[6], differential display (DD) [7], suppression subtractive hybridization (SSH) [8], and cDNA microarray analysis [9]. Among them, SSH can be performed with a small amount of clinical sample and allows us to use needle biopsy materials, circumventing the need for large mass of sample, such as surgically resected liver tissue. Thus, SSH is a suitable method to analyze gene expression profiles of liver diseases for which surgically resected livers or explants prior to transplantation usually are unavailable. Using SSH, we and other groups have successfully identified characteristic gene expression profiles in hepatocellular carcinoma [10], chronic hepatitis C [11], and autoimmune hepatitis [12].

In the present study, we took advantage of SSH to analyze the gene expression profile of a PBC liver and found over-expressed genes encoded by mitochondrial DNA. The expression of mitochondrial genes is regulated by molecules encoded by nuclear DNA, in particular, mitochondrial transcription factor A (mtTFA), which is known to principally promote mitochondrial replication and transcription [13,14], nuclear respiratory factor-1 (NRF-1) [15] that binds to and activates the mtTFA promoter [16], and the NRF-1 coactivator, PGC-1 [17], which is involved in thermogenesis or glucogenesis induced by increased energy demands from external stimuli, such as cold or fasting, respectively. To date, little is known about the expression of these mitochondria regulatory genes in human liver diseases, including PBC. Thus, we examined the expression of these molecules controlling mitochondrial biogenesis in various liver diseases, in order to define the mechanism of mitochondrial gene up-regulation, found in PBC liver by SSH.

2. Methods

2.1. SSH for screening of overexpressed genes in PBC liver

The patient was a 53-year-old female without a history of viral hepatitis, excess ethanol intake, or administration of hepatotoxic drugs. Her laboratory data showed an elevated serum alkaline phosphatase value and a strongly positive test for anti-mitochondrial antibodies. Histological diagnosis of a liver biopsy specimen was PBC at Scheuer's stage II. The liver biopsy sample of about 3-mm long with a net weight of ~50 mg) was used for SSH analysis. As a control, histologically normal liver tissue (more than 5 cm from the tumor) was obtained at surgery from a patient with metastatic liver cancer. Written informed consent was obtained from each patient for liver biopsy and the study protocol, which conformed to the ethical guidelines of the 1995 Declaration of Helsinki, and for which institutional approval also was obtained.

RNA was extracted by the acid–guanidium–phenol–chloroform method. Briefly, 150 μl of serum was mixed with 700 μl of ISOGEN (Wako Pure Chemical Industries, Osaka, Japan), and the aqueous phase was collected with 140 μl of chloroform. The RNA was precipitated with isopropanol, and the pellet was washed with ethanol, and finally dissolved in 10 μl of double distilled water at a final concentration of 1 μg/μl. Subsequently, double-strand full-length cDNA was synthesized using a SMART™ PCR cDNA synthesis kit (CLONTECH, Tokyo, Japan), according to the manufacturer's instruction.

SSH was performed using a PCR-Select™ cDNA subtraction kit (CLONTECH, Tokyo, Japan) according to the manufacturer's instructions. Briefly, after two different adaptors were ligated to *Rsa*I-digested SMART™ cDNA from the patient (tester), 2.5 ng of each adaptor-ligated SMART™ cDNA was hybridized with 1.5 μg of *Rsa*I-digested SMART™ cDNA from the normal liver (driver). In this process, cDNA sequences specific to the tester were enriched.

2.2. Quantitative competitive RT-PCR of the genes for the mitochondrial regulatory system

We analyzed the mRNA expression of three genes: nuclear respiratory factor-1 (NRF-1), mitochondrial transcription factor A (mtTFA), and PPAR gamma coactivator 1(PGC-1) in 60 liver biopsy samples from various liver diseases, which were obtained before initiations of any medications. In all cases, the histological stages of the lever were in the chronic stage of cholangitis or hepatitis, and had not developed cirrhosis. Quantification of hepatic gene expression was performed by competitive RT-PCR. Because conventional RT-PCR needs a relatively large amount of sample, we utilized SMART cDNA reported to reflect the sample's original complexity and the relative abundance of the original RNA sample [18]. Primer sequences for amplification and synthesis of the competitors are as follows: NRF-1 sense primer, 5′-CATGCGTTGAGCTACTGACA-3′; NRF-1 antisense primer, 5′-TATACGAGCAATTCAGGACT-3′; NRF-1 competitor sense primer, 5′-CATGCGTTGAGCTACTGACA-CTGCTGGACTCACCTGAGGA-3′. mtTFA sense primer, 5′-TTTACCGAGGTGGTTTTCATCTGTC-3′; mtTFA antisense primer, 5′-GATAACGAGTTTCGTCCTCTTTAGC-3′; mtTFA competitor sense primer, 5′-TTTACCGAGGT-GGTTTTCATCTGTCACAGAACTAATTAGAAGAATTGC-3′. PGC-1 sense primer, 5′-CGGGCACCAGAATGC-TAAAGTTTCC-3′; PGC-1 antisense primer, 5′-AAGGCTGCATTTACAGTGCATAGCT-3′; PGC-1 competitor sense primer, 5′-CGGGCACCAGAATGCTAAA-GTTTCCCGGGCACCAGAATGCTAAAGTTTCCGTCC-AAAATGTTGATCTTCCA-3′. PCR products were electrophoresed on 2.5% agarose gels and stained by ethidium bromide. Gel images were captured, and the intensity of each band was analyzed by computer software (Diversity Database, ver. 1.1, pdi, New York, NY) to calculate of the signal ratio. The expression of each gene in PBC liver was compared with that in other liver disease using the Mann–Whitney *U*-test (version 4.5, Statview, Abacus, Berkeley, CA).

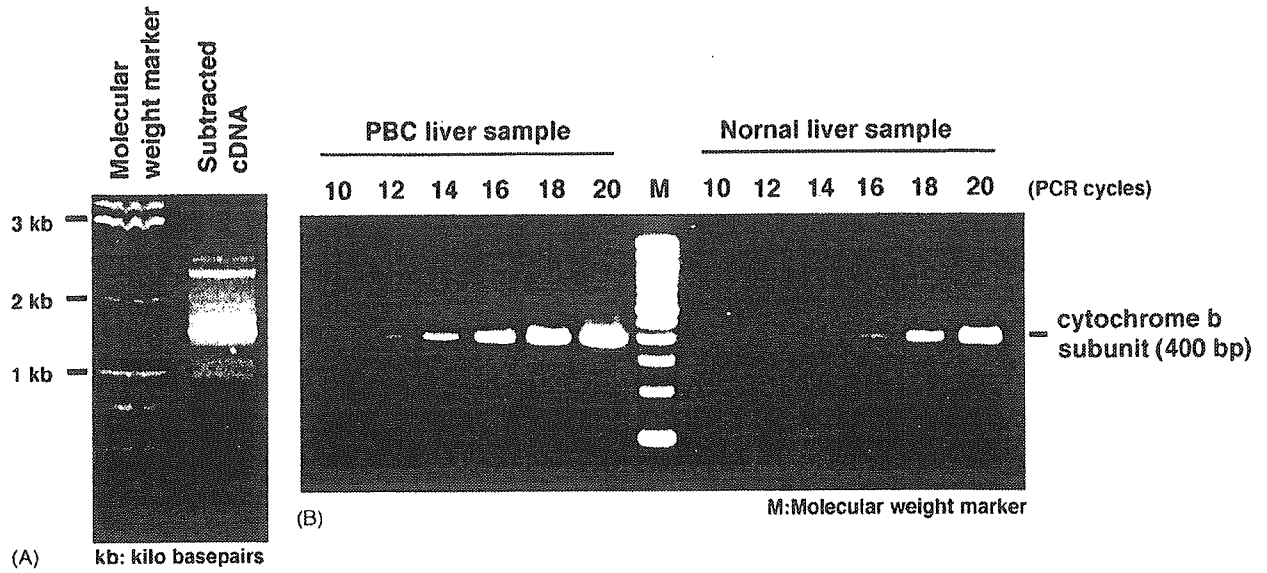

Fig. 1. *Panel A*: electrophoretic image of the subtracted PBC cDNA. Genes overexpressed in PBC are shown as oligoclonal bands. *Panel B*: semi-quantitative PCR to the expression of mitochondrial cytochrome *b* subunit. PCR signal of the PBC is clearly seen earlier than that of the normal. The signal of the PBC is seen in 12 cycles of PCR amplification, and that of the normal is clearly seen in 16 cycles.

3. Result

3.1. Overexpressed genes in a PBC liver sample

Using SSH with PBC and normal liver, we obtained several oligoclonal bands (Fig. 1A) and analyzed 100 clones obtained from the PCR products (Table 1). These genes were recognized as overexpressed in PBC liver. To confirm overexpression of these genes, we used semi-quantitative RT-PCR for the expression of the mitochondrial cytochrome *b* subunit. A PCR signal from the PBC liver was clearly seen after 12 cycles of amplification, but that from the normal liver could be seen only after 16 cycles (Fig. 1B). This result showed that the target mRNA was overexpressed by four cycles because the expression levels of a house-keeping gene, glycelaldehyde-3 phosphate dehydrogenase, were similar between these two samples (data not shown). It may be seen that mRNA transcribed from mitochondrial DNA was more abundant in the PBC liver than the normal liver. Thus, we subsequently proceeded to evaluate the expression of genes that control the transcription and replication of mitochondrial DNA.

Table 1
Sequence analysis of 100 clones isolated from subtracted PBC cDNA

Name of the clones	No.
Mitochondrial 12s rRNA[a]	13
Cytochrome *b*[a]	12
Mitochondrial tRNA (Val)[a]	6
Heat shock protein 90	3
Cytochrome *c* oxidase (II)[a]	2
HLA-DR alpha chain	2
Miscellaneous	62
Total	100

[a] cDNA derived from mitochondrial DNA-encoded genes.

3.2. Analysis by quantitative competitive PCR of expression of genes regulating mitochondrial transcription in liver tissues

We analyzed the mRNA expression of three genes: NRF-1, mtTFA, and PGC-1 in 60 liver biopsy samples from various liver diseases. NRF-1 and mtTFA expression levels were significantly higher in PBC liver than in other liver diseases (Fig. 2A and B). In contrast, PGC-1 mRNA expression was significantly suppressed in the PBC liver compared to that in the other liver diseases (Fig. 2C).

4. Discussion

In our present study, following the results of subtraction cloning that a variety of mitochondrial DNA-encoded genes were overexpressed in the PBC liver, we demonstrated that the expression of mtTFA, which are encoded by nuclear DNA and directs mitochondrial transcription and replication, and its transactivator NRF-1 was increased in the PBC liver compared to other liver diseases, while that of the NRF-1 coactivator PGC-1 was suppressed. These results indicate that the mitochondrial transcriptional factors mtTFA and NRF-1 are up-regulated in PBC liver, which may lead to the overexpression of mitochondrial genes.

Among 100 cDNA clones obtained from PBC cDNA subtracted from normal liver cDNA, six genes, i.e., mitochondrial 12S rRNA, cytochorome *b*, mitochondrial tRNA-Val,

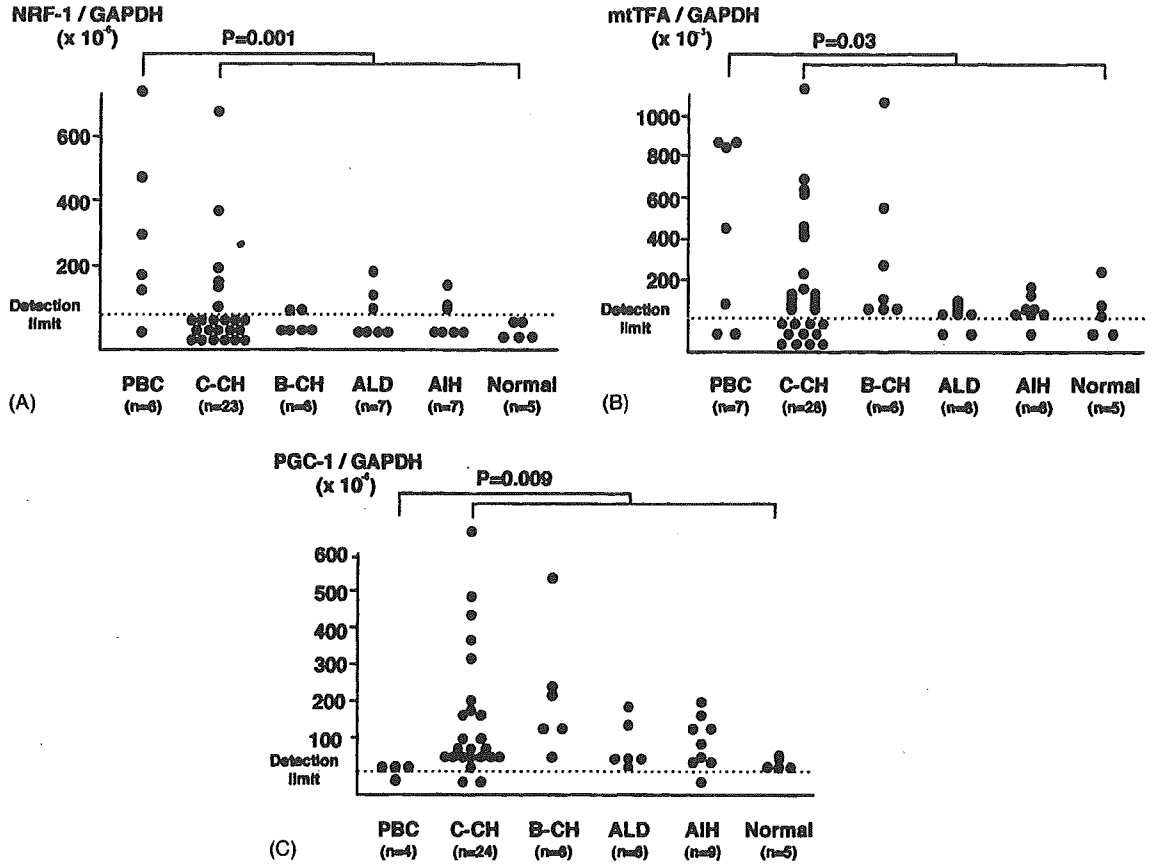

Fig. 2. Expression levels of mRNA, which are related to mitochondrial regulatory genes in PBC and other liver diseases. *Panel A*: the expression levels of NRF-1 mRNA; NRF-1 mRNA in the PBC liver are significantly higher than that in other liver diseases ($P=0.0015$). *Panel B*: the expression levels of mtTFA mRNA; mtTFA mRNA in the PBC liver is significantly higher than that in other liver diseases ($P=0.031$). *Panel C*: the expression levels of PGC-1 mRNA; PGC-1 mRNA is suppressed in the PBC liver, as it compared with other liver diseases ($P=0.009$). Due to the usage of biopsied materials, very small amounts of samples were available for each experiment limitting the number of repeated assays. In some cases, reproducible data for all of the three genes could not be obtained, and the number of cases is not uniform for these three genes.

cytochrome *c* oxidase, heat shock protein 90, and HLA-DR were found repeatedly indicating that these transcripts were abundant in the subtracted cDNA. Notably, the first four genes are encoded by mitochondrial DNA, and increased expression of these genes was confirmed by semi-quantitative RT-PCR. Heat shock proteins recently were found to be over-expressed in the PBC liver using a cDNA microarray and immunohistochemistry [19,20], and enhanced expression of the HLA-DR molecule in PBC liver is well established [21]. Thus, the result of SSH of PBC was not only consistent with earlier works but also revealed the increased expression of mitochondrial genes that previously was not appreciated.

Each human cell contains hundreds to thousands of mitochondria, carrying 2–10 copies of the 16 kb double-stranded circular mitochondrial DNA, which encodes the 13 mRNAs, 2 rRNAs, and 22 tRNAs, which are necessary for its function, i.e., energy production by ATP synthesis [22]. Increase in the number of mitochondria has been observed in a variety of physiological states demanding energy, such as cell growth and proliferation, aging, muscle contraction, and thermogenesis in response to cold [17]. Replication of mitochondrial DNA and transcription of mtDNA-encoded genes are tightly linked and result in mitochondrial proliferation or as it is called biogenesis, because mtDNA replication needs RNA transcripts as primers, and both are controlled by a single nuclear DNA-expressed transcriptional factor, mtTFA which binds to the mitochondrial promoter [14]. Moreover, expression of each mitochondrial gene is not regulated individually because large polycistronic transcripts are produced from each strand and are processed to generate mature RNAs [14]. Therefore, the increase in transcription of the four mitochondrial RNAs observed in PBC seems to be a reflection of an overall increase of polycistronic mtRNA transcription, rather than through other mechanisms, such as decreased degradation of individual mitochondrial transcripts. In the previous reports, electron microscopic observation of hepatocyte in PBC liver showed increased mass of mitochondria [23,24] confirming enhanced mitochondrial biogenesis.

Consequently, to determine the mechanism of the increase of mitochondrial gene transcription in PBC, we have analyzed

the expression levels of nuclear-encoded regulatory factors in liver biopsy specimens from various diseases, and found that the mtTFA and NRF-1 genes were increased in PBC liver. It is well known that the sensitivity of the subtraction cloning is not high enough to detect overexpressed genes completely. Therefore, it is not surprising that SSH did not identify NRF-1, mtTFA, and PGC-1 from PBC cDNA as overexpressed genes. Instead, subtraction of many mitochondrial genes strongly suggests that mitochondrial biogenesis, which is regulated by the above mentioned transcription factors, are enhanced. Unfortunately, we could not investigate the expression levels of mtDNA except for the index case due to the limitation of the samlple amounts, mtTFA known to be essential for the maintenance, replication and transcription of mtDNA [25] since mtTFA knockout mice exhibit a severe depletion of mtDNA, and an association between mitochondrial gene expression and mtTFA has been assumed in the literature [26]. Taken together, although direct evidences that mtTFA itself increases the mitochondrial mass is still lacking, the present results suggest that mtTFA overexpression may be a cause of the increased mitochondrial transcription detected in the PBC liver.

The increased NRF-1 mRNA levels in PBC than in other liver diseases, which we have found in the present study, suggest that the increased expression of NRF-1 may be responsible for the transcriptional activiation of mtTFA, leading to over-expression of mitochondrial genes. Although expression levels of mtTFA and NRF-1 in several PBC were lower than other liver diseases, the results of SSH for an index PBC case have shown that mtTFA or NRF-1 are not necessarily overexpressed in all cases of PBC investigated. On the other hand, we could not detect the up-regulation of the coactivator PGC-1, which binds and enhances NRF-1 through recruiting other transcriptional activators, and which leads to enhanced mitochondrial gene expression and biogenesis [17]. External stimuli, such as cold exposure or fasting, markedly upregulate PGC-1 mRNA [27]. However, PGC-1 mRNA levels were rather lower in PBC, suggesting that mitochondrial genes may be induced by a PGC-1 independent pathway, and that its expression seems to be suppressed in a compensatory manner. The question whether the PGC-1-regulated mitochondrial gene expression pathway is specific for PBC remains further verification. When Mann–Whitney *U*-test was used to test differences among disease groups, *P* values for the difference between PBC and B–CH ($P=0.2$) or C–CH ($P=0.1$) did not reach a statistical significance (Fig. 2B). Although expression levels of these three genes were not uniform among various diseases, the number of sample for each disease group seems too small to draw any firm conclusion, whether the expression levels are different between PBC and each disease group. Similarly, comparison of mitochondrial gene expression in PBC and other cholestatic diseases remains unanswered in the present study. We have preliminarily examined one case of primary sclerosing cholangitis in which mRNA levels of mtTFA and NRF-1 were lower (data not shown).

What is the mechanism of NRF-1 and mtTFA overexpression in the PBC liver? In pathological conditions, proliferation of mitochondria is known to be stimulated by reactive oxygen species (ROS) [28], which are produced mostly in the mitochondria as by-products of cellular respiration [29]. ROS production is associated with liver damage and has been reported to be increased in chronic liver diseases, especially in PBC [30]. Therefore, one possible explanation for the increased mitochondrial gene expression is stimulation of mitochondrial biogenesis by ROS. This is consistent with the finding that a proportion of the patients in the present study with chronic hepatitis and alcoholic liver disease also showed an increase in mtTFA and NRF-1 to the same level as in PBC [31]. In addition, mtDNA is particularly susceptible to mutation [32]. Its replication accelerated by ROS leading to impaired respiratory function and resulting in further production of ROS and accumulation of mutations as seen in the aging process [33,34] or in cancer cells [35]. Moreover, recently, tissue-specific mtDNA mutations has been reported in human buccal cells and cardiomyocytes, raising the possibility that tissue specific clonal expansion of mtDNA with somatic mutations is associated with the pathophysiological state in human [36]. Thus, if mtDNA in PBC liver primarily has some somatic mutations causing impaired function, increased ROS production might promote mitochondrial biogenesis. In this respect, mutational analysis of mtDNA in the PBC liver is of interest.

The abnormal mitochondrial biogenesis in PBC in the present study is noteworthy because there are two kinds of inexplicable association between PBC and mitochondria. First, in most PBC patients, anti-mitochondrial antibodies (AMA) are specifically positive [3], targeting components of mitochondrial proteins, such as PDH-E2 [4,5]. The cellular immune response against the major histocompatibility complex (MHC) bound PDH-E2-derived peptide on biliary epithelial cells is postulated to be involved in biliary epithelial cell injury [2]. Therefore, mitochondrial proteins act as autoantigens in PBC, but the mechanism of the immune reaction directed to mitochondrial proteins remains unclear. UDCA is thought to act as an anti-apoptotic agent, reducing mitochondrial permeability transition (MPT) and cytochrome *c* release and inhibiting mitochondria mediated apoptosis by hydrophobic bile acids [23,37–40]. This suggests that mitochondrial injury plays an important role in the pathogenesis of PBC, and this may be related to abnormal mitochondrial biogenesis.

As gene expression analyses in this study utilized homogenized needle biopsy samples of liver, it is unknown which type of cells in the liver are responsible for the changes observed in the expression of mitochondria related genes: i.e., hepatocytes, biliary epithelial cells, inflammatory cells, or other lineages of cells. The present study was conducted under the assumption that the major part of the overexpressed mitochondrial gene is derived from hepatocytes since most of liver cells are hepatocytes. However, to address this issue, analysis of separated cell populations by laser-capture

microdissection or density centrifugation, in situ hybridization or other technology may be necessary. Recently, two reports of comprehensive analysis of gene expression in the PBC liver were published, one used cDNA microarray analysis with a whole liver homogenate [20], and the other used SSH with isolated biliary epithelial cells derived from an explanted liver [41]. These studies have also reported overexpression of mitochondrial genes. Both reports mentioned the possible involvement of other genes, especially of the Wnt and Notch pathway, for which we did not demonstrate overexpression. This may be due in part to differences in disease stage between these studies and ours. The analysis of explanted liver could be influenced by non-specific pathological damage in end-stage liver disease. In contrast, our study using biopsy samples from an earlier stage is capable of detecting changes more essential and specific to the manifestation of PBC.

In conclusion, increased expression of nuclear DNA-encoded mitochondrial DNA transcription factor mtTFA, and its transactivator NRF-1 was detected in the biopsy samples of PBC liver, along with overexpression of mitochondrial DNA-encoded mRNAs. The significance of this abnormality in mitochondrial biogenesis in the pathogenesis of PBC warrants further investigation.

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Characteristic sequence changes of hepatitis C virus genotype 2b associated with sustained biochemical response to IFN therapy

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SUMMARY. In hepatitis C virus (HCV) genotype 2b infection, viral eradication (sustained viral response; sVR) is obtained in about 40% by interferon monotherapy, whereas a considerable proportion of non-sVR patients exhibit sustained biochemical response (sBR) showing normal biochemical values despite persistent viraemia. However, the mechanism of sBR has not yet been established. In this study, we analysed serial changes in full-length sequences of HCV genotype 2b before and after interferon (IFN) therapy in five patients with sBR and five with no response (NR; persistent viraemia and abnormal biochemical values after IFN therapy). The overall substitution rate of amino acids in the full-length HCV genome was higher in the sBR group than in the NR group [2.22 ± 0.48 (10^{-3} changes/site/year) *vs* 1.04 ± 0.30: $P = 0.002$]. When the genetic changes were analysed for individual HCV proteins, the sBR group had significantly higher substitution rates of amino acid in NS4A [8.82 ± 2.80 (10^{-3} changes/site/year) *vs* 0: $P = 0.001$]. These amino acid changes in sBR were mainly located in the binding motifs of HLA class I molecules including those frequently found in the Japanese population. These results demonstrated that the greater amino acid changes of HCV arising during interferon therapy are associated with the establishment of sBR. Although functional significance of these changes awaits further investigation, the finding that amino acid changes in NS4A in sBR patients are mainly located in the HLA class I binding motifs illustrated the potential roles of the escape mutations of HCV genome from CTLs in the decreasing activities of hepatitis in sBR.

Keywords: chronic hepatitis C, direct sequencing, hepatitis C virus genotype 2b, NS4A, sustained biochemical response.

INTRODUCTION

Hepatitis C virus (HCV) is a single-stranded RNA virus that consists of approximately 9500 nucleotides [1–3] and causes chronic hepatitis in humans and chimpanzees. Chronic HCV infection can result in liver cirrhosis and hepatocellular carcinoma (HCC) over the course of 20–30 years [4]. To date, interferon (IFN) alone or combined with ribavirin is the only curative therapy for chronic hepatitis C [5]. However, its efficacy is as low as 30%, even when a sufficient amount of IFN is administered [6].

A sustained virological response (sVR) to IFN therapy is defined as the elimination of the virus with sustained normal serum alanine aminotransferase (ALT) levels more than 6 months after therapy. Among the remaining patients with persistent viraemia, some demonstrate sustained normal ALT values over the long-term follow-up period, known as a sustained biochemical response (sBR). On the other hand, nonresponse (NR) is defined as persistently abnormal ALT values and viraemia after IFN therapy. The risk of the development of HCC is reported to be reduced greatly for patients with sBR, as low as those with sVR, suggesting the essential role of continuous necro-inflammation of the liver parenchyma in hepatocarcinogenesis in chronic hepatitis C [7,8]. Thus, the pathophysiology of sBR seems to be important for better understanding and control of hepatitis and carcinogenesis caused by HCV.

Abbreviations: ALT, alanine aminotransferase; HCC, hepatocellular carcinoma; HCV, hepatitis C virus; HVR, hypervariable region; IFN, interferon; NCR, noncoding region; NR, nonresponse; PCR, polymerase chain reaction; sBR, sustained biochemical response; sVR, sustained virological response.

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The mechanism of sBR has not been clarified to date, but we have already demonstrated the association between variation in the HCV genome and the severity of hepatitis in various clinical settings [9–11]. These findings indicate that specific sequence elements in the HCV genome could affect the severity of hepatocyte injury through different mechanisms, such as modulating virus replication [12], viral–host protein interaction [13–19], and the immune response to infected hepatocytes [20,21]. Therefore, determination of changes in the HCV genome in sBR during IFN treatment should be helpful in establishing the mechanisms of sBR and hepatocyte injury. Although HCV genotype 2b is responsible for only 8% of chronic hepatitis C in Japan [22], sBR is observed frequently with this genotype. Thus, to define the virological characteristics associated with sBR, we analysed the changes in full-length sequences of HCV genotype 2b during an initial course of IFN therapy.

PATIENTS AND METHODS

Patients

Ten patients with chronic HCV infection were studied. All were positive for serum HCV antibodies (third-generation assay) and HCV-RNA of genotype 2b [23,24]. Concentrations of serum HCV-RNA were determined by branched chain DNA assay [25] (Quantiplex HCV-RNA, Chiron, Emeryville, CA, USA). The limit of detection of the assay was 0.5 million-genome equivalents per millilitre. The patients were negative for serum hepatitis B surface antigen, anti-hepatitis B core antibodies and antinuclear antibodies and had no other causes of hepatitis including excessive alcohol intake or hepatotoxic drugs.

Liver biopsies were performed on all patients before IFN therapy, and the presence of chronic active hepatitis was confirmed histologically. Written informed consent to liver biopsy was obtained from each patient, and the study protocol conformed to the ethical guidelines of the 1975 Declaration of Helsinki. Institutional approval was obtained.

All patients received IFN for 6 months at a dose of 6–10 million units intramuscularly, the total amount of IFN ranged from 480 to 800 MU. The patients were divided into two groups according to their response to IFN: five patients with sBR and the remaining five with NR. Serum samples were obtained just before the start of IFN therapy. ALT values were monitored at least once a month during the study period. The serum-sampling interval used for sequencing was 1.7–4.5 years, and there was no significant difference between the two groups (Table 1). In the sBR group, all ALT values determined after therapy were normal. In this study, sBR is defined as the maintenance of normal ALT values during the observation period, following the final administration of IFN.

RNA extraction

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

Table 1 Clinical background of the patients

	sBR group					NR group				
Patient	1	2	3	4	5	6	7	8	9	10
Age (years)	58	33	56	65	55	55	47	47	44	48
Sex (M/F)	F	F	M	F	F	M	M	M	F	M
Sampling interval (years)	3.1	3.3	1.7	3.5	3.7	4.5	3.7	3.1	3.3	3.1
Serum ALT (IU/L)										
Before IFN therapy	119	71	108	110	294	188	134	86	58	186
After IFN therapy	9	18	25	12	12	378	169	91	46	62
Plt ($\times 10^4/mm^3$)	8.8	13.4	14.8	12.4	22.9	15.3	20.1	16.9	18.4	19.1
HCV-RNA* (Meq/mL)										
Before IFN therapy	3.1	11	8.3	10	25	2.5	5.5	3.5	1.4	12
After IFN therapy	8.5	12	15	12	7.2	2.3	4.3	3.2	8.5	4.3
Histological findings										
Activity	2	2	1	1	2	2	2	1	2	1
Fibrosis	2	1	2	1	2	2	1	1	2	2

*HCV-RNA levels were determined by a branched chain DNA assay.
ALT, alanine aminotransferase; HCV, hepatitis C virus; IFN, interferon.

cDNA synthesis

Five microlitres of the reverse transcription mixture were adjusted to contain 1 μL of the RNA solution, 50 U of Moloney murine leukaemia virus reverse transcriptase (Invitrogen, Carlabad, CA, USA) diluted to the appropriate concentration, 10 units of RNase inhibitor (Promega Corp., Madison, WI, USA) and 50 pg of random hexamers (Takara Biochemicals, Shiga, Japan). The mixture was incubated at 37 °C for 40 min.

Polymerase chain reaction (PCR)

The full-length HCV genome was amplified by nested PCR with 21 partially overlapping primer sets. We used an automatic hot start PCR with TaqStart antibodies (Advantage cDNA Polymerase Mix, CLONTECH, Alto, CA, USA), according to the manufacturer's instructions. The PCR parameters were as follows: for the first-round PCR, after denaturing at 94 °C for 30 s, 50 cycles of denaturing at 94 °C for 15 s, annéaling at 52 °C for 15 s and polymerization at 72 °C for 45 s; for second-round PCR, after denaturing at 94 °C for 30 s, 40 cycles of denaturing at 94 °C for 15 s, annealing at 55 °C for 15 s and polymerization at 72 °C for 45 s.

Sequence determination

Each PCR product was purified and the residual primers were removed using a spin column (Suprec 02, Takara, Japan) according to the manufacturer's instructions. Thereafter, both strands of the PCR products were cycle sequenced using the Big Dye Terminator Cycle Sequencing Ready Reaction Kits (Applied Biosystems, Foster, CA, USA) according to the manufacturer's instructions. The sequencing primers were the second PCR forward primer for the sense strand and the second PCR reverse primer for the antisense strand. The products were purified on a column (Quickspin column, Boehringer Mannheim, Indianapolis, IN) and sequenced using an automated DNA sequencer (model 373S, Applied Biosystems, Foster, CA, USA). The nucleotide and deduced amino acid sequences were compared using the sequence of HC-J8 [24] as a reference.

Statistical analysis

The rates of change of nucleotide and amino acid residues were determined for each patient by pair-wise comparison of sequences data obtained at the intervals for each patient. Mutation rates (10^{-3} changes/site/year) of both nucleotides and amino acids were calculated for each sample. The differences in sampling intervals among the patients were corrected by site-per-year expression. Mutation rates were calculated for each fragment of the HCV genome [i.e. 5′ noncoding region (5′ NCR), core (C), envelope (E)1, HVR-1, HVR-2, E2, NS2, NS3, NS4A, NS4B, NS5A, NS5B, 3′ NCR]. Both nucleotide and amino acid mutation rates were compared between the two groups by Mann–Whitney's *U*-test. Bonferroni's correction was used as a *post hoc* test of the Kruskall–Wallis test. All tests of significance were two-tailed with *P* values less than 0.05 considered to indicate statistical significance.

RESULTS

Clinical course during and after IFN therapy

Longitudinal variations of ALT values during the study period are shown schematically for representative cases in Fig. 1. In the sBR patients, ALT normalization (<40 IU/L) occurred shortly after the start of IFN therapy and normal ALT levels were sustained despite persistent viraemia. In the NR patients, ALT normalization occurred transiently, but became elevated after IFN therapy. In three of five sBR patients and one of five NR patients, HCV-RNA became

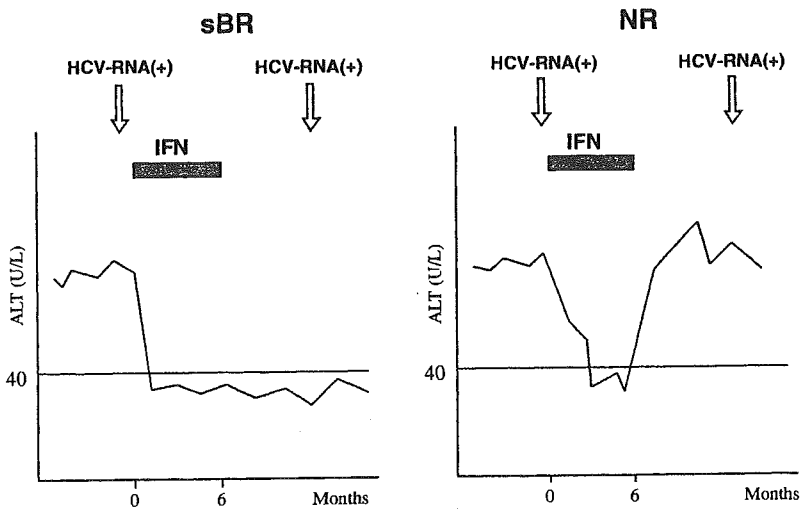
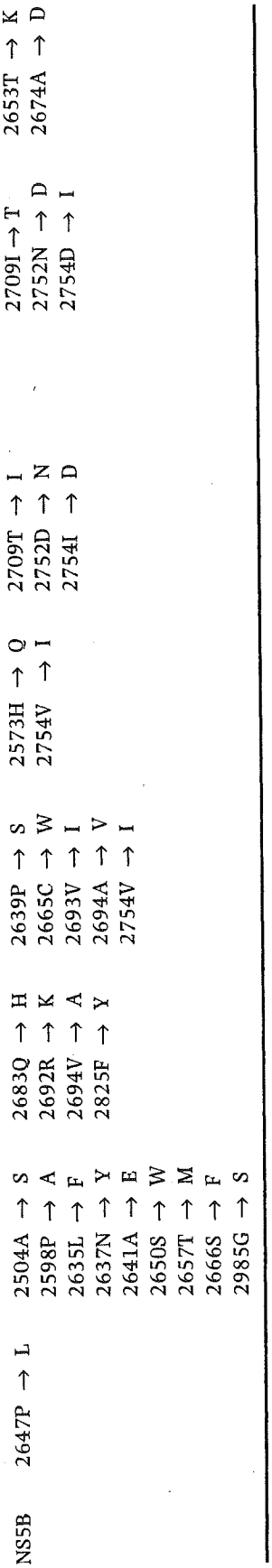


Fig. 1 Schematic diagram of the longitudinal course of serum alanine aminotransferase (ALT) values in representative cases during and after interferon (IFN). Normal ALT values <40 IU/L. In sustained biochemical response (sBR), normal ALT values were sustained after IFN therapy despite persistent viraemia. In non-response, (NR), ALT normalized transiently followed by a flare up after IFN therapy. In all patients, viraemia persists before and after IFN therapy without significant differences in levels. Arrows indicate times of the serum sampling.

Table 2 Changed amino acid residues before and after IFN therapy

	sBR group					NR group				
	1	2	3	4	5	6	7	8	9	10
Core	119L → F			179L → H 180A → D 181L → H	10K → I 15T → I 121R → K			94A → T		
E1	257N → H 296A → E 369A → S			193E → K 263D → Y 268A → T 299N → T		240I → V	279D → N		264A → V 280M → I 291L → F	356M → V
E2	392A → V 407A → P 639A → G	388T → I 408R → S 438I → L 456L → M	461G → A 725H → L	405P → A 530S → N	395A → G 456L → M	395S → L 407P → A 412H → Q 446K → R 667R → G	400A → T 401G → R 405R → F 445K → N	397S → G 595G → D 613I → V 614D → E 735I → V	404T → S	390A → G 400A → V 446N → S 562F → Y
p7	777A → T				789M → V					
NS2	826V → M	925I → V 940M → L			932I → L	853M → L 945M → I 1023A → I	828A → V		828V → A	
NS3	1062E → Q	1278I → V 1283T → R 1390R → K 1502I → T 1555C → H		1092K → N 1316V → I 1388S → F 1649I → T 1650A → G	1286V → A 1316V → I 1421Y → F 1451D → N 1537A → S 1587L → F 1610V → M 1659V → I	1204S → T		1299I → T	1245V → A	
NS4A	1663S → T	1698M → V	1695D → N	1663T → A 1665V → G	1694N → D 1696V → A					
NS4B		1745K → R			1753A → V 1879T → S 1966A → T	1722I → L	1806 P → S			1869S → A 1880I → V
NS5A	2307D → E 2313I → G	2283S → P 2298I → M 2302K → N 2304P → S	2163Q → H 2166C → W 2252V → I 2383P → A	2150T → K 2254L → H 2327H → P	2045M → L 2140I → N 2159G → V 2229E → D 2236S → F 2237R → M 2320L → P 2438A → S	2059L → M 2297M → V 2355L → P 2412A → T 2439V → I	2095V → I 2322T → A		2057L → W 2204N → S 2287P → S 2319A → T	2260I → T



undetectable during IFN treatment. In the other patients, HCV-RNA remained positive throughout IFN treatment. In all the patients, HCV-RNA was positive after the end of IFN administration.

Clinical background of the patients

The clinical characteristics of the five sBR patients and the five NR patients before IFN therapy are shown in Table 1. Except for the male-to-female ratio, there were no significant differences between two groups in parameters such as age, platelet counts and HCV-RNA levels. In the sBR group, ALT values at the second sampling point after IFN therapy were normal (<40 IU/L). In both groups, no significant changes in serum HCV-RNA levels were observed between the two sampling points.

Substitutions of residues during the observation period

We determined the HCV sequences in each patient except for the x-tail and poly-U–C regions of the 3′ UTR. The HCV genomes from all samples consisted of 9478 nucleotides with a single open reading frame of 3033 amino acids, and with similar genomic organizations to that of HC-J8 [24], the prototype HCV-2b clone. In the E2 protein of HCV-2b, hypervariable regions (HVR)-1 (amino acids 384–412) and HVR-2 (amino acids 437–446) were identified as for HCV-1b [26].

Serial changes of nucleotides were observed throughout the genome except for the 3′ UTR that was stable in all patients. The sequences of the 5′ UTR also were highly stable, and only one nucleotide change was observed: nucleotide176 (G to A) in Patient 9. As shown in Table 2 and Fig. 2, the serial changes in amino acid residues were distributed over most regions of the HCV genome, and there were no changes of single residues common to all sBR patients. In accordance with previous reports, clusters of amino acid changes were observed in HVR-1 and HVR-2 in E2 [27]. The overall substitution rate of codons in the entire HCV genome was higher in the sBR group than in the NR group [2.22 ± 0.48 (10^{-3} changes/site/year) *vs* 1.04 ± 0.30: $P = 0.002$].

The rate of amino acid substitutions in each HCV protein

Analysing the changes of each HCV protein individually (Table 3), the sBR group showed a higher rate of amino acid substitutions in NS4A than the NR group [8.82 ± 2.80 (10^{-3} changes/site/year) *vs* 0: $P = 0.001$] (Fig. 3). Recently, we have found that the amino acid substitution rates of NS5A and NS5B correlate with hepatitis activity in chronic hepatitis C caused by genotype 1b HCV [9]. In this study, although there was a tendency that the substitution rates in NS5A or NS5B was high in the sBR group it did not reach statistical significance.

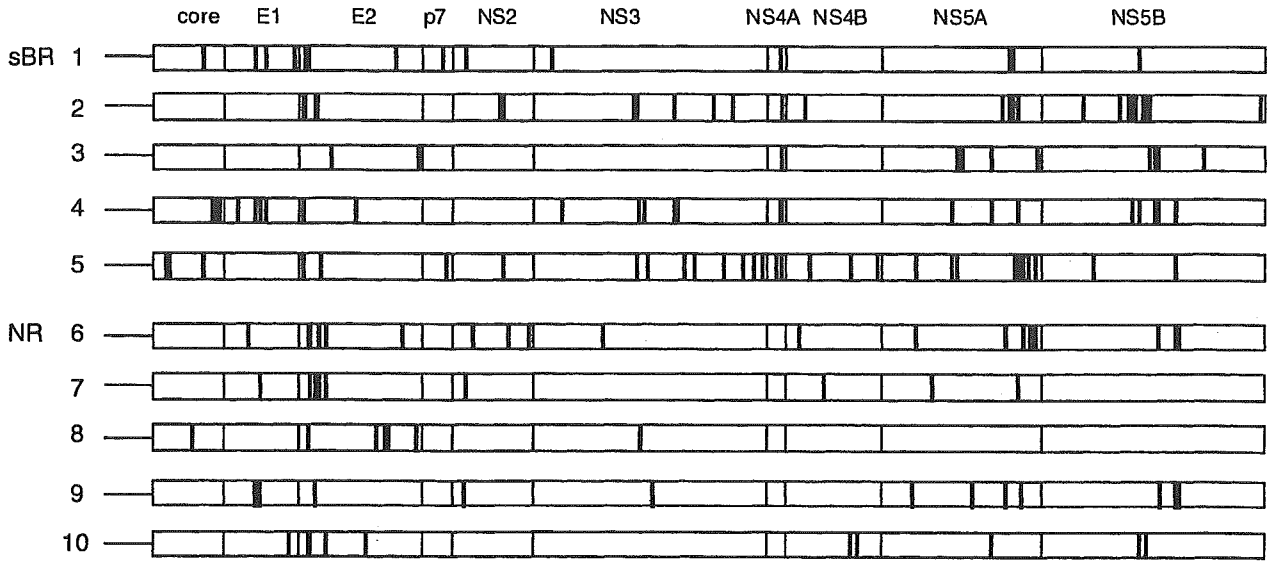


Fig. 2 Scheme of distribution of amino acid differences before and after interferon (IFN) therapy in five patients with sBR and five with NR. Vertical lines within the rectangles denoting the HCV proteins indicate the positions of amino acid differences between HCV pairs of each patient. Sequence data of these HCV isolates are deposited with the GenBank Data Libraries under Accession Nos AY232730–AY232739 for BRs 1–5 and Nos AY232740–AY232749 for NRs 6–10.

Table 3 The rate of amino acid substitutions (10^{-3} changes/site/year) in each protein coding region

Region	Core	E1	E2	P7	NS2	NS3	NS4A	NS4B	NS5A	NS5B	Total
sBR											
1	0.17	0.51	0.26	0.51	0.15	0.05	0.59	0	0.14	0.05	0.15
2	0	0	0.33	0	0.28	0.24	0.56	0.11	0.26	0.51	0.27
3	0	0	0.32	0	0	0	1.09	0	0.50	0.40	0.21
4	0.43	0.58	0.15	0	0	0.22	1.03	0	0.18	0.23	0.22
5	0.40	0	0.14	0.41	0.12	0.32	0.94	0.30	0.43	0.09	0.26
NR											
6	0	0.12	0.30	0	0.31	0.03	0	0.08	0.24	0.11	0.14
7	0	0.14	0.29	0	0.12	0	0	0.10	0.12	0	0.08
8	0.16	0	0.43	0	0	0.05	0	0	0	0	0.07
9	0	0.48	0.08	0	0.14	0.05	0	0	0.26	0.15	0.13
10	0	0.16	0.34	0	0	0	0	0.24	0.07	0.11	0.10

The rates of amino acid substitutions in each protein coding region are shown. The difference of sampling intervals among the patients was corrected by site-per-year expression.

Changes in amino acid residues of NS4A protein in sBR

The predicted amino acid sequences of the entire NS4A protein from all samples are aligned in Fig. 4. This region was completely stable in all NR patients, while different amino acid changes were found in all sBR patients after IFN therapy. Most of the amino acid changes found in sBR patients were located in potential epitopes restricted by the various HLA class I molecules found in the Japanese population. The central domains of NS4A (amino acids 1678–1690) were reported to be involved directly in NS3–NS4A complex formation, and the HLA-A2 restricted epitope was postulated at amino acids 1671–1680, partially overlapping this domain of NS4A in genotype 1b [30–33]. This domain was conserved and stable over time in all the sBR patients as well as the NR patients, indicating that its structure is highly restricted to retain functional integrity despite the potential advantages of escape mutations from the protective cytotoxic T lymphocyte (CTL) response against this region. Alternatively, there is substantial sequence diversity in the central domain among various HCV genotypes raising the possibility that the HLA-A2 epitope does not possibly exist in HCV-2b.

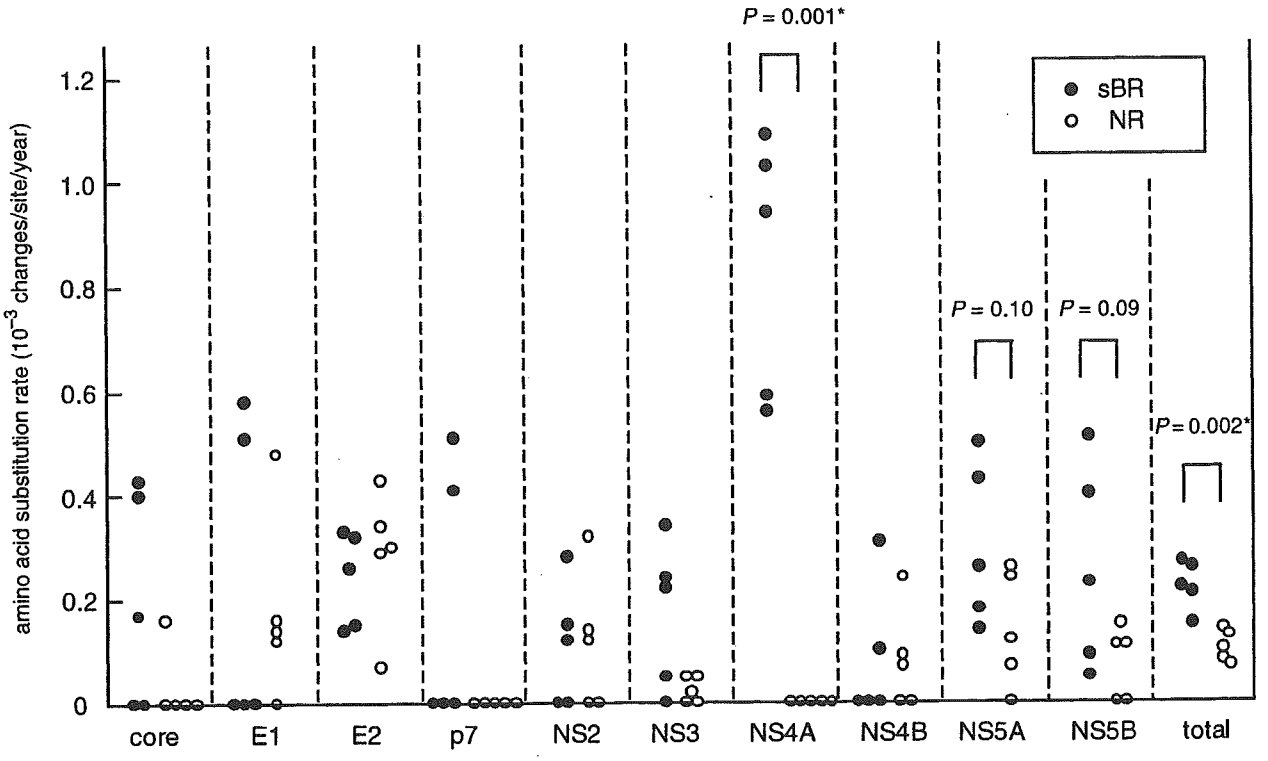


Fig. 3 The rate of amino acid substitution in each protein coding region, comparing sBR and NR groups. *P value <0.01.

DISCUSSION

In this study, we showed that the overall substitution rate of nucleotides and amino acids in the full-length HCV genome was greater during IFN treatment in sBR patients than in NR patients. Analysing each HCV protein separately, serial changes of amino acids in the NS4A protein were found exclusively in all the sBR patients, located predominantly in the putative HLA class I restricted motifs, while no such changes were found in the NR patients. These findings indicate that the subsiding of hepatitis in sBR is associated with changes of the HCV genome, especially with potential escape mutations from cytotoxic T lymphocytes as exemplified by the changes of NS4A protein observed in sBR patients.

The mechanism of sBR has not been understood clearly and sequencing the whole HCV genome before and after IFN therapy allows us to elucidate the genetic features of HCV in relation to sBR. As a result, we revealed for the first time that the genetic changes of HCV were scattered throughout the whole genome, and were greater in the sBR group than in the NR group. The HCV population in a patient with chronic hepatitis comprises a large number of closely related HCV variants, quasispecies, and it was reported that major quasispecies disappeared after IFN therapy in sBR patients, following analysis by of the HVR 1 SSCP pattern [34]. This finding, together with ours, indicates that the mechanism of sBR may be the eradication of quasispecies that can provoke hepatocyte injury. Virological heterogeneity of the different HCV quasispecies [21] could result in the modulation of virus replication, viral–host protein interaction [35] or immune reaction against infected hepatocytes leading to changes in the severity of hepatocyte injury, such as apoptosis, inflammation, direct cytotoxicity [36,37]. It should be determined which types of genetic features in each HCV quasispecies are associated with the severity of hepatocyte damage.

The amino acid changes found in sBR varied considerably in their distribution and no characteristic residues were found to be common to all the sBR patients. Severity of hepatocyte injury reflected by serum ALT levels varies greatly among HCV infected patients, from an asymptomatic carrier state to rapid progressive disease, but the underlying mechanism of this heterogeneity is not understood completely. Recently, we reported that amino acid substitutions in NS5A and NS5B are found more frequently in chronic hepatitis C patients with fluctuating hepatitis activity [9], and that the amino acid sequence of NS5A of genotype 1b (ARE; ALT response-related element: amino acids 2154–2172) is associated with the a transient biochemical response to IFN [11]. In addition, seven amino acid residues scattered throughout the HCV-1b genome are associated with the progression of chronic hepatitis C to cirrhosis and hepatoma [10]. Although these specific HCV variations seem to be involved in hepatocyte injury in each situation, they are also heterogeneous and exactly the same amino acid residues were not found in sBR patients in the present study.

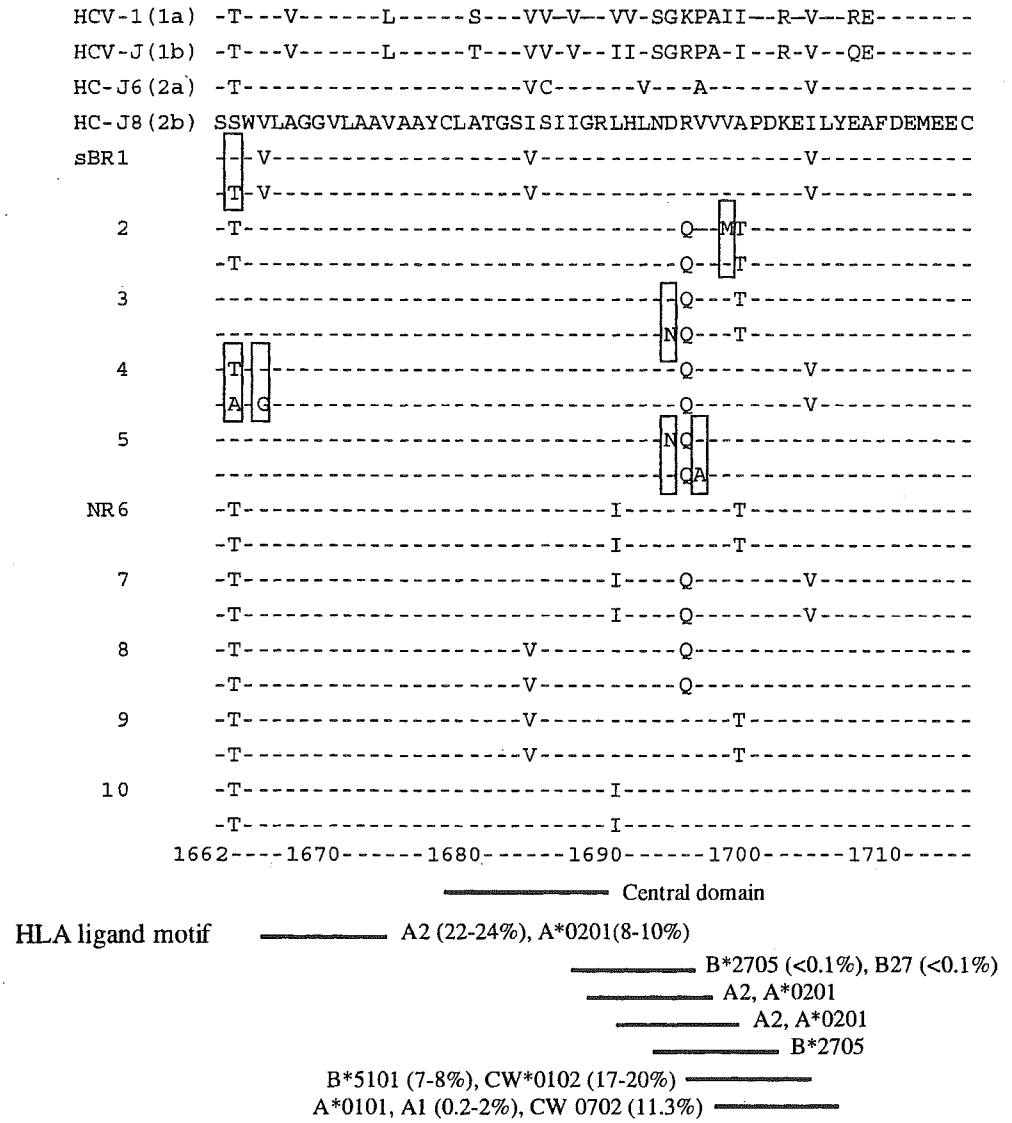


Fig. 4 Comparison of the amino acid sequences in NS4A before and after interferon (IFN) therapy, and with HCV isolates of genotypes 1a, 1b, 2a, 2b. The amino acids changed in sBR are boxed. The central domain of NS4A directly involved in NS3–NS4A complex formation and the HLA-A2 restricted epitope postulated in amino acids 1671–1680 partially overlapping this domain of NS4A in genotype 1b are indicated by solid lines below the sequence alignment, along with potential HLA class I binding motifs identified by HLA Ligand/Motif DATABASE (http://hlaligand.ouhsc.edu/LigandDB). Numbers in parentheses indicate the gene frequency of each HLA in the Japanese population [28,29].

These diversities may be partly because the mechanism of hepatocyte injury is complex and a relatively large number of amino acid residues or domains may be involved in different disease conditions or patients. In addition, previous studies have mostly analysed HCV-1b, in which genetic structures critical for inducing hepatitis might be different from HCV-2b, analysed in the present study. The roles of this genetic variation in relation to hepatocyte injury awaits analysis using newly developed *in vitro* systems, such as the HCV replicon [38,39] or infectious models using human hepatocytes transplanted into immunodeficient mice [40].

Although the amino acid changes in sBR are observed throughout the entire HCV polyproteins, NS4A mutations were found exclusively in the sBR patients, suggesting the potential importance of NS4A in hepatocyte injury. NS4A is an amphipathic peptide of 54 amino acid residues with a hydrophobic N-terminus and a hydrophilic C-terminus [41]. NS4A is necessary for the efficient cleavage of the nonstructural region of the HCV protein by NS3, assisting the membrane localization of NS3 and other viral replicase components [1,42]. The amino-terminal of NS3 and the central domain (amino acids 1679–1690) of NS4A are

involved directly in NS3–NS4A complex formation, and single amino acid substitutions in NS4A (amino acids 1682–1695) inhibited the enhancement of NS3 protease activity [43]. In this study, however, this central domain was well conserved in NR and sBR patients, and invariant in sBR, indicating that its activity seems to be intolerant of substitutions. Rather, the changes in NS4A were found in the regions flanking the central domain, raising the possibility that they might have some impact on hepatocyte injury influencing the NS3–NS4A interaction without a detrimental effect on HCV replication. This should be investigated further using an *in vitro* system such as the HCV replilcon.

Another possible explanation for the wide variety of genetic changes of HCV in sBR is that they are associated with HCV escape from the host's immune response, defined by the immunogenetic diversity, such as HLA, of each patient. Hepatocyte injury and viral clearance are thought to be mediated by HCV-specific CD8+ cytotoxic T lymphocytes directed to the HCV-infected hepatocytes, on which viral-derived peptides are presented bound to HLA class I molecules [44,45], and these CTLs are regulated by the CD4+ Th1 cells restricted by HLA class II molecules [46]. Thus, sBR might be mediated by the attenuation of interaction between CTL and the infected hepatocytes, presumably because of the mutations of HLA class I or II restricted epitopes in the HCV genome. Actually, several reports demonstrated the escape mutations in HLA class I epitopes specified by the HCV genome in patients with acute hepatitis who eventually develop chronic hepatitis [21], and the specific types of HLA class II molecules are linked to the prognosis of chronic HCV infection [47–49]. Thus, it could be assumed that a larger number of HCV mutations including in HLA restricted epitopes can be associated with a broader escape from the host's CTL attacks resulting in reduced hepatocyte injury, i.e. sBR, although the HLA haplotypes of the patients studied were not determined and we could not identify which mutations contribute to the CTL escapes. This notion is supported by the fact that most mutations found in NS4A of sBR patients were located in the HLA class I binding motifs as shown in Fig. 4 and that NS4 was known to be the most immunogenic for CD4+ T cells, indicating the presence of HLA class II restricted motifs [50,51].

The driving force for HCV genetic changes during IFN therapy is not understood completely. Direct suppression of HCV replication by IFN could be influenced by the specific amino acid sequence, such as the interferon sensitivity determining region (ISDR) in NS5A of HCV-1b [12,52,53], although the ISDR itself has not been confirmed in HCV-2b [54] and no amino acids changes were observed in the corresponding region in this study. Based on the quasispecies nature of the HCV genome, if selected IFN-resistant quasispecies coincidentally had escape mutations in HLA-restricted epitopes, this will give rise to sBR. Otherwise, IFN enhances the cellular immune response by upregulating HLA molecules on hepatocytes [55] and promoting the selection of HLA-restricted epitopes in HCV. In any case, functional analysis of CTL directed to HCV in sBR patients is needed to clarify these points.

In conclusion, this study provides new information about the increased genetic changes of HCV in sBR to IFN therapy taking advantage of the analysis of full-length HCV genotype 2b genomes, and escape mutations from immune responses were suggested to be related to reduced hepatocyte injury. As sBR after IFN therapy is associated with a lower risk of hepatocarcinogenesis with improved survival of patients, understanding the mechanisms of sBR that facilitate the prediction of sBR before IFN therapy is important for clinical treatment as well as basic research.

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Suppression of Hepatitis C Virus Replication by Cyclosporin A Is Mediated by Blockade of Cyclophilins

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Background & Aims: **Cyclosporin A specifically suppresses hepatitis C virus (HCV) replication in vitro at clinically achievable concentrations. In this study, we investigated the mechanisms of action of cyclosporin A against HCV replication.** ***Methods:*** **The in vitro effects of cyclosporin A on HCV replication were analyzed using an HCV replicon system that expresses chimeric luciferase reporter protein.** ***Results:*** **The significant effects of cyclosporin A on expression of an HCV replicon and the absence of such effects of FK506, which shares mechanisms of action with cyclosporin A, suggested the involvement of intracellular ligands of cyclosporin A, the cyclophilins. Transient and stable knockdown of the expression of cytoplasmic cyclophilins A, B, and C by short hairpin RNA–expressing vectors suppressed HCV replication significantly. A cyclosporin analogue, cyclosporin D, which lacks immunosuppressive activity but exhibits cyclophilin binding, induced a similar suppression of HCV replication. Furthermore, cyclosporin A treatment of Huh7 cells induced an unfolded protein response exemplified by expression of cellular BiP/GRP78. Treatment of cells with thapsigargin and mercaptoethanol, which induce the unfolded protein responses, suppressed HCV replication, suggesting that the cyclosporin-induced unfolded protein responses might contribute to the suppression of HCV protein processing and replication.** ***Conclusions:*** **The anti-HCV activity of cyclosporin A is mediated through a specific blockade of cyclophilins, and these molecules may constitute novel targets for anti-HCV therapeutics.**

Hepatitis C virus (HCV) infection, which affects 170 million people worldwide, is characterized by chronic liver inflammation and fibrogenesis, leading to end-stage liver failure and hepatocellular malignancy.[1] The difficulty in eradicating HCV is attributable partly to limited treatment options. Therapies with interferon alfa, with or without ribavirin in combination, are the only approved regimens that have shown efficacy.[2–4] The success rates of treatment, however, are at best 50%, with the most effective regimen being pegylated interferon alfa and ribavirin.[5] Furthermore, these therapies carry a substantial risk of serious side effects and in quite a considerable proportion of patients require premature discontinuation because of side effects.[6] Given this situation, the development of safe and effective therapies against HCV is our high-priority goal.

Cyclosporin A (CsA), a neutral cyclic undecapeptide that was isolated from the fungus *Hypocladium inflatum gams*, has been used widely for the treatment of allograft rejection, graft-versus-host disease, and various inflammatory diseases.[7,8] We and other researchers have reported that CsA substantially and specifically inhibits intracellular HCV replication in vitro.[9,10] Using our chimeric reporter HCV replicon system,[11] the 50% inhibitory concentration of CsA was found to be ~0.5 μg/mL, which is within clinically achievable concentrations. FK506 and rapamycin, which share pharmacologic mechanisms that suppress T-cell activation, did not show any inhibitory effect on HCV replication, suggesting that the anti-HCV effect of CsA is distinct from its immunosuppressive activity.

In this study, we have investigated further the mechanisms of action of CsA against HCV replication. Here, we show that the antiviral action of CsA is mediated by blockade of actions of cellular CsA-binding proteins, the cyclophilins.

Abbreviations used in this paper: BSD, blasticidin S; CsA, cyclosporin A; CsD, cyclosporin D; CypA, cyclophilin A; CypB, cyclophilin B; CypC, cyclophilin C; ER, endoplasmic reticulum; Fluc, firefly luciferase; His, polyhistidine; IRES, internal ribosome entry site; ISRE, interferon stimulation response element; MTS, dimethylthiazol carboxymethoxyphenyl sulfophenyl tetrazolium; NFAT, nuclear factor of activated T cells; PPIase, peptidyl prolyl *cis-trans* isomerase; Rluc, *renilla* luciferase; shRNA, short hairpin RNA.

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Materials and Methods

Drugs and Chemicals

CsA was purchased from Sigma Chemical Co (St Louis, MO). FK506 was from Alexis Biochemicals (Lausen, Switzerland). Recombinant human interferon alfa-2b was obtained from Schering-Plough (Kenilworth, NJ). Cyclosporin D (CsD) is a cyclosporin analogue that has valine at position 2 of CsA instead of L-α-aminobutyric acid. CsD was provided by Novartis Pharma Inc (Tokyo, Japan).

Cell Culture

Huh7 and 293 cells were maintained in Dulbecco's modified Eagle medium (Sigma Chemical Co) supplemented with 10% fetal calf serum at 37°C under 5% CO_2. Huh7 cells expressing HCV replicon were cultured in medium containing 500 μg/mL G418 (Wako, Osaka, Japan).

HCV Replicons

An HCV subgenomic replicon plasmid, pRep-Feo (Figure 1*A*), was derived from pRep-Neo (originally pHCVIb-neo-delS[12]). The pRep-Feo expresses a fusion gene comprising firefly luciferase (Fluc) and neomycin phosphotransferase, as described elsewhere.[11,13] Another replicon plasmid, pRep-BSD, expresses the blasticidin S (BSD) resistance gene. Replicon RNA was synthesized in vitro by T7-RNA polymerase (Promega, Madison, WI) and transfected into Huh7 cells by electroporation.[11] After culture in the presence of G418 (Wako), cell lines stably expressing the replicons were established (Huh7/Rep-Neo and Huh7/Rep-Feo, respectively).

Retrovirus Vectors Expressing Short Hairpin RNA

Oligodeoxyribonucleotides encoding short hairpin RNA (shRNA) sequences were synthesized and cloned just downstream of human U6 promoter in the plasmid pUC19, and the U6-shRNA cassette was subcloned into a retrovirus plasmid vector, pLNCX2 (Clontech, Palo Alto, CA). Sequences of the shRNAs were as follows: cyclophilin A (CypA) shRNA#3, 5′- GCA ATG TCG AAG AAC ACG GTG GGG TTG ACG GAG CTC GGT CAG CCT CAT CGT GTT CTT CGG CAT TGC TTT TTT -3′; CypA shRNA#441, 5′- GTG ATC TTC TTG CTG GTC TTG CCA TTC CTG GAG CTC GAG GAG TGG CAG GAT CAG CAG GAA GAT CAC TTT TTT -3′; cyclophilin B (CypB) shRNA#294, 5′- GTG AAG TCT CCG CCC TGG ATC ATG AAG TCG GAG CTC GGG CTT CAT GAT CCG GGG CGG AGG CTT CAT TTT TTT -3′; CypB shRNA#467, 5′- GCT TGC CAT CTA GCC AGG CTG TCT TGA CTG GAG CTC GAG TCG AGG CAG CCT GGT TAG ATG GCG AGC TTT TTT -3′; cyclophilin C (CypC) shRNA#0, 5′- GGT AGC AGC AGC CGA GGA CCC GGG CCC ATG GAG CTC GAT GGG CCT GGG TCT TCG GCT GCT GCT GCT TTT TTT -3′; and CypC shRNA#291, 5′- GTG CCA TCT CCA GTG GTG ATG TCA CCT CCG GAG CTC GGG AGG TGG CAT CAT CAT TGG AGG TGG CAC TTT TTT -3′.

Six negative control shRNA vectors were used that had reverse sequences of the respective targets: CypA shRNA#3 rev, CypA shRNA#441 rev, CypB shRNA#294 rev, CypBi#467 rev, CypCi#0 rev, and CypCi#291 rev. Another negative control, shRNA control, directed toward an unrelated target, the Machado–Joseph disease gene. A positive control shRNA vector, HCV shRNA, directed toward the 5′-untranslated region of HCV RNA.[13] The retrovirus plasmids were transfected into a packaging cell line, Retro Pack PT67 (Clontech), and the culture supernatant was applied to Huh7 cells with 4 μg/mL polybrene (Sigma Chemical Co). Huh7 cell lines stably expressing shRNA were established by culture in the presence of 300 μg/mL G418.

Reporter and Expression Plasmids

A plasmid, pISRE-TA-Luc (Invitrogen, Carlsbad, CA), expressed the Fluc gene under the control of the interferon stimulation response element (ISRE).[14] A nuclear factor of activated T cells (NFAT) reporter plasmid, pNFAT-Luc, was purchased from Stratagene (La Jolla, CA). A plasmid, pRL-CMV (Promega), which expresses the *renilla* luciferase (Rluc) gene under the control of the cytomegalovirus early promoter/enhancer, was used to normalize transfection efficiency. A plasmid, pCIneo-Rluc-IRES-Fluc, was constructed to analyze HCV internal ribosome entry site (IRES)-mediated translation efficiency (Figure 1*B*). The plasmid expressed a bicistronic RNA, in which Rluc was translated in a cap-dependent manner and Fluc was translated by HCV IRES-mediated initiation. A plasmid, pcDNA-NS3-5, expressed the HCV nonstructural gene spanning from NS3 to 5 of the HC-J4 clone (Figure 1*C*).[15]

Cyclophilin-Expressing Plasmid Vectors

The coding sequences of human cytoplasmic CypA, CypB, and CypC were amplified by reverse-transcription polymerase chain reaction using the following polymerase chain reaction primers: CypA-S, 5′- CAC CAT GGT CAA CCC CAC CGT GTT CTT CGA -3′; CypA-AS, 5′- TTC GAG TTG TCC ACA GTC AGC AAT GGT GAT -3′; CypB-S, 5′- CAC CAT GAA GGT GCT CCT TGC CGC CGC CCT -3′; CypB-AS, 5′- CTC CTT GGC GAT GGC AAA GGG CTT CTC CAC -3′; CypC-S, 5′- CAC CAT GGG CCC GGG TCC TCG GCT GCT GCT -3′; CypC-AS, 5′- CCA ATC AGC GAT CTC AAC CAC AAA AGG CGT -3′. The amplicons were cloned into pcDNA 3.1 using the Directional TOPO Expression Kit (Invitrogen) to make pcDNA-CypA, -CypB, and -CypC, respectively. The expressed proteins contained a C-terminal polyhistidine (His) tag, which allowed their detection by anti-His antibodies (Invitrogen).

Transfection of Plasmids

Transfection of plasmids was performed using Lipofectamine 2000 reagent (Invitrogen) according to the manufacturer's instructions.

Luciferase Assays

Luciferase activities were measured using a luminometer (Lumat LB9501; Promega) using the Bright-Glo Luciferase Assay System (Promega) or the Dual-Luciferase Reporter Assay System (Promega). Assays were performed in triplicate.

Northern Blotting

Total cellular RNA was extracted from cells using Isogen (Wako). The RNA was separated by denaturing agarose-formaldehyde gel electrophoresis and transferred to a Hybond-N+ nylon membrane (Amersham Biosciences Corp, Piscataway, NJ). The upper part of the membrane, which contained the HCV replicon RNA, was hybridized with a digoxigenin-labeled probe that was specific for the full-length replicon sequence, and the lower part of the membrane was hybridized with a probe specific for β-actin. The signals were detected in a chemiluminescence reaction using the Digoxigenin Luminescent Detection Kit (Roche, Mannheim, Germany) and were visualized using a Fluoro-Imager (Roche).

Western Blotting

Ten micrograms of total cell lysate was separated using NuPAGE 4%–12% Bis-Tris gels (Invitrogen) and blotted onto an Immobilon polyvinylidene difluoride membrane (Roche). The membrane was incubated with antibodies against NS5A (BioDesign, Saco, ME), anti-His (C Term; Invitrogen), β-actin (Sigma Chemical Co), and BiP/GRP78 (BD Biosciences, Franklin Lakes, NJ) and then incubated with peroxidase-conjugated secondary antibodies. Immunoreactions were performed using a BM Chemiluminescence Blotting Substrate (Roche) and visualized by Lumi-Imager F1 (Roche) according to the manufacturer's protocol.

Stable Colony Formation Assays

Cells were transfected with a replicon, Rep-BSD, and cultured in the presence of 150 μg/mL BSD (Invitrogen) in the medium. BSD-resistant cell colonies were obtained after ~2 weeks in culture. The colonies were stained with neutral red solution (Invitrogen) and counted.

Dimethylthiazol Carboxymethoxyphenyl Sulfophenyl Tetrazolium Assays

To evaluate cell viability, dimethylthiazol carboxymethoxyphenyl sulfophenyl tetrazolium (MTS) assays were per-

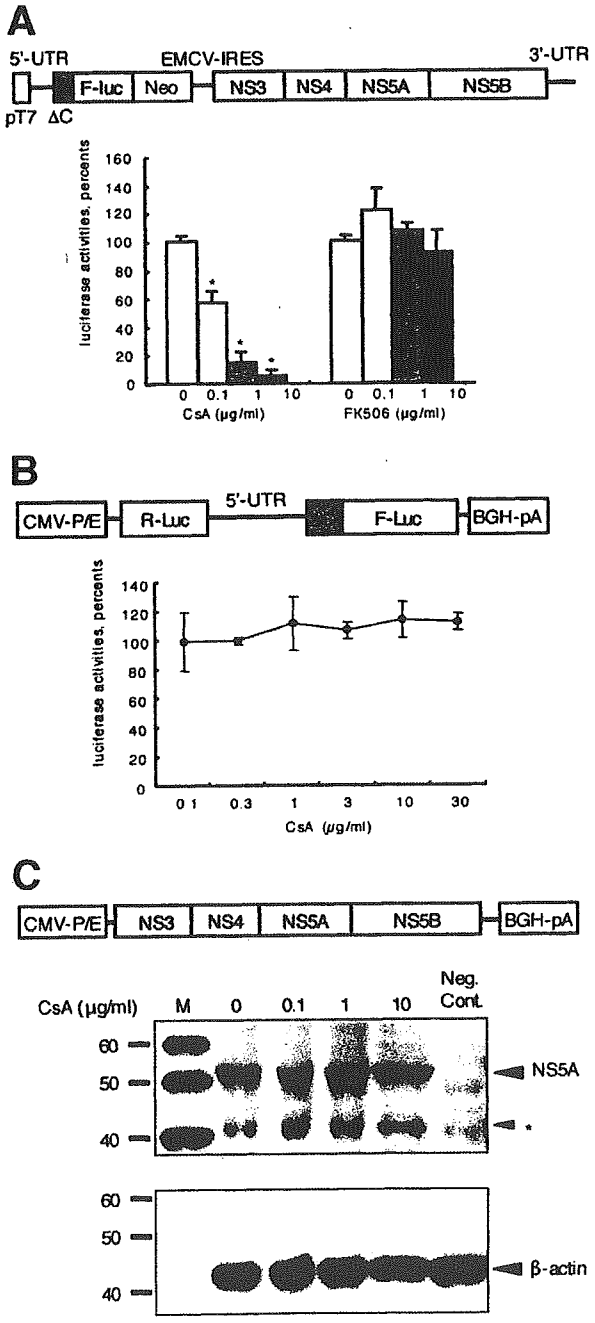

Figure 1. CsA does not influence HCV IRES-mediated translation or the processing of HCV nonstructural proteins. (*A*) Huh7/Rep-Feo cells that constitutively express the HCV Feo replicon (*top*) were cultured in the presence of CsA and FK506 at concentrations of 0, 0.1, 1, and 10 μg/mL. The internal luciferase activities were measured after 48 hours of culture. Assays were performed in triplicate. *Error bars* indicate mean ± 2 SD. **P* values of less than .05. (*B*) A bicistronic reporter gene plasmid, pCIneo-Rluc-IRES-Fluc (*top*), was stably transfected into Huh7 cells (Huh7/neo-Rluc IRES-Fluc). The cells were cultured with CsA at the concentrations indicated, and dual luciferase activities were measured after 48 hours of treatment. Values are displayed as ratios of Fluc to Rluc. *Error bars* indicate mean ± 2 SD. (*C*) Western blotting. An expression plasmid, pcDNA-NS3-5 (*top*), which expressed HCV nonstructural genes NS3–NS5, was used. The plasmid was transfected into 293 cells. At 24 hours after transfection, the cells were treated with the amounts of CsA indicated (0, 0.1, 1, and 10 μg/mL). The cells were harvested after 48 hours of treatment. The cell lysate was separated on NuPAGE 4%–12% Bis-Tris gels, transferred onto a polyvinylidene difluoride membrane, and incubated with a monoclonal anti-NS5A antibody or an anti–β-actin antibody. *Lane 1*, protein size markers. The pcDNA-NS3-5 was transfected into 293 cells and cultured in the absence (*lane 2*) or the presence of CsA at 0.1, 1 and 10 μg/mL, respectively (*lanes 3–5*). Neg. Cont., untransfected 293 cells. The *asterisk* indicates partially cleaved NS5A protein.