamoto N, Enomoto N, et al. Inhibition of intracellular hepatitis C virus replication by synthetic and vector-derived small interfering RNAs. EMBO Rep 2003; 4:602–8.
16. Kimball PM, Kerman RH, Kahan BD. Sensitivity of intracellular signals responsible for cell cycle progression to cyclosporine. Transplantation 1990; 49:186–91.
17. Colombani PM, Bright EC, Wells M, Hess AD. Drug-drug interaction between cyclosporine and agents affecting calcium-dependent lymphocyte proliferation. Transplant Proc 1989; 21:840–1.
18. Nagayama K, Kurosaki M, Enomoto N, et al. Time-related changes in full-length hepatitis C virus sequences and hepatitis activity. Virology 1999; 263:244–53.
19. Tanabe Y, Nagayama K, Enomoto N, et al. Characteristic sequence changes of hepatitis C virus genotype 2b associated with sustained biochemical response to IFN therapy. J Viral Hepatitis (in press).
20. Crotty S, Cameron CE, Andino R. RNA virus error catastrophe: direct molecular test by using ribarivin. Proc Natl Acad Sci USA 2001; 98: 6895–900.
21. Kurosaki M, Enomoto N, Marumo F, Sato C. Rapid sequence variation of the hypervariable region of hepatitis C virus during the course of chronic infection. Hepatology 1993; 18:1293–9.
22. Sakamoto N, Enomoto N, Kurosaki M, Marumo F, Sato C. Sequential change of the hypervariable region of the hepatitis C virus genome in acute infection. J Med Virol 1994; 42:103–8.
23. Peters M. Mechanisms of action of interferons. Semin Liver Dis 1989; 9: 235–9.
24. Blight KJ, Kolykhalov AA, Rice CM. Efficient initiation of HCV RNA replication in cell culture. Science 2000; 290:1972–4.
25. Reichard O, Andersson J, Schvarcz R, Weiland O. Ribavirin treatment for chronic hepatitis C. Lancet 1991; 337:1058–61.
26. Di Bisceglie AM, Shindo M, Fong TL, et al. A pilot study of ribavirin therapy for chronic hepatitis C. Hepatology 1992; 16:649–54.
27. Dusheiko G, Main J, Thomas H, et al. Ribavirin treatment for patients with chronic hepatitis C: results of a placebo-controlled study. J Hepatol 1996; 25:591–8.
28. Bodenheimer HC Jr, Lindsay KL, Davis GL, Lewis JH, Thung SN, Seeff LB. Tolerance and efficacy of oral ribavirin treatment of chronic hepatitis C: a multicenter trial. Hepatology 1997; 26:473–7.
29. Lanford RE, Guerra B, Lee H, et al. Antiviral effect and virus-host interactions in response to alpha interferon, gamma interferon, poly(I)-poly(C), tumor necrosis factor alpha, and ribavirin in hepatitis C virus subgenomic replicons. J Virol 2003; 77:1092–104.
30. Tallarida RJ, Stone DJ Jr, Raffa RB. Efficient designs for studying synergistic drug combinations. Life Sci 1997; 61:PL417–25.
31. Chung RT, He W, Saquib A, et al. Hepatitis C virus replication is

directly inhibited by IFN-α in a full-length binary expression system. Proc Natl Acad Sci USA 2001;98:9847–52.
32. Lanford RE, Sureau C, Jacob JR, White R, Fuerst TR. Demonstration of in vitro infection of chimpanzee hepatocytes with hepatitis C virus using strand-specific RT/PCR. Virology 1994;202:606–14.
33. Lanford R, Chavez D, Guerra B, et al. Ribavirin induces error-prone replication of GB virus B in primary tamarin hepatocytes. J Virol 2001; 75:8074–81.
34. Contreras AM, Hiasa Y, He W, Terella A, Schmidt EV, Chung RT. Viral RNA mutations are region specific and increased by ribavirin in a full-length hepatitis C virus replication system. J Virol 2002;76:8505–17.
35. Samuel C. Antiviral actions of interferons. Clin Microbiol Rev 2001; 14:778–809.
36. Sato C, Enomoto N. Mutations in the NS5A region of HCV and interferon efficacy [letter]. Gastroenterology 1998;115:796.
37. Enomoto N, Sakuma I, Asahina Y, et al. Mutations in the nonstructural protein 5A gene and response to interferon in patients with chronic hepatitis C virus 1b infection. N Engl J Med 1996;334:77–81.
38. Katze MG, He Y, Gale M Jr. Viruses and interferon: a fight for supremacy. Nat Rev Immunol 2002;2:675–87.
39. Beard MR, Abell G, Honda M, et al. An infectious molecular clone of a Japanese genotype 1b hepatitis C virus. Hepatology 1999;30:316–24.

Available online at www.sciencedirect.com
SCIENCE DIRECT
Hepatology Research 29 (2004) 89–96
Hepatology Research
www.elsevier.com/locate/ihepcom

Changes of HCV quasispecies during combination therapy with interferon and ribavirin

Eri Ueda[a], Nobuyuki Enomoto[a,*], Naoya Sakamoto[a], Kosei Hamano[a], Chifumi Sato[b], Namiki Izumi[c], Mamoru Watanabe[a]

[a] Department of Gastroenterology and Hepatology, Tokyo Medical and Dental University, 1-5-45 Yushima, Bunkyo-ku, Tokyo 113-8519, Japan
[b] Department of Analytical Health Science, Tokyo Medical and Dental University, 1-5-45 Yushima, Bunkyo-ku, Tokyo 113-8519, Japan
[c] Department of Gastroenterology and Hepatology, Musashino Red Cross Hospital, 1-26-1 Kyonan-cho, Musashino city, Tokyo 180-8610, Japan

Received 18 July 2003; received in revised form 14 January 2004; accepted 27 February 2004

Available online 22 April 2004

Abstract

Treatment of chronic hepatitis C virus (HCV) infection with interferon (IFN) and ribavirin improves the rate of eradication of HCV, but only about 13–14% of non-responders (NR) with HCV of genotype 1b previously treated with IFN achieve a sustained virological response (SVR). To determine whether HCV quasispecies diversity correlates with the outcome of therapy with IFN and ribavirin, we studied 13 patients undergoing combination therapy with IFN-α2b and ribavirin after failure of IFN monotherapy. HCV quasispecies diversity was assessed by cloning and sequencing before and during combination therapy. During therapy, quasispecies diversity diminished in NR patients, both in the hypervariable region (HVR) 1 of the envelope 2 (E2) domain and in the interferon sensitivity-determining region (ISDR) in the NS5A. Pre-treatment nucleotide quasispecies diversity was lower in SVR and end-of-therapy viral response (ETR) patients than in NR patients. Resistance to ribavirin was associated with high pre-treatment heterogeneity and the selection of quasispecies of the HCV genome. HVR quasispecies may be a predictor of efficacy of combination therapy with IFN and ribavirin.

Keywords: Chronic hepatitis C; Interferon (IFN); Ribavirin; Quasispecies; Hypervariable region (HVR); Interferon sensitivity-determining region (ISDR)

1. Introduction

Since its discovery in 1989, hepatitis C virus (HCV) has been recognized as a major cause of acute and chronic hepatitis, leading to liver cirrhosis and hepatocellular carcinoma [1,2]. The development of effective treatments to eradicate HCV and halt progression to cirrhosis and hepatocellular carcinoma is of great medical importance. Combination therapy with interferon (IFN)-α2b plus ribavirin results in a higher rate of sustained virological response (SVR) (35–45%) than IFN monotherapy, but only about 13–14% of non-responders and relapsers previously treated with IFN achieve a SVR [3,4]. It is important to address the question why combination therapy with IFN and ribavirin cannot eliminate HCV replication in these patients.

The precise mechanism of the action of ribavirin (1-β-D-ribofuranosyl-1,2,4-triazole-3-carboxamide), a purine nucleoside analogue, is not understood completely. To date, the enhancement of host immunity or direct antiviral mechanisms have been proposed as the modes of the action of ribavirin [5]. A number of studies have shown that ribavirin acts during the immune response as a modulator of the type 1/type 2 cytokine balance in favor of type 1 [6,7]. Monotherapy with ribavirin does not affect significantly HCV titers or HCV quasispecies [8], suggesting that ribavirin may enhance IFN antiviral activity through an immune modulatory mechanism [9,10]. Ribavirin also may have a direct anti-HCV effect through its incorporation into newly synthesized RNA transcripts by the NS5B polymerase [11]. More recently, Crotty et al. [12] proposed that ribavirin may act as an RNA mutagen that drives a rapid mutation of an RNA virus, leading to accumulation of defective viral genomes and suppression of viral replication or "error catastrophe" [13,14].

HCV is an RNA virus whose genome exhibits significant genetic heterogeneity as a result of the accumulation of mutations during viral replication. The resultant swarm of viruses with genetically heterogeneous but closely related

* Corresponding author. Tel.: +81-3-5803-5877; fax: +81-3-5803-0268.
E-mail address: nenomoto.gast@tmd.ac.jp (N. Enomoto).

1386-6346/$ – see front matter © 2004 Elsevier B.V. All rights reserved.
doi:10.1016/j.hepres.2004.02.014

genomes is referred to as a quasispecies [15–17]. Such quasispecies provide the flexibility for rapid adaptation of HCV to adverse environments, such as drug therapy and to evade host immune responses. The hypervariable region 1 (HVR1, amino acid position 384–410) of the envelope 2 (E2) domain of the HCV genome, which encodes the major neutralizing epitope, has been considered responsible for the generation of escape mutants [15–17]. It also has been reported that mutations in the IFN sensitivity-determining region (ISDR, amino acid position 2209–2248) in the NS5A gene are correlated closely with the response to IFN in patients with HCV 1b [18–22]. Therefore, determination of changes of HCV quasispecies in these regions from ribavirin-resistant patients should be helpful in elucidating the mechanism of action of ribavirin and resistance of HCV to ribavirin.

In this study, we examined the pre-treatment HCV quasispecies of E2–HVR and NS5A–ISDR, as well as whether the HCV quasispecies diversity decreased during combination therapy with IFN and ribavirin in patients with HCV 1b who were resistant to IFN monotherapy.

2. Patients and methods

2.1. Patients

The study group comprised 13 patients infected with HCV RNA of genotype 1b who had not responded to previous IFN monotherapy. They were negative for serum HBsAg (hepatitis B surface antigen), anti-HBc (hepatitis B core antibodies) and antinuclear antibodies and had no other causes of hepatitis, including excessive alcohol intake and hepatotoxic drugs. Liver biopsies were performed on all patients before therapy and the presence of chronic active hepatitis was confirmed histologically. Written informed consent was obtained from each patient for liver biopsy, and the study protocol conformed to the ethical guidelines of the 1975 Declaration of Helsinki as reflected in a priori approval by the institution's human research committee. They were treated with IFN-α2b (6 MU daily for the first 2 weeks followed by 6 MU three times a week for 22 weeks) and ribavirin (600–800 mg per day, according to body weight) in combination for 24 weeks. Five patients achieved a SVR with eradication of HCV, a further five patients showed an end-of-therapy virological response (ETR) but developed recurrent HCV viremia after treatment and the other three patients did not respond to the IFN–ribavirin combination therapy, with persistent viremia during treatment (no response) NR. Serum samples were obtained from each patient just before the commencement of combination therapy, and thereafter at several time points, and stored at −70 °C until use. We analyzed by cloning and sequencing the quasispecies of the HVR1 in E2 and the ISDR in NS5A in HCV RNA extracted from the serum samples taken from each patient just before therapy and 12 weeks later for the three NR patients.

2.2. RNA extraction

Serum RNA was extracted using a modified acid–guanidium–phenol–chloroform method. Briefly, 150 μl of serum were mixed with 700 μl of ISOGEN (Wako, Osaka, Japan), and the aqueous phase was extracted once with 140 μl of chloroform. The RNA was precipitated with isopropanol using 20 μg of glycogen (Boehringer Manheim, Manheim, Germany) as a carrier. The resultant RNA pellet was washed once with ethanol and finally dissolved in 10 μl of double distilled water and stored at −70 °C until use.

2.3. cDNA synthesis

Five μl of the reverse transcription mixture were adjusted to contain 1 μl of the RNA solution. 50 U of Moloney murine leukemia virus reverse transcriptase (MMLV-RT, Invitrogen, Carlsbad, CA) diluted to the appropriate concentration, 10 U of RNase inhibitor (Promega, Madison, WI) and 50 pg of random hexamers (Takara, Shiga, Japan). The mixture was incubated at 37 °C for 45 min.

2.4. PCR and sequencing of cloned cDNA

The fragments of E2–HVR and NS5A–ISDR of HCV genome were amplified by nested PCR with the following primer sets. The nucleotide sequences of the primers were:

HVR 1st-sense: GCCATTTATCAGGTCACCGCATGGC;
HVR 1st-antisense: GCTCCGGGCACCCGGACGAGTTGAA;

HVR 2nd-sense: TGTAAAACGACGGCCAGT TGGTGGCGGGGGCCCACTGG;
HVR 2nd-antisense: CAGGAAACAGCTATGACC AACCTGTGTGTGTAGAACAG;

ISDR 1st-sense: TGGATGGAGTGCGGTTGCACAGGTA;
ISDR 1st-antisense: TCTTTCTCCGTGGAGGTGGTATTGG;

ISDR 2nd-sense: TGTAAAACGACGGCCAGT CAGGTACGCTCCGGCGTGCA;
ISDR 2nd-antisense: CAGGAAACAGCTATGACC GGGGCCTTGGTAGGTGGCAA.

We used an automatic hot start PCR with TaqStart antibodies (Advantage cDNA Polymerase Mix, CLONTECH, Alto, CA), according to the manufacturer's instructions. PCR schedules were as follows: for the first PCR, denaturing at 94 °C for 60 s, followed by 40 cycles of denaturing at 94 °C for 10 s, annealing at 55 °C for 10 s, and polymerization at 72 °C for 30 s. We transferred 1 μl of the first PCR product to the upper mixture of the second round PCR assay. Other conditions of the second round PCR were the same as for the first. The amplicon was purified using Mini

quickspin DNA columns (Roche, Mannheim, Germany) and ligated into the pGEM-T vector (Promega, Madison, WI). The plasmids with HCV cDNA inserts were transformed into competent *Escherichia coli* XL-2 blue cells and plated onto agar plates containing ampicillin (100 μg/ml) and incubated overnight at 37 °C. Ten clones on average per sample were picked and subjected to colony PCR.

Thereafter, both strands of the PCR products were cycle sequenced using Big Dye Terminator Cycle Sequencing Ready Reaction Kits (Applied Biosystems, Foster, CA) according to the manufacturer's instructions. The products were purified on Mini Quickspin DNA column (Roche) and sequenced using an automated DNA sequencer (model 373S, Applied Biosystems, Chiba, Japan). The nucleotide and deduced amino acid sequences were compared using the prototype sequence of HCV 1b, HCV-J [23].

Sequences were aligned and edited using CLUSTAL W [24]. Final fragments of E2–HVR were 281 bp in length (part of E1/E2; amino acids, aa, 354–446; 93 aa) including the HVR1 (located in the first 81 bp of the E2 region: aa 384–410; 27 aa) and those of NS5A–ISDR were 532 bp in length (aa 2139–2314; 176 aa) including the ISDR (aa 2209–2248; 40 aa). The consensus sequence is the sequence of amino acids the most frequently shown before and after combination therapy.

2.5. Definitions and statistical analysis

Quasispecies intrasample diversity was defined as the degree of homogeneity of sequences from the same sample. We calculated the average number of nucleotide or amino acid differences between every sequenced clone of each sample and their respective consensus sequence. First, we calculated the mean change rates (percentage changes per site per clone) of nucleotides or amino acids of the total fragments of E2–HVR and NS5A–ISDR and then we calculated the mean change rates (percentage changes per site per clone) of nucleotides or amino acids of fragments of E2–HVR only and NS5A–ISDR only. We observed that both change rates generated a similar pattern of intrasample diversity. We used the former method [25].

Distributions of continuous variables were analyzed by the Mann–Whitney *U*-test for two groups and by the Kruskal–Wallis test for three groups, using StatView-J 5.0 software (Hulinks, Tokyo, Japan). A P-value <0.05 was considered to be statistically significant.

3. Results

The clinical features of the patients, in relation to the outcome of combination therapy with IFN and ribavirin after the failure of IFN monotherapy, are summarized in Table 1. There were no statistically significant differences between the groups in terms of age, sex, alanine aminotransferase (ALT) levels, platelet counts, liver histology, and the number of amino acid mutations in the ISDR. In the NR patients, HCV levels rapidly decreased to one-tenth to one-hundredth of pre-treatment levels 4 weeks after the start of combination therapy, and then remained stable. Also, the decrease in ALT levels was not sufficient in the NR patients. On the other hand, in the SVR patients, HCV RNA levels decreased immediately after the start of treatment and were below the limit of detection 12 weeks later. Finally, ALT levels normalized in all SVR patients and were sustained. As for the ETR patients, HCV RNA levels were below the limit of detection in four of the five patients 12 weeks later, and in one patient decreased under 100 international units (IU)/ml. In all ETR patients, HCV RNA became undetectable at the end of therapy but increased later.

Fig. 1 shows the pre-treatment amino acid consensus sequences of the HVR and ISDR for each patient. The HVR showed a variety of differences in each patient, but no distinct correlation between the sequences and the effectiveness of therapy was recognized. Meanwhile, the ISDR contained no amino acid changes (wild type) or fewer than two changes

Table 1
Clinical features of the patients in relation to the response to IFN–ribavirin therapy

	NR ($n = 3$)	ETR ($n = 5$)	SVR ($n = 5$)	P-value
Median age (range)	48 (32–52)	55 (52–62)	45 (35–60)	NS
Sex (male/female)	2/1	4/1	5/0	NS
Laboratory data				
Median serum ALT (IU/L) (range)	63 (31–72)	53 (45–160)	117 (43–327)	NS
Median platelet ($\times 10^4$/mm^3) (range)	18.0 (15.6–20.1)	16.0 (13.8–20.8)	16.9 (11.4–19.9)	NS
Median HCV RNA (kcopy/ml) (range)	850 (380–980)	690 (610–850)	780 (31–850)	NS
Liver histology before treatment				
Median activity score (range)	1 (0–1)	1 (1–2)	2 (1–2)	NS
Median fibrosis score (range)	2 (1–2)	1 (1–3)	1 (1–3)	NS
Median number of amino acid				
Changes in ISDR (range)	0 (0–2)	1 (0–2)	1 (0–1)	NS

Abbreviations: NR, no response; ETR, end-of-therapy virological response; SVR, sustained virological response; ALT, alanine aminotransferase; ISDR, interferon sensitivity-determining region.

```
                      384                                410
HCV-J HVR       GVDG HTHVTGGRVASSTQSLVSWLSQGPSQK IQLVNT

NR
 Patient 1      ---- S-Y---AAAGR--S-IA-LFTP-A--- ------
 Patient 2      ---- E-R-S--SQGRT-FR-TQFFTL--Q-- V--I--
 Patient 3      ---- D---S--TA-YNARG-STLF-F-A--- ------
ETR
 Patient 4      ---- Q-R----AA-FT-S--T-LF-P-SR-- ------
 Patient 5      ---- E-----AAAS-T--RFT-LF-L-SA-R ---I--
 Patient 6      ---- G-RI---QQ-RAASG-T-LFTP--T-- L--I--
 Patient 7      ---- E-YT---KAGRV-S-FT-LF----T-- ---I--
 Patient 8      ---- ---T---TA-HT-RG-T-LF-P----N ------
SVR
 Patient 9      S--- D-N-M--TAGKD-FGFA-LF-S-A--- ---I--
 Patient 10     ---- Q------N--RGA-G-N-LFAA----- ------
 Patient 11     ---- T---A--AAGRTAFR-A-IF-S-S--N ------
 Patient 12     ---- G-YT---TA-RT-RG-A-LF-S-AQ-- ---I--
 Patient 13     ---- R-YT---AQ-RT-SG-T-LF-T----- ---I--


                      2209                               2248
HCV-J ISDR      PSLKATCTTHHDSPDADLIEANLLWRQEMGGNITRVESEN

NR
 Patient 1      --------AR------------------------------
 Patient 2      --------------------------------------
 Patient 3      --------------------------------------
ETR
 Patient 4      ---------Y-G-----------------------------
 Patient 5      ---------R-------------------------------
 Patient 6      ---------R-------------------------------
 Patient 7      --------------------------------------
 Patient 8      ---------R-------------------------------
SVR
 Patient 9      ---------R-------------------------------
 Patient 10     ---------H-------------------------------
 Patient 11     --------------------------------------
 Patient 12     --------I-------------------------------
 Patient 13     --------------------------------------
```

Fig. 1. Amino acid (aa) consensus sequences of the E2–HVR (aa 384–410) and the NS5A–ISDR (aa 2209–2248) before treatment of 13 patients infected with HCV 1b. Amino acid residues are indicated by the standard single-letter codes, and dashes indicate residues identical to those in HCV-J, the prototype strain of HCV 1b.

(intermediate type), and that is consistent with the clinical course of a poor response to previous IFN monotherapy [18].

The amino acid sequences of the E2–HVR quasispecies from the 13 patients are shown in Fig. 2. For the NR patients (Patient 1–3), the changes of quasispecies between pre-treatment (0W) and post-treatment (12W) samples are illustrated. Before treatment, several quasispecies were observed in each patient, but 12 weeks later the amino acid heterogeneity had decreased. In Patients 1 and 2, some HVR quasispecies were selected during combination therapy, and in Patient 3, the emergence of quasispecies quite different to those at pre-treatment was observed. Consequently, the HVR quasispecies became homogenous in each patient during treatment. However, the increase in amino acid changes, which leads to viral "error catastrophe" and is suggested to be one of the mechanism of action of ribavirin, was not seen in these patients. Pre-treatment (0W) amino acid sequences of 10 clones are shown for the ETR and SVR patients. Patients 6 and 7 of the ETR group and Patients 9, 12 and 13 of the SVR group had a variety of mutations in the HVR, and the other patients had relatively homogenous HVR quasispecies. In Patients 12 and 13, one and two amino acid insertions were observed, respectively. Collectively, there was no definite tendency for amino acid changes in the HVR in patients in the ETR and SVR groups.

Fig. 3 shows the amino acid sequences of in the NS5A–ISDR in the 13 patients. In Patient 1 of the NR group, several quasispecies were observed before treatment (0W) as seen in the HVR. The mutant type quasispecies with six or seven amino acid changes disappeared during combination therapy and the intermediate type quasispecies with one or two amino acid changes persisted. This was considered to be a phenomenon of selection of the ISDR quasispecies by the IFN treatment. Patients 2 and 3 of the NR group had homogeneous quasispecies before treatment and the amino acid differences reduced during treatment, but the type of the ISDR did not change (wild type). Consequently, the effect of acceleration of mutation by ribavirin

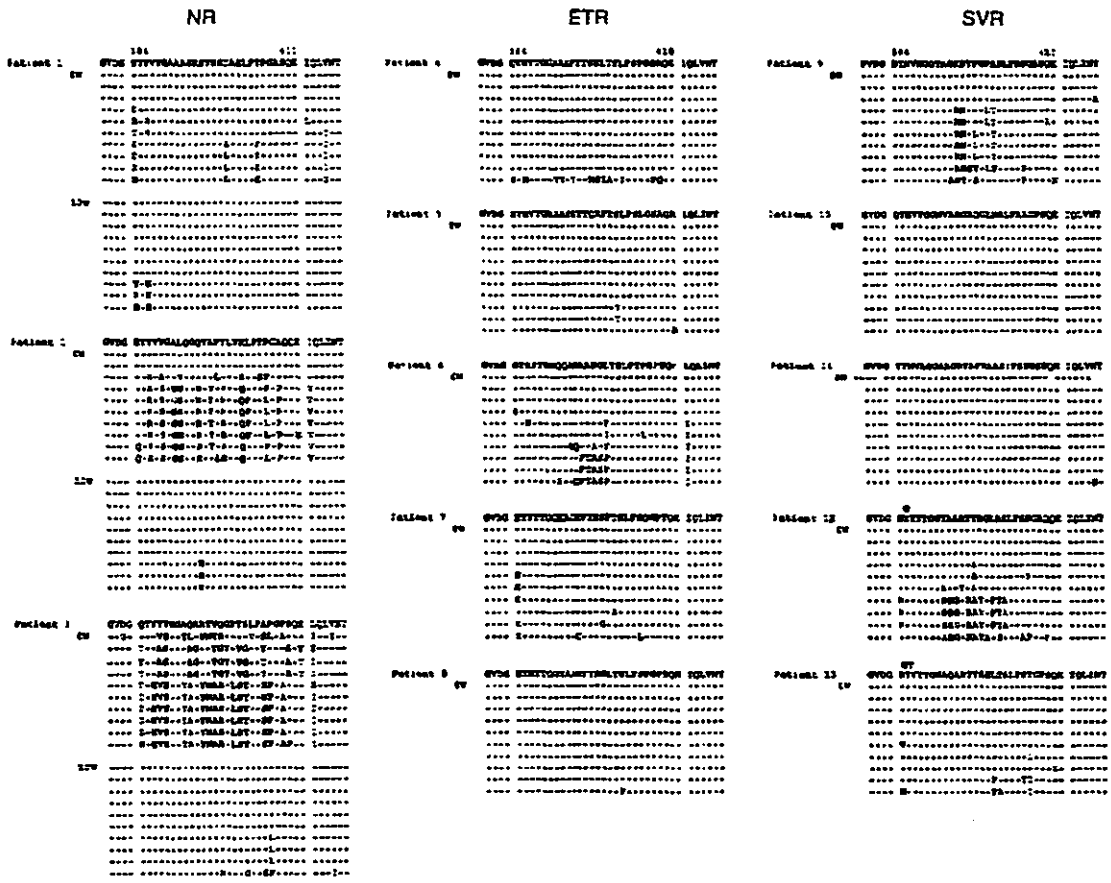

Fig. 2. Sequences of hypervariable region (HVR, aa 384–410) in 13 patients infected with HCV 1b and treated with interferon α-2b and ribavirin. The consensus sequences for Patients 1–13 are shown consecutively. The consensus sequence is the sequence of amino acids the most frequently shown before and after combination therapy. Amino acid residues are indicated by the standard single-letter codes, and dashes indicate residues identical to each consensus sequence. The v symbols above the consensus sequences of the Patients 12 and 13 indicate the positions of the insertions shown above.

was not observed and the amino acid heterogeneity around the ISDR reduced, as seen in the HVR. In the SVR and ETR patients, the variation in mutations in the ISDR was smaller than those in the HVR.

The mean rates of change of nucleotides or amino acids for each patient before and during combination therapy are summarized in Fig. 4. In the NR patients, the change rate of: (A) nucleotides and (B) amino acids for the E2–HVR reduced during combination therapy (12W) compared to pre-treatment (0W) (A. $P = 0.046$; B. $P = 0.049$). Similar trends also were observed in (C) and (D) of the NS5A–ISDR, although the results did not reach statistical significance (C, $P = 0.275$; D. $P = 0.275$). On the other hand, the pre-treatment rates of change of the HVR of the ETR and SVR patients were lower than those of the NR patients. This tendency is more distinct for the NS5A–ISDR. The pre-treatment rates of change of nucleotides in the HVR and ISDR in the ETR and SVR patients were significantly lower than those of the NR patients ($P = 0.014$ and 0.022) (Table 2). The rates of change of amino acids in both the HVR and ISDR did not differ significantly between the NR and the ETR plus SVR groups.

Table 2
Comparison of the change rates before combination therapy between the NR and the ETR plus SVR groups

	Rate of change (%)		
	NR median (range)	ETR + SVR median (range)	*P*-value
E2–HVR nucleotides	2.40 (2.1–5.1)	0.90 (0.2–2.1)	0.014*
E2–HVR amino acids	5.20 (2.8–8.3)	1.25 (0.2–5.1)	0.063
NS5A–ISDR nucleotides	1.40 (1.1–3.3)	0.55 (0.1–1.4)	0.022*
NS5A–ISDR amino acids	0.60 (0.5–3.6)	0.45 (0.2–1.5)	0.172

Change rate = percentage changes of nucleotide or amino acid per site per clone.

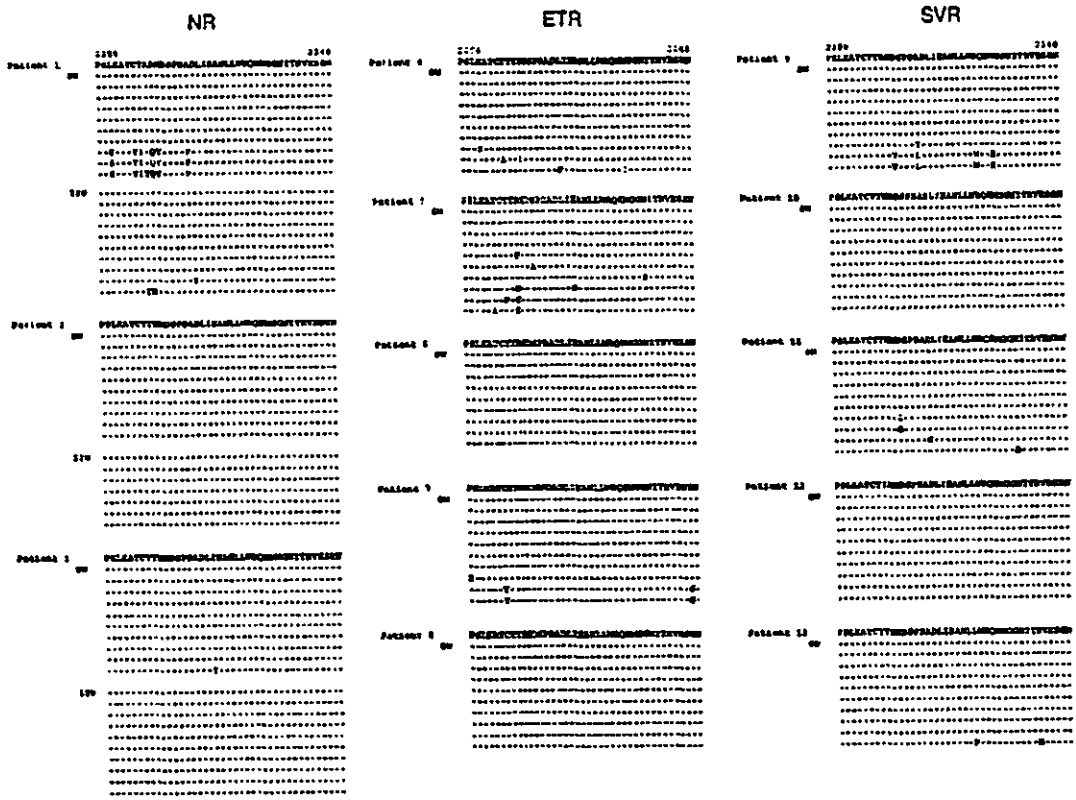

Fig. 3. Sequences of the interferon sensitivity-determining region (ISDR, aa 2209–2248) in 13 patients infected with HCV 1b and treated with interferon α-2b and ribavirin. The consensus sequences for Patients 1–13 are shown consecutively. Amino acid residues are indicated by the standard single-letter codes, and dashes indicate residues identical to each consensus sequence.

4. Discussion

In this study, we found that E2–HVR quasispecies diversity reduced significantly in NR patients during combination therapy with IFN and ribavirin. In addition, pre-treatment nucleotide changes in E2–HVR and NS5A–ISDR are more common in NR patients than SVR and ETR patients. These findings indicate that HCV quasispecies play an important role in resistance to combination therapy.

Reduction of HVR quasispecies diversity in NR patients indicates that quasispecies selection occurs during combination therapy. The HVR shows a high degree of variation in the amino acid sequences constituting a neutralizing epitope probably presented on the surface of the virion as a part of viral envelope, which is susceptible to various kinds of pressure such as host immune pressure or antiviral agents. Therefore, the changes in HVR quasispecies composition reflect the selection of escape mutants [10,26]. In fact, previous studies have also confirmed the reduction of HVR quasispecies during IFN monotherapy and the correlation between quasispecies diversity and IFN responsiveness [9,16,17]. Although the mechanism of action of combination therapy has not been elucidated completely, enhancement of a HCV-specific T-helper (Th)1 response [6,7], or more recently, the mutagenic effects of ribavirin on the HCV-RNA genome resulting in "error catastrophe" [12,14] have been postulated. Regarding the former possibility, enhancement of the immune response could accelerate the selection of immune escaping quasispecies, leading to reduced quasispecies diversity [5,6,10]. In the latter case, ribavirin sensitive quasispecies would be mutated and eliminated rapidly, because turnover of HCV replication in vivo is assumed to be less than several hours, so that detection of mutated HCV genomes by PCR cloning from serum may be difficult.

More importantly, HCV quasispecies that are resistant to the effects of ribavirin survive preferentially and selection will take place. In this case, HVR variation is merely a surrogate marker of various quasispecies with distinct genetic features outside the HVR. What kind of genetic features confer susceptibility to ribavirin has not been elucidated yet, but ribavirin is a nucleoside analogue acting during RNA synthesis and substantial evidences has been reported that the HCV–NS5B protein, which is the RNA-dependent RNA polymerase in HCV replication, has an affinity for ribavirin and ribavirin is incorporated into nascent RNA [11]. NS5B variations may be the primary determinant of ribavirin susceptibility for each HCV strain. In this regard, genetic analysis of the HCV–NS5B region and correlation with sensitivity to ribavirin is required.

On the other hand, a tendency for a reduction of ISDR quasispecies in NR patients was observed in our study. IFN monotherapy also influences the quasispecies as a result of

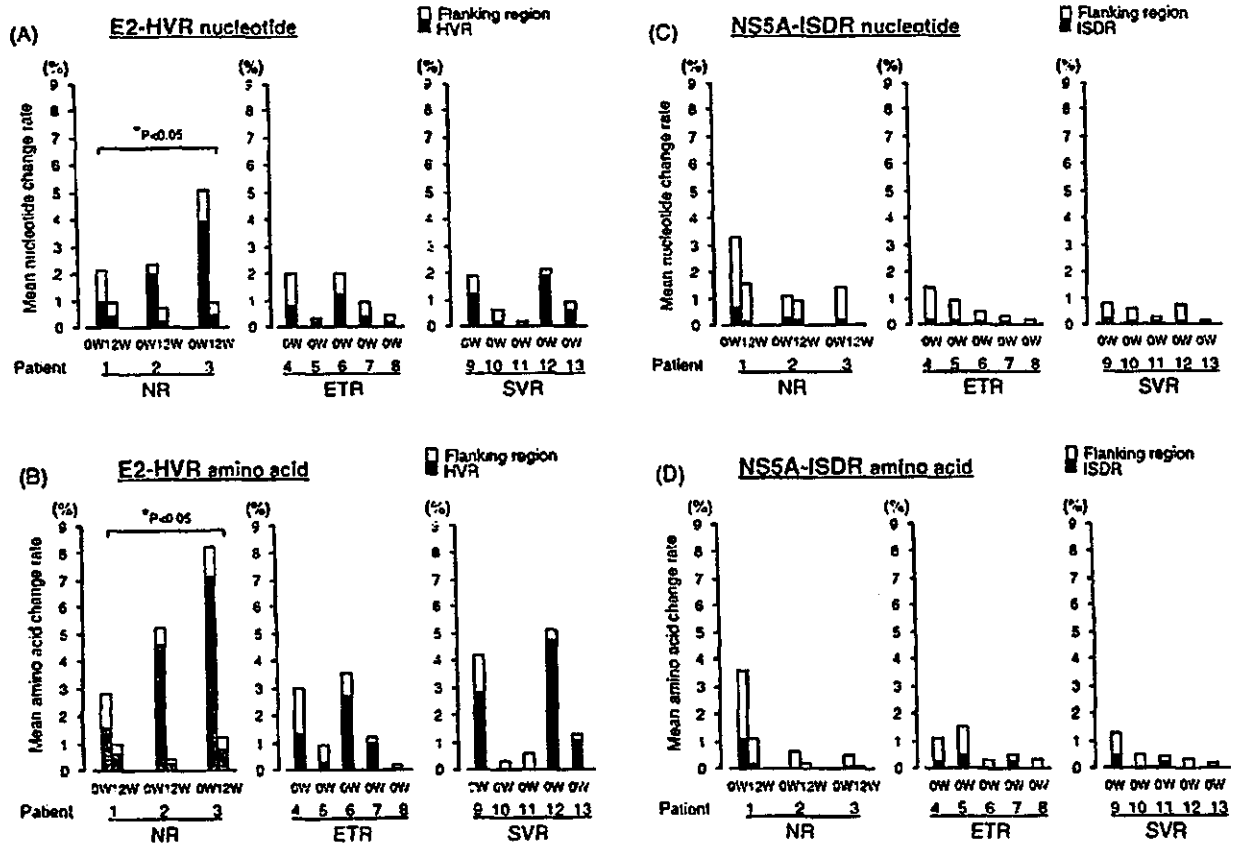

Fig. 4. Mean rates of change of nucleotides and amino acids of the E2–hypervariable region (HVR) and NS5A–interferon sensitivity-determining region (ISDR) for each patient. Change rate = percentage changes of nucleotide or amino acid per site per clone. The entire E2–HVR fragment is 281 bp in length (93 amino acids, aa, 354–446) including the HVR1 (aa 384–410) and the entire NS5A–ISDR fragment is 532 bp in length (aa 2139–2314; 176 aa) including the ISDR (aa 2209–2248). The flanking regions are excluding HVR or ISDR from the entire fragment. (A) The pre-treatment (0W) mean nucleotide change rates of E2–HVR were 2.1, 2.4 and 5.1% in the NR patients, and decreased to 0.9, 0.7 and 0.9% 12 weeks after the beginning of IFN–ribavirin therapy ($P = 0.046$). (B) The pre-treatment mean amino acid change rates of E2–HVR were 2.8, 5.2 and 8.3% in the NR patients, and decreased to 1.0, 0.4 and 1.2% 12 weeks after the beginning of IFN–ribavirin therapy ($P = 0.049$). (C) and (D) In the NR patients, mean rates of change of nucleotides and amino acids of the NS5A–ISDR also were decreased 12 weeks after the beginning of IFN–ribavirin therapy, but not significantly (C, $P = 0.275$; D, $P = 0.275$).

its direct antiviral and immunomodulatory activities [27,28]. It is well established that the ISDR in the NS5A protein is a determinant of IFN sensitivity [18,29]. A homogeneous viral population before combination therapy might be the result of prior selection of an IFN-resistant strain during the first cycle of IFN therapy. However, because no patients in this study responded to IFN monotherapy, these results indicate the additional effect of ribavirin. To investigate the effect of ribavirin, single-strand conformation polymorphism (SSCP) was used to analyze the evolution of the genetic heterogeneity of HCV in relation to the anti-HCV humoral response in patients treated with ribavirin alone [30]. The conclusion was that ribavirin monotherapy had no impact on the ISDR sequences. Collectively, resistance to combination therapy in NR patients results from the evasion of error catastrophe or enhanced immune pressure by some mechanism and the selection of the ISDR quasispecies.

We also showed that pre-treatment mean rates of nucleotides change in both the HVR and ISDR are higher in NR patients than SVR and ETR patients. This finding indicates that more complex quasispecies exist in the HCV population of NR patients and more strains with resistance to antiviral agents are present. No statistical significance in pre-treatment amino acids change rates means that synonymous substitutions were dominant in the process of selection. In addition to the well known pre-treatment variables associated with resistance to combination therapy, such as genotype 1, high serum HCV RNA levels, and severe fibrosis [3], the genetic heterogeneity of HCV may influence the response to treatment. Since all of the patients analyzed in the present study were non-responders to the previous IFN monotherapy and had HCV with IFN-resistant wild or intermediate ISDR, the HVR heterogeneity seems an important predictor of the response to combination therapy in such clinical settings.

The changes in quasispecies composition during combination therapy with IFN and ribavirin have been investigated clinically, principally by indirect methods, such as SSCP analysis [31] or heteroduplex tracking assay [32]. These studies reported somewhat conflicting data, but many of

them showed that no significant changes occur during combination therapy. The discrepancy between this study and those could be attributable to the different methods used. Our PCR cloning quantifies directly the quasispecies diversity, but other methods based on electrophoresis have a lower sensitivity because HCV fragments with different sequences may have the same electrophoretic pattern, especially in one-dimensional systems. Thus, these methods are merely qualitative surrogate analysis, underestimating the changes in quasispecies, and cloning and sequencing of each quasispecies, as in this study, is the gold standard. Only a relatively small number of patients were evaluated in this study. further studies of a large number of patients with a large number of serial serum samples are needed to draw firm conclusions about the impact of HCV quasispecies on IFN–ribavirin combination therapy.

In conclusion, during combination therapy with IFN and ribavirin the HCV quasispecies diversity diminished significantly in NR patients. This suggests that resistance to ribavirin is associated with the selection of HCV quasispecies under greater immune pressure with no incidence of error catastrophe. Also, the heterogeneity of HCV quasispecies could be a predictor of resistance to combination therapy.

References

[1] Choo QL, Kuo G, Weiner AJ, Overby LR, Bradley DW, Houghton M. Isolation of a cDNA clone derived from a blood-borne non-A, non-B viral hepatitis genome. Science 1989;244:359–62.

[2] Hoofnagle JH. Course and outcome of hepatitis C. Hepatology 2002;36:S21–9.

[3] Di Bisceglie AM, Hoofnagle JH. Optimal therapy of hepatitis C. Hepatology 2002;36:S121–7.

[4] Chander G, Sulkowski MS, Jenckes MW, et al. Treatment of chronic hepatitis C: a systematic review. Hepatology 2002;36:S135–44.

[5] Lau JY, Tam RC, Liang TJ, Hong Z. Mechanism of action of ribavirin in the combination treatment of chronic HCV infection. Hepatology 2002;35:1002–9.

[6] Cramp ME, Rossol S, Chokshi S, Carucci P, Williams R, Naoumov NV. Hepatitis C virus-specific T-cell reactivity during interferon and ribavirin treatment in chronic hepatitis C. Gastroenterology 2000;118:346–55.

[7] Kamal SM, Fehr J, Roesler B, Peters T, Rasenack JW. Peginterferon alone or with ribavirin enhances HCV-specific CD4 T-helper 1 responses in patients with chronic hepatitis C. Gastroenterology 2002;123:1070–83.

[8] Lee JH, von Wagner M, Roth WK, Teuber G, Sarrazin C, Zeuzem S. Effect of ribavirin on virus load and quasispecies distribution in patients infected with hepatitis C virus. J Hepatol 1998;29:29–35.

[9] Zeuzem S. Hepatitis C virus: kinetics and quasispecies evolution during anti-viral therapy. Forum (Genova) 2000;10:32–42.

[10] Hu KQ, Vierling JM, Redeker AG. Viral, host and interferon-related factors modulating the effect of interferon therapy for hepatitis C virus infection. J Viral Hepat 2001;8:1–18.

[11] Maag D, Castro C, Hong Z, Cameron CE. Hepatitis C virus RNA-dependent RNA polymerase (NS5B) as a mediator of the antiviral activity of ribavirin. J Biol Chem 2001;276:46094–8.

[12] Crotty S, Cameron CE, Andino R. RNA virus error catastrophe: direct molecular test by using ribavirin. Proc Natl Acad Sci USA 2001;98:6895–900.

[13] Eigen M. Error catastrophe and antiviral strategy. Proc Natl Acad Sci USA 2002;99:13374–6.

[14] Graci JD, Cameron CE. Quasispecies, error catastrophe, and the antiviral activity of ribavirin. Virology 2002;298:175–80.

[15] Forns X, Purcell RH, Bukh J. Quasispecies in viral persistence and pathogenesis of hepatitis C virus. Trends Microbiol 1999;7:402–10.

[16] Gomez J, Martell M, Quer J, Cabot B, Esteban JI. Hepatitis C viral quasispecies. J Viral Hepat 1999;6:3–16.

[17] Farci P, Purcell RH. Clinical significance of hepatitis C virus genotypes and quasispecies. Semin Liver Dis 2000;20:103–26.

[18] Enomoto N, Sakuma I, Asahina Y, et al. Mutations in the nonstructural protein 5A gene and response to interferon in patients with chronic hepatitis C virus 1b infection. N Engl J Med 1996;334:77–81.

[19] Polyak SJ, Gerotto M. The molecular basis for responsiveness to anti-viral therapy in hepatitis C. Forum (Genova) 2000;10:46–58.

[20] Tan SL, Katze MG. How hepatitis C virus counteracts the interferon response: the jury is still out on *NS5A*. Virology 2001;284:1–12.

[21] Katze MG, He Y, Gale Jr M. Viruses and interferon: a fight for supremacy. Nat Rev Immunol 2002;2:675–87.

[22] He Y, Katze MG. To interfere and to anti-interfere: the interplay between hepatitis C virus and interferon. Viral Immunol 2002;15:95–119.

[23] Kato N, Hijikata M, Ootsuyama Y, et al. Molecular cloning of the human hepatitis C virus genome from Japanese patients with non-A, non-B hepatitis. Proc Natl Acad Sci USA 1990;87:9524–8.

[24] Thompson JD, Higgins DG, Gibson TJ. CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. Nucl Acids Res 1994;22:4673–80.

[25] Lyra AC, Fan X, Lang DM, et al. Evolution of hepatitis C viral quasispecies after liver transplantation. Gastroenterology 2002;123:1485–93.

[26] Ciurea A, Klenerman P, Hunziker L, et al. Viral persistence in vivo through selection of neutralizing antibody-escape variants. Proc Natl Acad Sci USA 2000;97:2749–54.

[27] Polyak SJ, McArdle S, Liu SL, et al. Evolution of hepatitis C virus quasispecies in hypervariable region 1 and the putative interferon sensitivity-determining region during interferon therapy and natural infection. J Virol 1998;72:4288–96.

[28] Farci P, Strazzera R, Alter HJ, et al. Early changes in hepatitis C viral quasispecies during interferon therapy predict the therapeutic outcome. Proc Natl Acad Sci USA 2002;99:3081–6.

[29] Witherell GW, Beineke P. Statistical analysis of combined substitutions in nonstructural 5A region of hepatitis C virus and interferon response. J Med Virol 2001;63:8–16.

[30] Querenghi F, Yu Q, Billaud G, Maertens G, Trepo C, Zoulim F. Evolution of hepatitis C virus genome in chronically infected patients receiving ribavirin monotherapy. J Viral Hepat 2001;8:120–31.

[31] Sookoian S, Castano G, Frider B, Cello J, Campos R, Flichman D. Combined therapy with interferon and ribavirin in chronic hepatitis C does not affect serum quasispecies diversity. Dig Dis Sci 2001;46:1067–71.

[32] Gerotto M, Sullivan DG, Polyak SJ. Effect of retreatment with interferon alone or interferon plus ribavirin on hepatitis C virus quasispecies diversification in nonresponder patients with chronic hepatitis C. J Virol 1999;73:7241–7.

Available online at www.sciencedirect.com
SCIENCE DIRECT
Virus Research 107 (2005) 73–81
Virus Research
www.elsevier.com/locate/virusres

cDNA microarray analysis to compare HCV subgenomic replicon cells with their cured cells

Ken-ichi Abe[a], Masanori Ikeda[a], Hiromichi Dansako[a], Kazuhito Naka[a], Kunitada Shimotohno[b], Nobuyuki Kato[a,*]

[a] *Department of Molecular Biology, Okayama University Graduate School of Medicine and Dentistry, 2-5-1 Shikata-cho, Okayama 700-8558, Japan*
[b] *Department of Viral Oncology, Institute for Virus Research, Kyoto University, 53 Kawara-cho Shogo-in, Sakyo-ku, Kyoto 606-8507, Japan*

Received 9 April 2004; received in revised form 21 June 2004; accepted 25 June 2004
Available online 12 August 2004

Abstract

The hepatitis C virus (HCV) replicon system carrying autonomously replicating HCV subgenomic RNA in human hepatocyte cells is a potent tool for basic studies of HCV, such as viral replication and drug development. Recently, we developed two HCV subgenomic replicons (50-1 and 1B-2R1) derived from two HCV strains, 1B-1 and 1B-2, respectively. Since the expression of HCV proteins is thought to affect the host cells' gene expression profiles, we attempted to identify target genes of HCV proteins using microarray analysis (9970 genes) by comparing 50-1 and 1B-2R1 replicon cells with their "cured cells", from which the replicons had been eliminated by prolonged treatment with interferon-α. The results showed that HCV replicons could have a variety of expression profiles in human hepatocytes. The results also showed that 2 and 6 genes were commonly up-regulated (more than 2.0-fold) and down-regulated (less than 0.50-fold), respectively, in both 50-1 and 1B-2R1 replicon cells compared with their cured cells. The differential expression profiles of genes selected by the microarray analysis were confirmed with standard RT-PCR and real-time LightCycler PCR. It was noteworthy that the commonly down-regulated genes contained large multifunctional proteases 2 and 7, which are known as catalytic subunits of immunoproteasome, and serine proteinase inhibitor clade C. Our microarray analysis demonstrated that HCV subgenomic replicons can change the gene expression profiles of host cells, and it allowed us to compile the first list of genes that the replicons transcriptionally regulate.

Keywords: Hepatitis C virus; HCV subgenomic replicon; Cured cells; cDNA microarray; Gene expression profile

1. Introduction

Hepatitis C virus (HCV) infection frequently causes chronic hepatitis (Choo et al., 1989; Kuo et al., 1989), which progresses to liver cirrhosis and hepatocellular carcinoma (Ohkoshi et al., 1990; Saito et al., 1990). HCV belongs to the family Flaviviridae, whose genome consists of a positive-stranded RNA molecule of about 9.6 kb and encodes a large polyprotein precursor of about 3000 amino acids (Kato et al., 1990; Tanaka et al., 1995). This precursor protein is cleaved by the host and viral proteases to generate at least ten proteins: the core, envelope 1 (E1), E2, p7, nonstructural protein 2 (NS2), NS3, NS4A, NS4B, NS5A, and NS5B. Although many hypotheses have been proposed over the past decade regarding the functions of the viral proteins (Bartenschlager and Lohmann, 2000; Kato, 2001), the lack of reproducible and efficient HCV proliferation in cell cultures (Kato and Shimotohno, 2000) has been a serious obstacle in understanding those proteins' actual functions.

However, in 1999, an HCV replicon system carring autonomously replicating HCV subgenomic RNA containing the NS3-NS5B regions was first established using a human hepatoma cell line, Huh-7 (Lohmann et al., 1999). Since then, several additional replicons have also been established (Blight et al., 2000, 2003; Ikeda et al., 2002; Kato et al., 2003b). In these systems, replicated HCV RNAs were detected by Northern blot analysis, and the HCV proteins pro-

* Corresponding author. Tel.: +81 86 235 7385; fax: +81 86 235 7392.
E-mail address: nkato@md.okayama-u.ac.jp (N. Kato).

0168-1702/$ – see front matter © 2004 Elsevier B.V. All rights reserved.
doi:10.1016/j.virusres.2004.06.013

duced were detected by Western blot analysis. Therefore, the system of HCV replicons has become a powerful tool for basic studies in HCV, such as viral replication and drug development (Bartenschlager, 2002).

Recently, we also established two HCV subgenomic replicons (50-1 and 1B-2R1) derived from two HCV strains, 1B-1 and 1B-2, respectively, using Huh-7 cells (Kato et al., 2003a; Kishine et al., 2002). We demonstrated that the 50-1 and 1B-2R1 subgenomic replicons (Kato et al., 2003a) were sensitive to interferon (IFN)-α, IFN-β and IFN-γ as are the other replicons (Frese et al., 2001, 2002). The nucleotide sequences of the NS3-NS5B regions in the 50-1 subgenomic replicon showed differences of 8.1% from those in the1B-2R1 subgenomic replicon (Kato et al., 2003a), although both the 1B-1 and 1B-2 strains belong to genotype 1b. Although the efficient replication of an HCV subgenomic replicon expressing HCV proteins is considered to affect the gene expression profiles of host cells (Bartenschlager and Lohmann, 2000; Kato, 2001), few reports have demonstrated inclusive searches for HCV's target genes (Zhu et al., 2003). Therefore, we thought a comprehensive search for HCV subgenomic replicon-regulated cellular genes would be important in understanding the molecular interplay exerted by HCV in vivo.

In the present study, to obtain the candidates of HCV's target genes, we performed cDNA microarray analysis by comparing two types of HCV subgenomic replicon cells with their "cured cells", from which the replicons had been eliminated by prolonged treatment with IFN-α. Here we report on the differential gene expression profiles in the replicon cells, and we first provide a list of genes that the replicons transcriptionally regulate.

2. Materials and methods

2.1. Cell cultures

50-1 and 1B-2R1 cells possessing 50-1 and 1B-2R1 subgenomic replicons, respectively, were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum and G418 (300 mg/ml; Geneticine, Invitrogen). The 50-1 and 1B-2R1 cells were known to possess the G418-resistant phenotype, because neomycin phophotransferase (NEOR) was produced by the efficient replication of HCV subgenomic replicon in the cells. When an HCV subgenomic replicon is excluded from the cells or its level is decreased, the cells are killed by the presence of G418. Therefore, the cured cells obtained from 50-1 and 1B-2R1 cells were maintained in the absence of G418.

2.2. IFN treatment

To prepare the cured cells, 50-1 and 1B-2R1 cells (each 1×10^6) were plated onto 10-cm plates and were cultured for 1 day immediately before IFN treatment. Human IFN-α (Sigma) was added to the cells at a final concentration of 3000 IU/ml as described previously (Kato et al., 2003a). The incubation in the absence of G418 was continued for 3 weeks with the addition of IFN-α (3000 IU/ml) at 4-day intervals. The cured cells obtained from 50-1 and 1B-2R1 cells were named 50-1C and 1B-2R1C cells, respectively.

2.3. Northern blot analysis

Total RNAs from the cultured cells were prepared using the RNeasy extraction kit (Qiagen). Three micrograms of total RNA was used to detect the HCV replicon RNA and β-actin. Northern blotting and hybridization were performed as described previously (Ikeda et al., 2002; Kato et al., 2003a). As a molecular length marker, replicon RNA synthesized in vitro from replicon cassette plasmid pNSS1RZ2RU (Kato et al., 2003a) was also utilized.

2.4. Western blot analysis

The preparation of cell lysates, sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), and immunoblotting analysis with a polyvinylidene difluoride membrane were performed as previously described (Hijikata et al., 1993). The antibodies used in this study were those against NS3 (Novacastra Laboratories, UK), NS5B (a generous gift from M. Kohara, Tokyo Metropolitan Institute of Medical Science), and β-actin (Sigma). Immunocomplexes on the membranes were detected by enhanced chemiluminescence assay (Renaissance; Perkin-Elmer Life Sciences).

2.5. cDNA microarray analysis

The 50-1, 50-1C, 1B-2R1, and 1B-2R1C cells (each 1×10^6 cells) were plated onto 10 cm plates, and each plate was cultured for 5 days in the absence of G418. The confluent cells were harvested and total RNAs were prepared using the RNeasy extraction kit (Qiagen). Using the obtained total RNAs, cDNA microarray analysis (CodeLink™, Uniset human I containing 9970 spots of 30-mer oligonucleotides; Amersham Biosciences) was performed by Kurabo Industries Ltd. (Osaka, Japan) with the authorization of Amersham Biosciences.

2.6. Analysis of mRNA expression by RT-PCR

The total RNAs (each 2 μg) that were the same as those subjected to cDNA microarray analysis were reverse-transcribed with Superscript II using an oligo dT primer (Invitrogen). One-tenth of the synthesized cDNA was subjected to PCR. The PCR primers are listed in Table 1. After 10 min at 98 °C, PCR was performed with Taq DNA polymerase (TaKaRa, Japan). Each cycle consisted of annealing at 60 °C (64 °C for LMP2 and LMP7 only) for 45 s, primer extension at 72 °C for 1 min, and denaturation at 94 °C for 20 s. The cycle numbers and the size of PCR products were also

Table 1
The primers used for RT-PCR analysis of mRNA expression

Genes	Orientation	Nucleotide sequence	Product (bp)	Cycles
Large multifunctional protease2 (LMP2)	Forward	ATGGAACCCTGGGAGGAATGCTG	145	30
	Reverse	GCAATAGCGTCTGTGGTGAAGCG		
Large multifunctional protease 7 (LMP7)	Forward	CTGGGATAAGAAGGGTCCTGGAC	293	27
	Reverse	TACTGGTGCAGCAGGTCACTGGAC		
Serine proteinase inhibitor (serpin) clade C	Forward	TGGATGAATTGGAGGAGATGATGC	249	25
	Reverse	CAATCACAACAGCGGTACTTGCAG		
S100-type calcium binding protein A14	Forward	CAGAGGATGCTCAGGAATTCAGTG	256	27
	Reverse	CTCTTGGCCGCTTCTCCAATGAG		
Latent transforming growth factor β binding protein 1 (LTBP1)	Forward	GCCTTGGTTGACTTCAGTGAACAG	325	27
	Reverse	CAGAAGGCACGTAGCCTGGCAG		
Weakly similar to zinc finger protein 91	Forward	CCAGAACCACATCCAAACCATCC	299	33
	Reverse	CCATCCCTTCGAAGCTGTGCTC		
Transgelin	Forward	GATTCTGAGCAAGCTGGTGAACAG	254	25
	Reverse	AGTGCCCATCATTCTTGGTCACTG		
Annexin A1	Forward	GATGCCAGGGCCTTGTATGAAGC	264	25
	Reverse	AACACCTTTCATGGCTTGATGAAGC		
Solute carrier family 7	Forward	AGTCCTTCGCTGGAAGAAGCCTG	314	27
	Reverse	CCATGTCCTCATTAGCCTCCTCTG		
Protein phosphatase 1 regulatory subunit 1A	Forward	CCACGGCAACGGAAGAAGATGAC	302	27
	Reverse	GCTCCCTTGGAATCCAGTGGTGG		
Phosphatidylserine-specific phospholipase A1α	Forward	GAGAAACAAGGACACCAACATCGAG	288	28
	Reverse	GTCACACTTGCTTGTAAGTTCACTG		
Oncostatin M receptor	Forward	CAGAAAAGAGTCACTCTGGCCCTG	292	27
	Reverse	GGTGCCTCTACTGGGTTTGTTGG		
Similar to interferon-induced protein 35	Forward	CCGTATGTGAATGGGGAGATCCAG	222	27
	Reverse	GCCTGACTCAGAGGTGAAGACTG		
Caspase 1	Forward	AGAAACACTCTGAGCAAGTCCCAG	278	30
	Reverse	AACATTATCTGGTGTGGAAGAGCAG		
Neutrophil cytosolic factor 2	Forward	GACATGGTGTCTAAGAAACTGGAG	277	27
	Reverse	CTCATAACTGAAGAGTGCCTCCAC		
Putative secred protein ZSIG13	Forward	CTGGTTATGACAATGACCGACCAG	272	25
	Reverse	GCAGATCTGGGCATATTTGAGAGG		
GAPDH	Forward	GACTCATGACCACAGTCCATGC	334	22
	Reverse	GAGGAGACCACCTGGTGCTCAG		

listed in Table 1. The PCR products were detected by staining with ethidium bromide after separation by electrophoresis on 3% agarose gels. RT-PCR was performed in duplicate experiments. The mRNA levels of target genes were monitored by a ChemiImager 4400 (Alpha Innotech), which measured the intensities of bands stained with ethidium bromide as described previously (Kato et al., 2003a). As an internal control, glyceraldehydes-3-phosphate dehydrogenase (GAPDH) mRNA was amplified by RT-PCR, and the products were used to normalize the mRNA levels of the target genes.

2.7. LightCycler PCR

One-twentieth of the cDNA synthesized above was subjected to real-time LightCycler PCR as described previously (Nozaki and Kato, 2002; Nozaki et al., 2003). The primers listed in Table 1 were also used for LightCycler PCR. Temperature cycling conditions for each primer set consisted of 10 min at 95 °C followed by 35 cycles for 1 s at 94 °C, 5 s at 60 °C (64 °C for LMP2 and LMP7 only), and 6–14 s (25 bp per second) at 72 °C. All reactions were performed in a LightCycler™ Quick System 330 (Roche) using Fast-Start DNA Master SYBR Green I mix (Roche) according to the manufacturer's instructions. The experiments were performed in at least triplicate. The relative mRNA expression ratios of the target genes were calculated based on crossing-point analysis using a second derivative maximum method (LightCycler analysis software version 3.5). To correct for differences in RNA quality and quantity between the samples, data were normalized using the ratio of the target cDNA concentration to that of GAPDH. This ratio was assessed by a different reaction in the same experimental round.

Table 4
Genes whose expression levels were down-regulated (less than 0.33-fold) in either 1B-2R1 or 50-1 cells compared with the cured cells

Genes	Relative mRNA expression ratio		Accession no.
	1B-2R1/1B-2R1C	50-1/50-1C	
Hephaestin	**0.14**	1.7	NM_014799
Solute carrier family 7[a]	**0.15**	0.62	NM_003982
Caspase 1[a]	**0.18**	0.65	NM_033292
Protease inhibitor 3	**0.19**	1.1	NM_002638
Collagen type II α1	**0.31**	1.6	NM_033150
C-terminal binding protein 2	**0.31**	0.71	NM_022802
ATPase α polypeptide (ATP 12A)	0.57	**0.26**	NM_001676
Hypothetical protein FLJ20043	0.79	**0.27**	NM_017637
CM2-HT0948-070900-368-D08 cDNA	1.0	**0.28**	BF089733
S100-type calcium binding protein A14[a]	0.62	**0.30**	NM_020672
Hypothetical protein MGC2827	0.65	**0.31**	NM_023940
EGFL6	2.4	**0.32**	NM_015507
ISL1 transcription factor	0.94	**0.32**	NM_002202
Pre-α globulin inhibitor	1.2	**0.32**	NM_002217
Regulator of G-protein signalling 16	0.65	**0.33**	NM_002928

The numbers of less than 0.33-fold were indicated by bold letters.
[a] RT-PCR analysis was performed to confirm the result of microarray analysis.

ther subjected to real-time LightCycler PCR analysis in order to obtain the actual ratios of mRNA expression. As shown in Table 5, the resultant relative mRNA expression ratios actually correlated with those obtained by our microarray analysis. Regarding the selected genes in this study, we confirmed by RT-PCR the reproducibility of the relative mRNA ratios using different lots of RNA specimens derived from 1B-2R1 and 1B-2R1C cells (data not shown). Taken together, our results suggest that these altered mRNA expressions are caused by the multiplication of HCV subgenomic replicons.

4. Discussion

This study yielded evidence of alterations in gene expression by HCV subgenomic replicons in human hepatocytes, as observed through microarray analysis (9970 genes), and first provided a list of genes including LMP2, LMP7, and serpin clade C that the replicons transcriptionally regulate.

To date, only one report of cDNA microarray analysis (832 cytokine-related genes) has been conducted by comparing HCV subgenomic replicon cells with parental Huh-7 cells (Zhu et al., 2003). That analysis obtained 14 up-regulated genes (those with ratios of more than 2.0) in the replicon cells. However, the parental Huh-7 cells may not be appropriate for use as control cells in such microarray analyses, because the HCV subgenomic replicon cells used are derived from a single cloned cell. Therefore, it is very important to avoid the clone-based differences for microarray analysis. From this principal reason, we used two types of cured cells derived from 50-1 and 1B-2R1 cells as the control cells for our microarray analysis. The cured cells are considered to have the same background as the replicon cells. The possibility remains that the genes selected in this study were obtained by the effect of IFN-α that was used to

Table 5
LightCycler RT-PCR analysis of genes whose expression levels were altered by HCV replicons

Genes	Relative mRNA expression ratio (mean ± S.D.)	
	1B-2R1/1B-2R1C	50-1/50-1C
Up-regulation		
Phosphatidylserine-specific phospholipase A1α	2.03 ± 0.09	3.09 ± 0.74
Oncostatin M receptor	2.58 ± 0.20	2.46 ± 0.49
Transgelin	0.83 ± 0.11	13.72 ± 0.56
Annexin A1	1.19 ± 0.17	4.23 ± 0.72
Down-regulation		
LMP2	0.06 ± 0.00	0.40 ± 0.12
LMP7	0.09 ± 0.02	0.33 ± 0.08
Serpin clade C	0.39 ± 0.11	0.37 ± 0.11
Solute carrier family 7	0.13 ± 0.08	0.77 ± 0.18
S100-type calcium binding protein A14	0.37 ± 0.21	0.32 ± 0.17

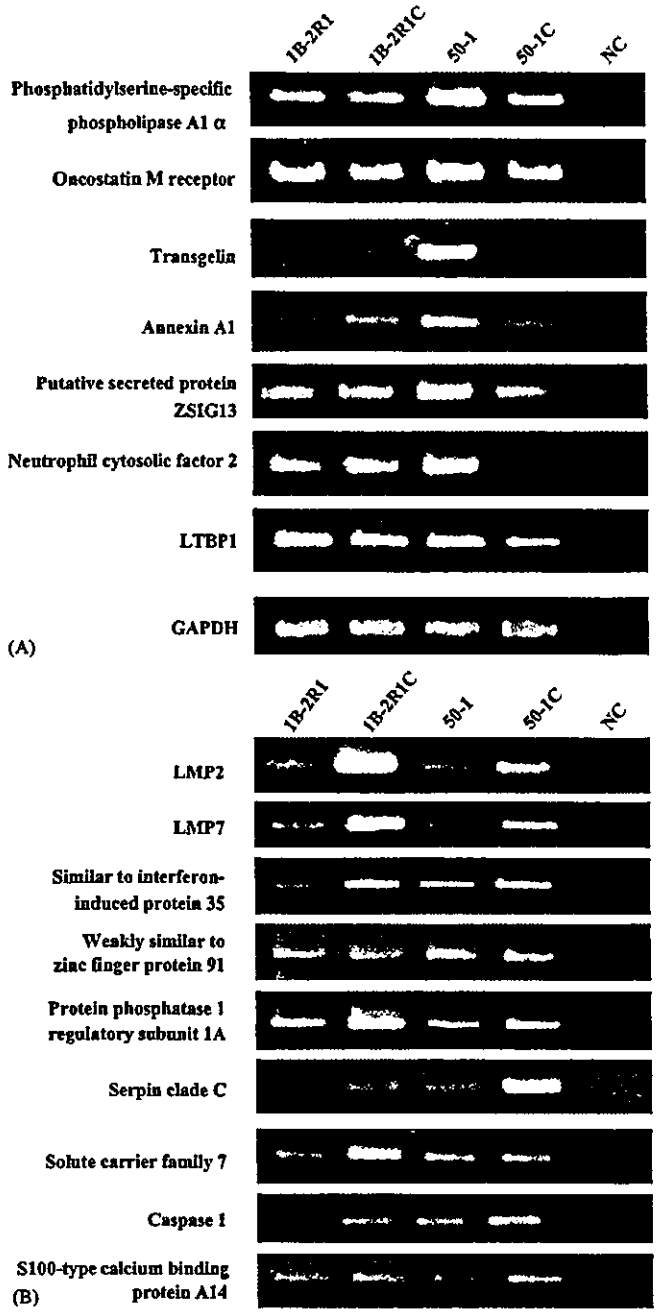

Fig. 2. RT-PCR analysis of mRNA expression of selected genes based on the microarray results. The total RNAs extracted from 50-1, 50-1C, 1B-2R1, and 1B-2R1C cells were subjected to RT-PCR, and the mRNA levels of target genes were monitored as described in Section 2. The primers used, PCR product lengths, and PCR cycle numbers are listed in Table 1. GAPDH was used as a control. (A) Up-regulated genes. (B) Down-regulated genes.

eliminate replicon RNA. However, this possibility is quite low because most of the genes regulated by IFN-α were not transcriptionally altered according to our microarray analysis, and it is unlikely that IFN-α treatment irreversibly altered the expression of genes that it either induced or suppressed. In actuality, most genes selected by our cDNA microarray analysis were not related to the genes regulated by IFN-α, although the selected genes in this study did not show any common characteristics involved in the progression of hepatic diseases. Very recently, after most of the present study was finished, a cDNA microarray analysis using full-length HCV RNA replicating cells and their cured cells was reported by Scholle et al. (2004). Although those authors found dozens of genes whose expression levels were altered by the replication of HCV RNA derived from HCV strain N, we had no information on the genes they had selected and therefore we could not compare them with ours. Therefore, we are currently establishing full-length HCV RNA replicating cells derived from 1B-1 and 1B-2 HCV strains. Further cDNA microarray analysis using such full-length HCV RNA replicating cells will help to identify HCV's target genes.

Among the genes selected in this study, LMP2 and LMP7 are quite interesting. Both are known as important catalytic subunits in immunoproteasome induced by IFN-γ (Akiyama et al., 1994; Tanaka and Kasahara, 1998). In the presence of IFN-γ, the three catalytic subunits of vertebrate proteasomes are replaced by their homologous subunits, LMP2, LMP7, and MECL1, to form immunoproteasome, which increased the ability to produce peptides with a proper motif for efficient MHC binding (Fehling et al., 1994; Van Kaer et al., 1994). A number of peptides that were poorly processed by the standard proteasome were recently found to be more effectively produced by the immunoproteasome (Van den Eynde and Morel, 2001). Therefore, down-regulation of LMP2 and LMP7 expressions in HCV subgenomic replicon cells will reduce the production efficiency of viral antigenic peptides presented to CD8+ T cells (Van den Eynde and Morel, 2001), and may subsequently help to cause the persistent viral infection. In contrast with the expression of LMP2 and LMP7, that of the MECL1 gene was not altered regardless of the presence of HCV replicon. The molecular mechanism by which HCV replicon cells suppress LMP2 and LMP7 remains unknown. This phenomenon is considered to be caused by one of the HCV NS proteins in the replicon cells. As a first step toward identifying the responsible NS protein, we carried out a preliminary experiment using 1B-2R1C cells that stably expressed NS3, NS4A, NS4B, NS5A, NS5B, or NS3-NS5B protein by retrovirus-mediated gene transfer. Unfortunately, however, this experiment failed to identify the responsible NS protein. This result suggests that either the replication of replicon RNA or replicon RNA itself is necessary to suppress LMP2 and LMP7 gene expression. To clarify this point, further analysis will be necessary, using HCV subgenomic replicon cells derived from the other HCV strains or HCV subgenomic replicon cells re-established by the transfection of 50-1 or 1B-2R1 subgenomic replicon RNA.

A third interesting gene obtained in this study was serpin clade C. Although the expression of the serpin clade C gene was down-regulated to approximately one-third in HCV subgenomic replicon cells, those of the other eight clades of the serpin family were not quite altered. Since serpins are a unique class of proteinase inhibitors that irreversibly neu-

tralize target proteinases by a mechanism that conformationally distorts the proteinase (Gettins, 2002), the relationship between serpin clade C and HCV serine proteinase is interesting. To clarify this relationship, further analysis, such as that of the compulsory expression of serpin clade C in the replicon cells, will be necessary.

In this study, we demonstrated that microarray analysis to compare HCV subgenomic replicon cells with their cured cells was useful for screening and selecting HCV's target genes. Also, we compiled the first list of genes transcriptionally regulated by the multiplication of HCV subgenomic replicons. Although we need to clarify the mechanisms underlying transcriptional regulation by HCV subgenomic replicons, we believe that the genes involved in viral replication and multiplication are among the genes listed in this study. Further analysis using new experimental systems, such as the full-length HCV RNA replicating system, will be useful to clarify this point.

Acknowledgements

We thank A. Nozaki, T. Nakamura, and A. Morishita for their helpful experimental assistance. This work was supported by grants-in-aid for research on hepatitis and BSE from the Ministry of Health, Labor, and Welfare of Japan, and by grants-in-aid for scientific research from the Organization for Pharmaceutical Safety and Research (OPSR).

References

Akiyama, K., Kagawa, S., Tamura, T., Shimbara, N., Takashina, M., Kristensen, P., Hendil, K.B., Tanaka, K., Ichihara, A., 1994. Replacement of proteasome subunits X and Y by LMP7 and LMP2 induced by interferon-gamma for acquirement of the functional diversity responsible for antigen processing. FEBS Lett. 18, 85–88.

Bartenschlager, R., 2002. Hepatitis C virus replicons: potential role for drug development. Nat. Rev. Drug Discov. 1, 911–916.

Bartenschlager, R., Lohmann, V., 2000. Replication of hepatitis C virus. J. Gen. Virol. 81, 1631–1648.

Blight, K.J., Kolykhalov, A.A., Rice, C.M., 2000. Efficient initiation of HCV RNA replication in cell culture. Science 290, 1972–1974.

Blight, K.J., McKeating, J.A., Marcotrigiano, J., Rice, C.M., 2003. Efficient replication of hepatitis C virus genotype 1a RNAs in cell culture. J. Virol. 77, 3181–3190.

Choo, Q.L., Kuo, G., Weiner, A.J., Overby, L.R., Bradley, D.W., Houghton, M., 1989. Isolation of a cDNA clone derived from a blood-borne non-A, non-B viral hepatitis genome. Science 244, 359–362.

Fehling, H.J., Swat, W., Laplace, C., Kuhn, R., Rajewsky, K., Muller, U., von Boehmer, H., 1994. MHC class I expression in mice lacking the proteasome subunit LMP-7. Science 265, 1234–1237.

Frese, M., Pietschmann, T., Moradpour, D., Haller, O., Bartenschlager, R., 2001. Interferon-a inhibits hepatitis C virus subgenomic RNA replication by an MxA-independent pathway. J. Gen. Virol. 82, 723–733.

Frese, M., Schwarzle, V., Barth, K., Krieger, N., Lohmann, V., Mihm, S., Haller, O., Bartenschlager, R., 2002. Interferon-g inhibits replication of subgenomic and genomic hepatitis C virus RNAs. Hepatology 35, 694–703.

Gettins, P.G., 2002. Serpin structure, mechanism, and function. Chem. Rev. 102, 4751–4804.

Hijikata, M., Mizushima, H., Tanji, Y., Komoda, Y., Hirowatari, Y., Akagi, T., Kato, N., Kimura, K., Shimotohno, K., 1993. Proteolytic processing and membrane association of putative nonstructural proteins of hepatitis C virus. Proc. Natl. Acad. Sci. U.S.A. 90, 10773–10777.

Ikeda, M., Yi, M., Li, K., Lemon, S.M., 2002. Selectable subgenomic and genome-length dicistronic RNAs derived from an infectious molecular clone of the HCV-N strain of hepatitis C virus replicate efficiently in cultured Huh7 cells. J. Virol. 76, 2997–3006.

Kato, N., 2001. Molecular virology of hepatitis C virus. Acta Med. Okayama 55, 133–159.

Kato, N., Hijikata, M., Ootsuyama, Y., Nakagawa, M., Ohkoshi, S., Sugimura, T., Shimotohno, K., 1990. Molecular cloning of the human hepatitis C virus genome from Japanese patients with non-A, non-B hepatitis. Proc. Natl. Acad. Sci. U.S.A. 87, 9524–9528.

Kato, N., Shimotohno, K., 2000. Systems to culture hepatitis C virus. Curr. Top. Microbiol. Immunol. 242, 261–278.

Kato, N., Sugiyama, K., Namba, K., Dansako, H., Nakamura, T., Takami, M., Naka, K., Nozaki, A., Shimotohno, K., 2003a. Establishment of a hepatitis C virus subgenomic replicon derived from human hepatocytes infected in vitro. Biochem. Biophys. Res. Commun. 306, 756–766.

Kato, T., Date, T., Miyamoto, M., Furusaka, A., Tokushige, K., Mizokami, M., Wakita, T., 2003b. Efficient replication of the genotype 2a hepatitis C virus subgenomic replicon. Gastroenterology 125, 1808–1817.

Kishine, H., Sugiyama, K., Hijikata, M., Kato, N., Takahashi, H., Noshi, T., Nio, Y., Hosaka, M., Miyanari, Y., Shimotohno, K., 2002. Subgenomic replicon derived from a cell line infected with the hepatitis C virus. Biochem. Biophys. Res. Commun. 293, 993–999.

Kuo, G., Choo, Q.L., Alter, H.J., Gitnick, G.L., Redeker, A.G., Purcell, R.H., Miyamura, T., Dienstag, J.L., Alter, M.J., Stevens, C.E., Tegtmeier, G.E., Bonino, F., Colombo, W.S., Lee, W.S., Kuo, C., Berger, K., Shuster, J.R., Overby, L.R., Bradley, D.W., Houghton, M., 1989. An assay for circulating antibodies to a major etiologic virus of human non-A, non-B hepatitis. Science 244, 362–364.

Lohmann, V., Korner, F., Koch, J., Herian, U., Theilmann, L., Bartenschlager, R., 1999. Replication of subgenomic hepatitis C virus RNAs in a hepatoma cell line. Science 285, 110–113.

Mizutani, T., Kato, N., Saito, S., Ikeda, M., Sugiyama, K., Shimotohno, K., 1996. Characterization of hepatitis C virus replication in cloned cells obtained from a human T-cell leukemia virus type I infected cell line MT-2. J. Virol. 70, 7219–7223.

Nozaki, A., Kato, N., 2002. Quantitative method of intracellular hepatitis C virus RNA using LightCycler PCR. Acta Med. Okayama 56, 107–110.

Nozaki, A., Ikeda, M., Naganuma, A., Nakamura, T., Inudoh, M., Tanaka, K., Kato, N., 2003. Identification of a lactoferrin-derived peptide possessing binding activity to hepatitis C virus E2 envelope protein. J. Biol. Chem. 278, 10162–10173.

Ohkoshi, S., Kojima, H., Tawaraya, H., Miyajima, T., Kamimura, T., Asakura, H., Satoh, A., Hirose, S., Hijikata, M., Kato, N., Shimotohno, K., 1990. Prevalence of antibody against non-A, non-B hepatitis virus in Japanese patients with hepatocellular carcinoma. Jpn. J. Cancer Res. 81, 550–553.

Saito, I., Miyamura, T., Ohbayashi, A., Harada, H., Katayama, T., Kikuchi, S., Watanabe, Y., Koi, S., Onji, M., Ohta, Y., Choo, Q.L., Houghton, M., Kuo, G., 1990. Hepatitis C virus infection is associated with the development of hepatocellular carcinoma. Proc. Natl. Acad. Sci. U.S.A. 87, 6547–6549.

Scholle, F., Li, K., Bodola, F., Ikeda, M., Luxon, B.A., Lemon, S.M., 2004. Virus-host cell interactions during hepatitis C virus RNA replication: impact of polyprotein expression on the cellular transcriptome and cell cycle association with viral RNA synthesis. J. Virol. 78, 1513–1524.

Tanaka, K., Kasahara, M., 1998. The MHC class I ligand-generating system: roles of immunoproteasomes and the interferon-gamma-inducible proteasome activator PA28. Immunol. Rev. 163, 161–176.

Tanaka, T., Kato, N., Cho, M.-J., Shimotohno, K., 1995. A novel sequence found at the 3' terminus of hepatitis C virus genome. Biochem. Biophys. Res. Commun. 215, 744–749.

Van den Eynde, B.J., Morel, S., 2001. Differential processing of class-I-restricted epitopes by the standard proteasome and the immunoproteasome. Curr. Opin. Immunol. 13, 147–153.

Van Kaer, L., Ashton-Rickardt, P.G., Eichelberger, M., Gaczynska, M., Nagashima, K., Rock, K.L., Goldberg, A.L., Doherty, P.C., Tonegawa, S., 1994. Altered peptidase and viral-specific T cell response in LMP2 mutant mice. Immunity 1, 533–541.

Zhu, H., Zhao, H., Collins, C.D., Eckenrode, S.E., Run, Q., McIndoe, R.A., Crawford, J.M., Nelson, D.R., She, J.X., Liu, C., 2003. Gene expression associated with interferon alfa antiviral activity in an HCV replicon cell line. Hepatology 37, 1180–1188.

Available online at www.sciencedirect.com
SCIENCE DIRECT®
Biochemical and Biophysical Research Communications 323 (2004) 299–309
BBRC
www.elsevier.com/locate/ybbrc

Establishment of hepatitis C virus replicon cell lines possessing interferon-resistant phenotype

Katsuyuki Namba[a,b,1], Kazuhito Naka[a,1], Hiromichi Dansako[a], Akito Nozaki[a], Masanori Ikeda[a], Yasushi Shiratori[b], Kunitada Shimotohno[c], Nobuyuki Kato[a,*]

[a] *Department of Molecular Biology, Okayama University Graduate School of Medicine and Dentistry, 2-5-1 Shikata-cho, Okayama 700-8558, Japan*
[b] *First Department of Internal Medicine, Okayama University Graduate School of Medicine and Dentistry, 2-5-1 Shikata-cho, Okayama 700-8558, Japan*
[c] *Department of Viral Oncology, Institute for Virus Research, Kyoto University, 53 Kawara-cho Shogo-in, Sakyo-ku, Kyoto 606-8507, Japan*

Received 21 June 2004
Available online 28 August 2004

Abstract

To clarify the mechanism underlying resistance to interferon (IFN) by the hepatitis C virus (HCV) in patients with chronic hepatitis, we attempted to develop an IFN-resistant HCV replicon from the IFN-sensitive 50-1 replicon established previously. By treating 50-1 replicon cells with a prolonged low-dose treatment of IFN-α and then transfecting the total RNA derived from the IFN-α-treated replicon cells, we successfully obtained four clones (named 1, 3, 4, and 5) of HCV replicon cells that survived against IFN-α (200 IU/ml). These cloned cells were further treated with IFN-α or IFN-β (increased gradually to 2000 or 1000 IU/ml, respectively). This led to four replicon cell lines (αR series) possessing the IFN-α-resistant phenotype and four replicon cell lines (βR series) possessing the IFN-β-resistant phenotype. Furthermore, we obtained an additional replicon cell line (αRmix) possessing the IFN-α-resistant phenotype by two rounds of prolonged treatment with IFN-α and RNA transfection as mentioned above. Characterization of these obtained HCV replicon cell lines revealed that the βR series were highly resistant to both IFN-α and IFN-β, although the αR series containing αRmix were only partially resistant to both IFN-α and IFN-β. Genetic analysis of these HCV replicons found one common amino acid substitution in the NS4B and several additional amino acid substitutions in the NS5A of the βR series, suggesting that these genetic alterations are involved in the IFN resistance of these HCV replicons. These newly established HCV replicon cell lines possessing IFN-resistant phenotypes are the first useful tools for understanding the mechanisms by which HCV acquires IFN resistance in vivo.

Keywords: Hepatitis C virus; Huh-7; Replicon; Interferon resistance

Persistent infection with the hepatitis C virus (HCV) is a major cause of chronic hepatitis (CH) [1,2], which progresses to liver cirrhosis (LC), and hepatocellular carcinoma (HCC) [3,4]. Since approximately 170 million individuals are estimated to be infected with HCV worldwide, this infection is a global health problem [5]. HCV belongs to the family *Flaviviridae*, whose genome consists of a positive-stranded 9.6 kilobase (kb) RNA and encodes a large polyprotein precursor of about 3000 amino acid residues [6,7]. This polyprotein is processed by a combination of the host and viral proteases into at least ten proteins: the core, envelope 1 (E1), E2, p7, nonstructural protein 2 (NS2), NS3, NS4A, NS4B, NS5A, and NS5B. Six major HCV genotypes have been classified as HCV-1a, -1b, -2a, -2b, -3a, and -3b [8].

To prevent the progression to CH, LC, and HCC, it is essential to eliminate HCV immediately from the

* Corresponding author. Fax: +81 86 235 7392.
E-mail address: nkato@md.okayama-u.ac.jp (N. Kato).
[1] Both authors contributed equally to this work.

0006-291X/$ - see front matter © 2004 Elsevier Inc. All rights reserved.
doi:10.1016/j.bbrc.2004.08.091

human body. Thus far, however, the only effective anti-HCV reagents used in current clinical therapy are interferon (IFN)-α and IFN-β. Moreover, IFN's effectiveness is limited to about 30% of the reported cases [9], although combined treatment of IFN and ribavirin has been found more effective (though still less than 50%) than treatment with IFN alone [10]. These clinical results suggest that HCV is rather resistant to the antiviral actions of IFN, and that HCV proteins directly or indirectly attenuate those actions [11].

Although many hypotheses have been proposed regarding the mechanisms of HCV's resistance to IFN [8,12], the lack of reproducible and efficient HCV proliferation in cell culture has been a serious obstacle to the clarification of such mechanisms [13].

In 1999, an HCV replicon system carrying autonomously replicating HCV subgenomic RNA containing the NS3–NS5B regions was first established using a human hepatoma cell line, Huh-7 [14]. Since then, several additional replicon systems, including ours (50-1 and 1B-2R1 replicons), have been established [15–20]. Recently, HCV replicons that autonomously replicate in human cervical carcinoma HeLa, human embryonic kidney 293, or mouse hepatoma cells have been introduced [21,22]. In these systems, replicated HCV RNAs and HCV proteins were detected by Northern and Western blot analyses, respectively. HCV replicon systems have become a powerful tool for basic studies of HCV, such as viral replication, virus–host interactions, and drug development [23]. Therefore, HCV replicon systems have been considered useful for clarifying the mechanisms underlying HCV's resistance to IFN.

However, unexpectedly, all HCV replicons established to date are found to be highly sensitive to IFN-α, IFN-β, and IFN-γ [19,24–27]. The mechanisms by which HCV replicons regulate the IFN-sensitive phenotype have not yet been clarified, although recent studies have proposed the involvement of proteasome subunits and ubiquitin-like proteins induced in replicon cells treated with IFN-α or IFN-γ [27,28]. The fact that HCV replicons are highly sensitive to IFNs seems to contradict the fact that more than 50% of patients with CH are resistant to current IFN therapy [10]. The elimination of this wide gap will contribute to the development of a method to eliminate HCV from the human body in vivo. Thus, we speculated that some stimuli might prompt IFN-sensitive HCV replicons to change into the IFN-resistant phenotype. According to this speculation, we attempted to develop IFN-resistant HCV replicons by a prolonged low-dose treatment of IFN against our established 50-1 replicon cells (termed 50-1 cells) [17].

Here, we report the successful establishment of HCV replicon cell lines possessing the IFN-resistant phenotype. We have also found several genetic alterations observed in only their HCV replicons.

Materials and methods

Cell cultures. Huh-7 and 50-1 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum. Our 50-1 cells were cultured in the presence of G418 (300 μg/ml; Geneticine, Invitrogen). The 50-1 cells were known to possess the G418-resistant phenotype, because neomycin phosphotransferase (Neo) was produced by the efficient replication of HCV replicon in the cells. Therefore, when an HCV replicon is excluded from the cells or its level is decreased, the cells are killed by the presence of G418.

IFN treatment. For the initial treatment with IFN-α, 50-1 cells were plated onto six-well plates (1×10^5 cells/plate) and were cultured for one day immediately before IFN treatment. Human IFN-α (Sigma) was added to the cells at a final concentration of 1, 10, 100, or 1000 IU/ml, as described previously [19]. When the cells reached condition of confluence, they were passaged with several-fold dilutions. These cell cultures were continued for five months with the further addition of IFN-α at 5–6 day intervals. For further treatment with IFN-α, the replicon cells were plated onto 10 cm plates (1×10^6 cells/plate) and were cultured for one day immediately before IFN treatment. IFN-α was added to the cells at 4-day intervals, and the concentration of IFN-α was increased step by step to 400, 600, 800, 1000, and 2000 IU/ml. Human IFN-β (a gift from Toray Industries, Tokyo, Japan) was also added to the cells step by step at 4-day intervals, from concentrations of 400–600, 800, and 1000 IU/ml. The incubation was continued until apparent IFN-resistant colonies formed on the culture plates (in general, approximately one month). The analysis of the HCV replicon's sensitivity to IFN was performed as described previously [19]. Briefly, HCV replicon cells were plated in duplicate onto six-well plates (1×10^5 cells/plate) and were cultured for one day immediately before IFN treatment. IFN-α or IFN-β was added to the cells at a final concentration of 1, 10, 100, 500, 1000, or 2000 IU/ml, and incubation was continued. The cells were harvested 48 h after IFN treatment for the semi-quantitative analysis of HCV replicon RNA, or they were harvested five days after IFN treatment for the Western blot analysis of HCV proteins.

RNA transfection and selection of G418-resistant cells. RNA transfection into Huh-7 cells was performed by electroporation as described by Lohmann et al. [14]. Cells were selected in complete DMEM containing 300 μg/ml G418 as described previously [19].

Northern blot analysis. Total RNAs from the cultured cells were prepared using the RNeasy extraction kit (Qiagen). Three micrograms of total RNA was used to detect the HCV replicon RNA and β-actin. Northern blotting and hybridization were performed as described previously [19,29]. RNA Ladder (Invitrogen) was used to mark molecular length.

Western blot analysis. The preparation of cell lysates, sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE), and immunoblotting analysis with a polyvinylidene difluoride membrane were all performed as previously described [30]. The antibodies used to examine the expression levels of HCV proteins were those against NS3 (Novacastra Laboratories, UK), NS5A [30], and NS5B (a generous gift from M. Kohara, Tokyo Metropolitan Institute of Medical Science). Anti-β-actin antibody (AC-15, Sigma) was also used to detect β-actin as the internal control. To monitor the expression levels and phosphorylation status of the components involved in the IFN signal transduction pathway, HCV replicon cells were cultured for 30 min with or without IFN-α (500 IU/ml), and then cell lysates were used for immunoblotting analysis. Anti-JAK1, Tyk2, STAT1, STAT2, and STAT3 antibodies (BD Transduction Laboratories, Lexington, KY) were used to detect JAK1, Tyk2, STAT1, STAT2, and STAT3, respectively. Anti-*p*-JAK1(Tyr1022/1023) (Sigma), *p*-Tyk2(Tyr1054/1055), *p*-STAT1(Tyr701) (Cell Signaling Technology, Beverly, MA), *p*-STAT2(Tyr689) (Upstate Biotechnology, Lake Placid, NY), and *p*-STAT3(Tyr705) (Cell Signaling Technology) antibodies were used to