Table 1 Markers of hepatic fibrosis and damage

	Hydroxyproline (mmol/mg)	AST (IU/l)	ALT (IU/l)
No treatment	0.8±1.1	71.7±3.2	46.0±2.3
DMN-PBS[a]	4.8±1.0[b]	662.0±171.4[c]	352.1±68.2[d]
DMN-AxCA-lacZ[a]	3.7±0.2[e]	844.6±270.2[f]	501.3±15.7[g]
DMN-AxCA-rIFN[h]	1.8±0.3[i]	182.5±11.9[j]	81.0±5.7[k]

Mean values and SDs are shown for five rats in each group.[a]Blood and liver tissues were collected 10 days after the vector transduction and DMN treatment. All the rates in PBS and AxCA-lacZ-injected groups died within 3 weeks after the gene transfer. b *versus* i, P=0.0315; e *versus* i, P=0.0064; f *versus* j, P=0.0329; c *versus* j, P=0.0346; g *versus* k, P<0.0001; d *versus* k, P<0.0001.[h]Blood and liver tissues were collected 70 days after the vector transduction and DMN treatment.

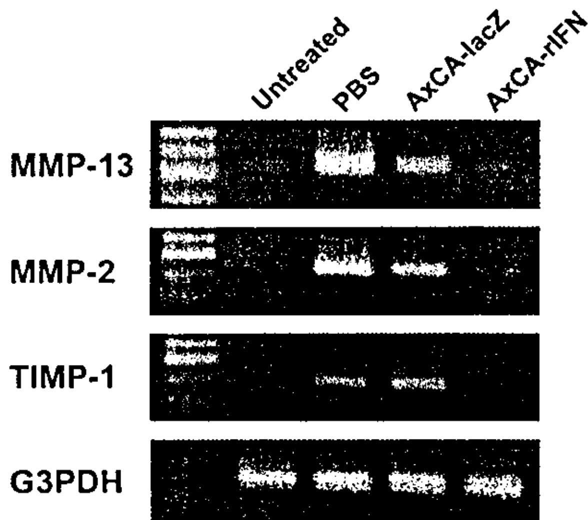

Figure 5 Expression of MMPs and TIMP in the liver. The expression of MMP-13, MMP-2 and TIMP-1 mRNA was analyzed in the DMN-treated rats injected with the vectors by the RT-PCR method.

increases in the process of liver fibrosis to promote progression of liver fibrosis by preventing degradation of secreted collagens and decreases in the recovery phase.[28] In this study, the expression of MMP-13, MMP-2 and TIMP-1 was downregulated in the liver treated with AxCA-rIFN compared with the PBS- or AxCA-lacZ-injected cirrhotic liver, supporting the antifibrotic action of the AxCA-rIFN.

Furthermore, since it is reported that overexpression of TGF-β plays a pivotal role in the progression of fibrosis,[9] we examined TGF-β expression in the liver. The control value of TGF-β in untreated normal rats was 5.5±1.0 ng/g (n=5). Among the rats with DMN-induced LC, AxCA-rIFN-transduced animals showed a lower TGF-β expression level (40.2±7.4 ng/g) than did the PBS-injected rats (71.3±6.0 ng/g). TGF-β immunoreactivity was detected in the liver of an AxCA-lacZ-transduced rat, showing evidence of LC, and by contrast, TGF-β was not detected in the AxCA-rIFN-transduced rat (data not shown). The data suggested that IFN-α gene transfer suppresses TGF-β induction in the process of DMN-induced hepatic fibrogenesis.

Regulation of IFN-α expression by Cre-loxP reaction

Although not apparent in our rat experiment, it is known from clinical experience that IFN protein treatment may cause acute adverse effects. Thus, to examine a shut-off system of IFN-α expression based on the Cre recombinase–loxP reaction in the adenovirus vector, an adenovirus vector harboring the rIFN-α gene between two loxP sites (AxCALNL–rIFN) was constructed (Figure 6a). The intravenous injection of AxCALNL–rIFN was able to produce approximately 120 IU/g of IFN-α in the liver similar to the AxCA-rIFN injection (Figures 2 and 6d), suggesting both vectors have equal potential for treating cirrhotic liver. First, NBT-2 cells were transfected with AxCALNL-rIFN, and 2 days later a 100 times larger amount of an adenovirus vector expressing the Cre recombinase (AxCAN-Cre) was added to the culture medium to excise the IFN-α gene from AxCALNL-rIFN DNA. As shown in Figure 6b, the 783 bp vector fragment containing the rat IFN-α gene disappeared, and the loxP-flanked excised fragment (225 bp) was detected by PCR analysis in NBT-2 cells only after the treatment of AxCAN-Cre. Rat IFN-α production was also significantly suppressed in the NBT-2 cells *in vitro* by the treatment of AxCAN-Cre (Figure 6c).

Next, normal rats were sequentially transduced via the tail vein, first with AxCALNL-rIFN and then 2 days later with AxCAN-Cre. Rat IFN-α expression was again significantly decreased in the liver of the rats injected with AxCAN-Cre as compared to the rats without AxCAN-Cre injection (Figure 6d).

Discussion

Among preclinical models of liver fibrosis, those induced by the chronic carbon tetrachloride (CCl_4) intoxication or ligation of bile duct are often used. However, the reversibility of hepatic fibrosis has been reported in these models.[24] Therefore, we employed a DMN-induced model, in which fibrosis and hepatic transaminase elevation do not spontaneously regress in rat LC.[16,18,22] This is the first preclinical study demonstrating the advantage and feasibility of IFN-α gene therapy for LC, and the efficacy and safety of the approach need to be reproduced in other LC models in different animals.

Several strategies have been recently proposed for the gene therapies of LC: a blockade of TGF-β signaling using the dominant negative form of the TGF-β receptor prevented liver fibrogenesis and dysfunction;[29] transduction with hepatocyte growth factor (HGF) gene inhibited fibrogenesis and hepatocyte apoptosis in cirrhotic liver;[30] and an adenovirus-mediated expression of telomerase RNA and urokinase-type plasminogen activator also exerted inhibitory effects against fibrogenesis.[31,32] However, none of those strategies has been examined for an antiviral effect on HCV. We found that the AxCA-rIFN vector inhibited HCV replication *in vitro* (unpublished data), and the anti-HCV effect may add an advantage to the IFN-α gene therapy for HCV-induced LC. It is also noted that HGF transgenic mice developed a broad array of tumors, suggesting caution should be used when considering HGF as a future therapeutic agent.[33]

The subcutaneous route of IFN protein injection in conventional IFN therapy is often associated with systemic toxicity, because it requires an approximate

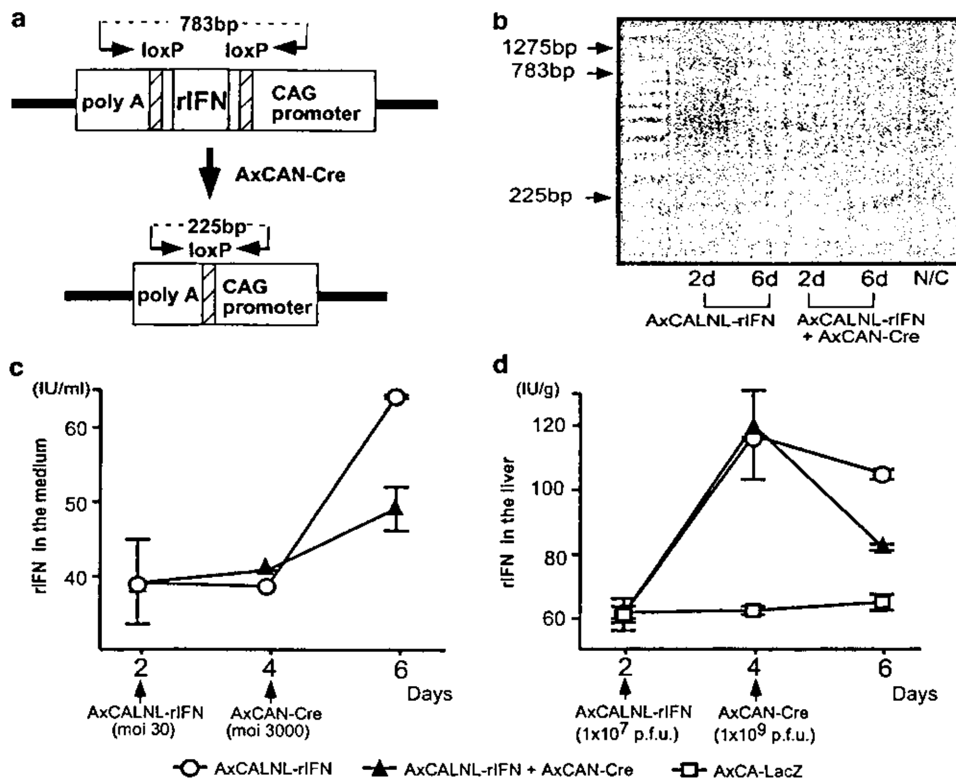

Figure 6 Regulation of IFN-α expression by Cre–loxP reaction. (a) Structure of AxCALNL-rIFN. PCR primers are shown by arrows together with the Cre recombination products. (b) PCR detection of the IFN-α transgene fragment cleaved by superinfection with AxCAN-Cre (moi 3000) 2 days after the transduction with AxCALNL-rIFN (moi 30). The PCR primers amplified a fragment containing Cre (1275 bp), a fragment containing rIFN-α flanked by two loxP sequences on both ends (783 bp) and the corresponding fragment with the loxP-rIFN-loxP portion excised off by Cre (225 bp). (c, d) Rat IFN-α expression measured by ELISA in NBT-2 cells in vitro (c) and in the rat liver in vivo (d) showing an effective suppression of the IFN-α transgene expression. Open circle, AxCALNL-rIFN (n=3); solid triangle, AxCALNL-rIFN+AxCAN-Cre (n=3); open square, AxCA-lacZ (n=3).

10–20-fold higher level of IFN concentration in the systemic circulation than in the liver.[14] In this study, we found that an adenovirus-mediated IFN-α gene delivery can lead to a significant IFN-α production in the liver but not in the serum. The locally produced IFN-α will rapidly bind to the receptor in the liver tissue, and only a few IFN-α may escape to the systemic circulation. We detected no significant systemic toxicity in the rats after injection of AxCA-rIFN. Moreover, Figure 2 suggested that a single injection of the IFN-α vector led to a more sustained IFN-α level and a larger area under the concentration curve than did the subcutaneous injection of the IFN-α protein. The cost of the standard IFN regimen in Japan is about 2 million yen (approximately 15 000 US dollars) per patient for 6 months under the Current National Health Insurance Program, which also limits access to therapy for patients who need more IFN because of a significant amount of virus in the serum. The IFN-α gene therapy may offer a better cost–benefit than the conventional IFN protein therapy.

Since it is known from clinical experience that IFN protein treatment may cause acute adverse effects, a safety device should be considered so that vector-induced IFN-α production can be terminated when a significant adverse effect appears. Several regulatory systems have been proposed for gene transfer: prokaryotic repressor–operator-based approaches (lac, tet) were applied to transgenic mice to control certain gene expression, and recently, a chemically induced dimerization-based approach (FKBP) was shown to be useful for *in vivo* gene regulation because of its high induction ratio. Among these methods, the Cre–loxP system has an advantage in its simplicity: only a Cre-expressing adenovirus vector is necessary to shut off the gene. To the best of our knowledge, this report is the first to demonstrate that a Cre-mediated regulation system is useful for shutting off gene expression in a gene therapy model *in vivo*.

Several delivery systems for targeting the liver were reported. Eto and Takahashi[34] reported that hepatitis B virus production was inhibited by asialoglycoprotein receptor-directed IFN, and Protzer *et al*[35] demonstrated that IFN gene transfer by a hepatitis B virus vector efficiently suppressed wild-type virus replication in a duck model of hepatitis B virus infection. However, the hepatitis B virus vector has a low gene transfer efficiency in the liver as compared with that of an adenovirus. When adenovirus vectors were systemically injected into rodents with normal liver, 80–90% of the vector is found in the liver and most vectors target hepatocytes.[36,37] Several reports showed that adenovirus-mediated transgene expression was preferentially shown in septal cells rather than in hepatocytes in cirrhotic rats,[15,38,39] and Nakamura *et al*[15] have hypothesized that the reduction of intralobular hemodynamics by the shunt formation between portal and central veins resulted in the shift of

gene expression from hepatocytes to septal cells in cirrhotic rats. In this study also, the expression of lacZ and AP genes was mainly detected in the fibrous septa of cirrhotic liver after the intravenous injection of vectors. Therefore, an adenovirus might be particularly suitable for gene therapy for LC, because it has a high efficiency of gene transduction into the connective tissues of fibrous septa by the systemic injection.

A major concern regarding the adenovirus vectors is their immunogenicity, because a repeated injection of the vector may be necessary to eradicate HCV infection. Adenoviral proteins can induce a toxic/anaphylactic reaction, which seems to depend on the amount of adenovirus vectors.[40,41] In our strategy, a large amount of adenovirus vector may not be required, because cytokines such as IFN are effective in a small dose. In fact, in this study the injection of 1×10^7 PFU (compatible with 2×10^9 PFU in human) AxCA-rIFN resulted in significant IFN-α production in the rat liver, a level comparable to that produced by subcutaneous injection of 100 000 IU of recombinant IFN-α protein. The intrahepatic arterial infusion, instead of a systemic administration, could further reduce the amount of adenovirus vectors. Less immunogenic vectors such as the 'gutless' adenoviral vector[42–44] and polycation-based synthetic nonviral vectors[45] are being developed in several laboratories at a rapid pace. The present study showed the feasibility and potential advantages of IFN-α gene therapy for HCV-associated liver diseases.

Materials and methods

Replication-defective recombinant adenoviruses

Replication-defective recombinant adenoviral vectors carrying either rat IFN-α cDNA (AxCA-rIFN), β-galactosidase (AxCA-lacZ) or alkaline phosphatase (ADVCA-AP) gene were prepared as described.[46,47] The recombinant adenoviruses are Ad5 defective with a deletion in the E3 region and have the CAG promoter, which is a hybrid of the cytomegalovirus immediate-early enhancer sequence and the chicken β-actin/rabbit β-globin promoter.[48] AxCALNL-rIFN, which carries the rat IFN-α cDNA between two loxP sequences downstream of the CAG promoter, was constructed to regulate the IFN-α expression by Cre–lox reaction (Figure 6a).

Animal model

As a model of LC, we used DMN-injected LC rats, an established animal model of persistent liver fibrosis with pathophysiological findings closely resembling those of human LC.[16–18,22] A measure of 1% DMN dissolved in saline was injected into 48 Sprague–Dawley male rats (4-week old, 120–130 g in body weight) intraperitoneally at 1 ml per kg weight for 3 consecutive days per week for 7 weeks. After the DMN injection for 3 weeks, five rats were killed for examination of LC development. Another three rats received subcutaneous injection of the recombinant IFN-α protein. The remaining 40 rats were injected via the tail vein with a single infusion of 200 μl of either PBS (n=13), 1×10^7 PFU of AxCA-rIFN (n=12), AxCA-lacZ (n=13) or ADVCA-AP (n=2). Three rats from each group (ADVCA-AP: n=2) were killed at day 2 for the analysis of vector distribution. Several organs including the liver, lung, heart, spleen, kidney, small intestine and testis were fixed in 10% formalin for histological examination and histochemical staining of AP or frozen immediately in liquid nitrogen for the measurement of hydroxyproline and TGF-β1, and for the histochemical staining of X-gal. The remaining rats in each PBS- (n=10), AxCA-rIFN- (n=9) and AxCA-lacZ-injected group (n=10) were observed for survival.

Organ distribution of lacZ gene expression

After DMN injection for 3 weeks, the Sprague-Dawley male rats were intravenously injected with 1×10^9 PFU of AxCA-lacZ. Total RNA was extracted 2 days later from various organs such as the lung, heart, liver, spleen, kidney and small intestine using Isogen® reagent (Nippon Gene, Tokyo, Japan) and 2 μg of RNA was used for cDNA synthesis. Amplification of the lacZ cDNA (250 bp) was carried out using 1 μl of synthesized cDNA in a 50 μl PCR mixture containing 20 pmol of the forward primer (5′-GATAGATCCCGTCGTTTTAC-3′), reverse primer (5′-TGAGGGGACGACGACAGTAT-3′), 1.5 mM $MgCl_2$, 0.2 mM dNTPs and 1 U of recombinant *Taq* DNA polymerase. In total, 30 cycles of the PCR were carried out at 95°C for 1 min, 60°C for 1 min and 72°C for 2 min. The PCR products were electrophoresed on a 2% agarose gel, transferred onto a nylon membrane (Hybond N, Amarsham Biosciences Corp., Piscataway, NJ, USA) and hybridized with a ^{32}P-labeled lacZ gene in 50% foramide, 5 × Denhardt's solution, 0.1% SDS, 5 × SSPE , and 100 μg/ml of salmon testis DNA at 42°C for 16 h. The membranes were then washed in 0.1 × SSC and 0.1% SDS. As a positive control for each RNA preparation and RT-PCR, the glyceraldehydes 3-phosphatase dehydrogenase (G3PDH) sequence was also amplified simultaneously in separate tubes using the forward (5′-TGCACCACCAACTGCTTAG-3′) and the reverse primers (5′-GGATGCAGGGATGATGTTC-3′). For amplification of G3PDH, PCR was performed for 25 cycles.

Measurement of rat IFN-α, 2′-5′-oligoadenylate synthetase (2′-5′AS) and TGF-β1

Rat IFN-α was measured by enzyme-linked immunosorbant assay (ELISA) using anti-rat IFN-α polyclonal antibody (Access Biomedical Diagnostic Research Laboratories Inc., San Diego, CA, USA). TGF-β1 was measured by enzyme assay at a clinical reference laboratory (SRL, Tokyo, Japan). 2′-5′ AS activity was determined by a radioimmunoassay kit (Eiken, Tokyo, Japan).

Immunohistochemical examination

Immunohistochemical analysis was performed using an antibody against rat IFN-α (Access Biomedical Diagnostic Research Laboratories, Inc., α-smooth muscle actin (American Research Product, Tokyo, Japan), or TGF-β1 (Promega, Tokyo, Japan) and developed using ABC kit (Nichirei, Tokyo, Japan).

Expression of MMPs and TIMP

Liver tissues were collected from DMN-treated rats 10 days after the injection of PBS or AxCA-lacZ, and 70 days after the injection of AxCA-rIFN. The expression of MMP-13, MMP-2 and TIMP-1 was examined by the RT-PCR method as described above using the following primer sets: MMP-13, forward (5′-TGACTATGCG-

TGGCTGGAA-3′) and reverse primers (5′-AAGCT-GAAATCTTGCCTTGGA-3′) (355 bp); MMP-2, forward (5′-ACCATCGCCCATCATCAAGT-3′) and reverse primers (5′-CGAGCAAAAGCATCATCCAC-3′) (348 bp); TIMP-1, forward (5′-CCGCAGACGGCGTTCT-GCAA-3′) and reverse primers (5′-TCGAGACCCAAGG-GATTGCC-3′) (525 bp). In all, 35 cycles (MMP-2: 30 cycles) of PCR were carried out at 94°C for 30 s, 58°C (MMP-13: 52°C) for 30 s and 72°C for 30 s, and the PCR products were electrophoresed on a 2% agarose gel.

Regulation of IFN-α expression by Cre-loxP reaction

NBT-2 cells (rat bladder cancer cell line, 1×10^5 cells) were transfected with AxCALNL-rIFN at an moi of 30, and 2 days later the cells were transfected with AxCAN-Cre at an moi of 3000. The Sprague-Dawley rats were transduced with 200 μl of AxCALNL-rIFN (1×10^7 PFU) through the tail vein, and 2 days later AxCAN-Cre (1×10^9 PFU) was transduced via the same route. Two days later, the rats were killed to examine rat IFN-α expression by ELISA. The status of the vector DNA was examined by PCR 2 and 6 days after the injection. The sequences of the forward and reverse primers were 5′-GTGGTATTTGTGAGCCAGGG-3′, and 5′-TACAGCTC-CTGGGCAACGTG-3′, respectively (Figure 6a). In total 35 cycles of the PCR were carried out at 94°C for 30 s, 57°C for 30 s and 72°C for 60 s, and the PCR products were electrophoresed on a 1% agarose gel.

Statistical analysis

Statistical differences between variables were analyzed by the unpaired *t*-test. Survival distributions were calculated by the Kaplan–Meier method and were analyzed using the log-rank test. P-value <0.01 was considered significant.

Acknowledgements

This work was supported in part by a grant-in-aid for the 2nd Term Comprehensive 10-year Strategy for Japan, and by grants-in-aid for Cancer Research from the Ministry of Health, Labour and Welfare of Japan.

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Biochemical and Biophysical Research Communications 307 (2003) 814–819

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Adenovirus-mediated gene transfer of interferon α inhibits hepatitis C virus replication in hepatocytes

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Received 16 June 2003

Abstract

Recently we reported that on-site interferon (IFN)-α production in the liver using an adenovirus vector can achieve a substantial confinement of IFN-α in the target organ and can improve liver fibrosis in a rat liver cirrhosis model. However, the major therapeutic effect of IFN for hepatitis C virus (HCV)-associated liver diseases is its antiviral effect on HCV. As a prelude to the in vivo HCV infection experiment using a primate animal model, here we examined the antiviral effect of IFN-α gene transfer into HCV-positive hepatocytes in vitro. The non-neoplastic human hepatocyte cell line PH5CH8 was inoculated with HCV-positive serum. Successful in vitro HCV replication and thus the validity of this model was confirmed by a strong selection for HCV variants determined by sequence analysis of the hypervariable region and an increase of HCV RNA estimated by real time TaqMan RT-PCR. One day after the inoculation of HCV, PH5CH8 cells were infected with adenoviral vectors encoding human IFN-α cDNA. HCV completely disappeared 9 days after the adenoviral infection, which is linked to the increase of 2′,5′-oligoadenylate synthetase activity, suggesting that IFN-α produced by gene transfer effectively inhibits HCV replication in hepatocytes. This study supports the development of IFN-α gene therapy for HCV-associated liver diseases.
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Keywords: Adenovirus; Gene transfer; Interferon; Liver cirrhosis; Hepatitis C virus

Hepatitis C virus (HCV) is a major cause of acute hepatitis and chronic liver diseases, including liver cirrhosis (LC) and liver cancer in Japan and some southern European countries. Chronic hepatitis progresses to LC and liver cancer at a high incidence within two or three decades after the infection. Globally, an estimated 170 million persons, 3% of the world's population, are chronically infected with HCV and 3–4 million persons are newly infected each year [1–3].

The most effective therapy for HCV is currently considered to be interferon (IFN) treatment [2,4,5]. However, the conventional administration route of IFN protein through a subcutaneous space requires a higher IFN level in the serum than in the target organ, the liver, due to the rapid degradation of IFN in blood circulation. Although dose escalation is expected to be effective in a resistant case, it is often impossible because of systemic toxicity such as flu-like symptoms. Our recent study showed that on-site, targeted IFN production in the liver using an adenovirus-mediated IFN-α gene delivery system can achieve a higher IFN concentration in the liver than in the serum and can improve the progression of liver fibrosis in a rat LC model [6], suggesting that IFN-α gene therapy may overcome the limitation of conventional IFN treatment. Although the anti-fibrosis effect is clinically significant, the major therapeutic effect we expect for IFN in the treatment of HCV-associated liver diseases is its direct antiviral effect on HCV. Many

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doi:10.1016/S0006-291X(03)01255-5

viruses, including HCV, can inhibit protein synthesis in their host cells by targeting translation [7]. Therefore, when considering the development of an IFN-α gene therapy for HCV-associated diseases, it is crucial to examine whether the IFN-α transgene is effectively translated and induces an antiviral effect in HCV-infected hepatocytes.

Any final preclinical demonstration of the in vivo efficacy of an IFN gene therapy on HCV infection would require an experiment on chimpanzees, but the ethical issues, high costs, and the inherent difficulties in working with large animals restrict their use [8]. The most convenient approach to study virus replication is based on the infection of a culture system. Many attempts have been undertaken to establish such a culture system for HCV, and with respect to liver cell lines, detailed studies have been performed on PH5CH cells [8,9]. The detection of intracellular HCV RNA for 70–100 days postinoculation and the shift to limited variants from the quasi-species of HCV populations demonstrated a successful replication of HCV in PH5CH cells [8,9]. In this study, by using this in vitro HCV replication culture system, we demonstrated for the first time that IFN-α protein produced by an adenovirus vector induces an anti-HCV effect.

Materials and methods

Cell line and virus inoculation. The non-neoplastic human hepatocyte cell line PH5CH was established by immortalization after transfection with a simian virus 40 large T antigen expression vector, pRSV-Tag [9,10]. PH5CH8 cells, which support an efficient and persistent HCV replication, were isolated from the parental PH5CH cells [9,10]. A total of 80 μl of undiluted serum 1B-2 (1×10^7 HCV/ml), which was obtained from an HCV-positive blood donor, was added to PH5CH8 cells (1×10^5 cells) suspended in 500 μl of a fresh culture medium (day -1) [9,10]. After incubation for 2 h at 37 °C, the PH5CH8 cells were maintained at 32 °C with an additional 500 μl of fresh culture medium.

RT-nested PCR and sequencing. RNA from PH5CH8 cells inoculated with serum 1B-2 was prepared using Isogen reagent (Nippon Gene, Japan). RNA samples were used as templates for the detection of the hypervariable region I (HVRI) of HCV RNA by RT nested PCR using specific primers as previously described [11–13]. An antisense primer, 145RA, 5′-GTCCCCACTACAACAGGGCT-3′ (corresponding to positions 1863–1882 of HCV-J) was used to prime cDNA synthesis. Primer 144, 5′-CTACTCCGGATCCCACAAGC-3′ (corresponding to positions 1338–1357 of HCV-J) and 145RA were employed in the first round of PCR. Primer 146A, 5′-ATTCCAT GGTGGGGAACTGG-3′ (corresponding to positions 1414–1433 of HCV-J) and 147RA, 5′-GGGGTGAAGCAATACACTGG-3′ (corresponding to positions 1842–1861 of HCV-J) were used in the second round of PCR. PCR products were cloned into a pTZ19R plasmid vector [9] and sequenced with an ABI Prism dye terminator cycle sequencing kit (Perkin–Elmer, Tokyo, Japan).

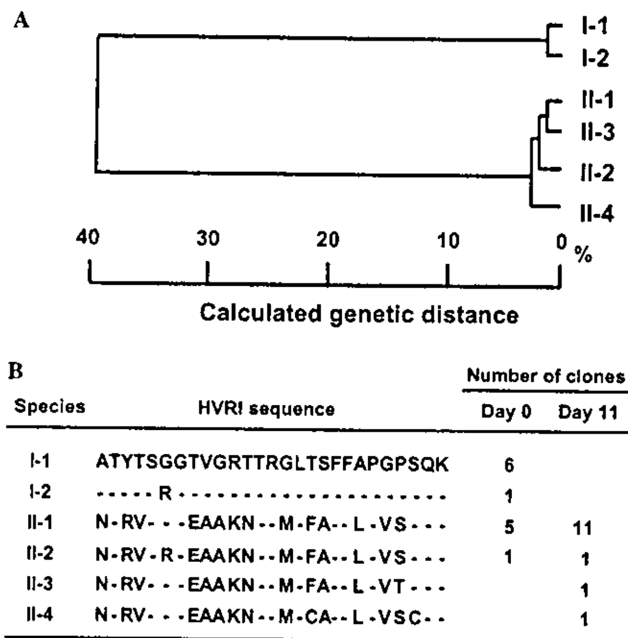
Replication-defective recombinant adenoviruses and transfection into the PH5CH8 cells. Replication-defective recombinant adenoviral vectors carrying either human IFN-α cDNA (AxCA-IFN) or β-galactosidase (AxCA-lacZ) gene were prepared as described [14,15]. The recombinant adenoviruses are serotype five derivative with a deletion in the E3 region and have a CAG promoter, which is a hybrid of the cytomegalovirus immediate–early enhancer sequence and the chicken β-actin/rabbit β-globin promoter [16]. In chronic viral hepatitis, only a few percent of hepatocytes are infected at a given time, and the liver tissue is characterized by the continued death and regeneration of hepatocytes. New infection with HCV into the uninfected and/or regenerating hepatocytes occurs persistently in vivo. To examine an inhibitory effect of AxCA-IFN at an early phase of the HCV infection, the PH5CH8 cells were infected with either AxCA-IFN or AxCA-lacZ at an moi (multiplicity of infection) of 30 one day after inoculation of HCV (day 0). Cells were harvested at day 11 for an analysis of the HVRI of HCV. At day 11, half of the cells were passaged and cultured up to day 18.

Quantification of HCV RNA by real-time detection (RTD)-PCR. RTD-PCR was performed using an EZ rTth RNA PCR kit (Perkin–Elmer, Tokyo, Japan) with an ABI Prism 7700 sequence detector system as previously described [17]. For the quantification of HCV RNA, a new set of primers and probe was designed as follows. The sequences of the forward and reverse primers and probe were 5′-CGGGAGAGCCATAGTGG-3′, 5′-AGTACCACAAGGCCTTT CG-3′ and 5′-CTGCGGAACCGGTGAGTACAC-3′, respectively. After amplification, real-time data acquisition and analysis were performed. Once the threshold was chosen, the point at which the amplification plot crossed the threshold was defined as the threshold cycle (Ct). The calculated Ct value is predictive of the quantity of target RNA copies present in the sample. The standard curve for this assay was calculated using a series of 10-fold dilutions of previously titrated synthetic HCV RNA [17]. The copy number of GAPDH mRNA was also quantified using the EZ rTth RNA PCR kit. The data were expressed as the relative HCV copy number (HCV/GAPDH copies), which is the copy number of HCV RNA divided by the copy number of GAPDH mRNA, to estimate the time course of HCV replication.

Results

Populations from PH5CH8 cells inoculated with sera 1B-2

It is well known that HCV shows a marked sequence heterogeneity, variability, and a quasi-species nature in a serum from an individual patient. HVRI is used as a good molecular marker for distinguishing HCV species [13,18,19]. It has been reported that limited HCV species became predominant during the culture of in vitro HCV-infected human lymphocytes [12,20–22]. To monitor the alteration of HCV species after inoculation, HVRI populations were analyzed by RT-nested PCR using RNA, which were extracted from HCV-infected PH5CH8 cells (days 0 and 11). Phylogenetic trees were constructed by multialignment analysis using the unweighted pair-grouping method with arithmetic mean [23] and each species was given a name as shown in Fig. 1A. The quasi-species nature of the HVRI populations in the HCV-infected PH5CH8 cells at day 0 was relatively simple. The amino acid sequence and frequency of each species in HCV-infected cells at day 0 and day 11 are shown in Fig. 1B. HVRI populations were I-1 dominant (46% frequency) at day 0, whereas they became almost homogeneous to species II-1 (78%) at day 11, suggesting that HCV can effectively replicate in vitro.



Species	HVRI sequence	Number of clones Day 0	Day 11
I-1	ATYTSGGTVGRTTRGLTSFFAPGPSQK	6	
I-2	·····R·····················	1	
II-1	N·RV···EAAKN··M·FA··L·VS···	5	11
II-2	N·RV·R·EAAKN··M·FA··L·VS···	1	1
II-3	N·RV···EAAKN··M·FA··L·VT···		1
II-4	N·RV···EAAKN··M·CA··L·VSC··		1

Fig. 1. The shift to distinct HVRI species after inoculation to hepatocytes. (A) HVRI populations from PH5CH8 cells inoculated with serum IB-2. The HVRI species were named according to the order of isolation and position in the phylogenetic tree [22] constructed by multialignment analysis using the unweighted pair-grouping method with arithmetic mean [23] of the GENETYX MAC program. (B) The deduced amino acid sequences of HVRI species obtained from the HCV-infected cells. The amino acid sequences are written with a single letter code. Dotted lines indicate amino acids identical to the amino acid sequence of I-1. The numbers on the right side indicate the actual number of plasmid clones obtained and sequenced.

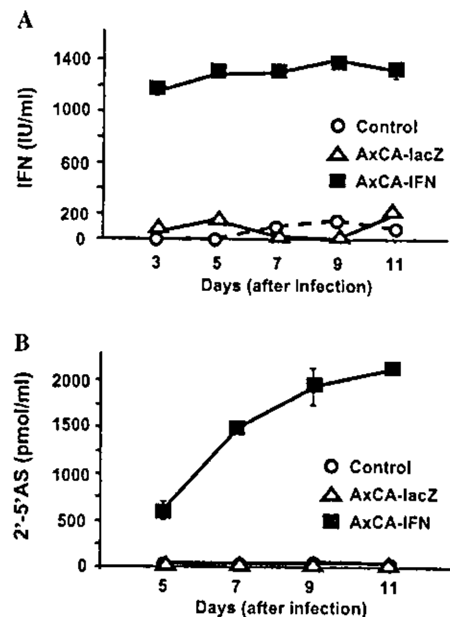


Fig. 2. AxCA-IFN produced IFN-α and activated 2′–5′AS in HCV-infected hepatocytes. (A) Human IFN-α concentration. Twenty-four hours after inoculation of HCV (8×10^5 HCV) into PH5CH8 cells, the cells were infected with either AxCA-IFN or AxCA-lacZ at an moi of 30. IFN-α was measured by enzyme-linked immunosorbent assay (ELISA) using human IFN-α measurement kit (Nippon Antibody Institute, Tokyo, Japan). (B) 2′–5′AS activity. 2′–5′AS activity was determined by a radioimmunoassay (SRL, Tokyo, Japan). The assays (carried out in triplicate) were repeated a minimum of two times and the means ± standard deviations were plotted.

These data were consistent with previous reports [9,24] and confirmed the validity of our experiment.

Continuous IFN production and induction of 2′–5′AS

Next, to examine whether the IFN-α-expressing adenovirus infection can effectively produce IFN-α protein in HCV-positive hepatocytes, the PH5CH8 cells were infected with AxCA-IFN at an moi of 30 one day after the inoculation of serum 1B-2. These infected cells produced more than 1000 IU/ml of IFN-α in the conditional medium for at least 11 days after the adenoviral infection, whereas the AxCA-lacZ-infected cells (moi 30) did not (Fig. 2A).

One of the major antiviral mechanisms of IFN protein is degradation of viral RNA. A 2′–5′AS induced by IFN is activated in the presence of viral double stranded RNA, and a 2′–5′oligoadenylate produced by an activated 2′–5′AS binds to ribonuclease L (RNase L), which results in degradation of viral RNA [25]. The activity of 2′–5′AS was gradually increased up to 2000 pmol/ml (Fig. 2B), demonstrating that IFN-α protein produced by gene transfer can effectively activate the 2′–5′AS/RNase L pathway in hepatocytes.

Inhibition of HCV replication by AxCA-IFN

To examine the anti-HCV effect of the IFN-α gene transfer in vitro, copy numbers of HCV were quantified by real-time detection (RTD)-PCR. Copy numbers of HCV gradually decreased for up to 7 days after the adenoviral infection and then started to increase in both the control and AxCA-lacZ-treated cells because it takes 3–4 days for HCV to start replication in PH5CH8 cells. By contrast, HCV RNA was not detected at days 9 and 11 in the AxCA-IFN-treated cells (Figs. 3A and B) (control vs AxCA-IFN; $p = 0.0007$, AxCA-lacZ vs AxCA-IFN; $p < 0.0001$). The disappearance of HCV RNA was linked to the increase of 2′–5′AS activity (2000 pmol/ml) (Fig. 2B), which suggests that the activated 2′–5′AS/RNase L pathway induces the degradation of HCV RNA. At day 11, half of the infected cells were passaged to a new culture dish and cultured up to day 18. Although HCV was still detected at levels of more than 200 HCV/GAPDH copies in both the control and AxCA-lacZ-treated cells, it was not detected in the AxCA-IFN-treated cells at day 18 (data not shown). Although the concentration of IFN-α in the conditional medium was relatively high compared with that in the serum of patients treated with IFN, there was no significant difference in cell growth and mor-

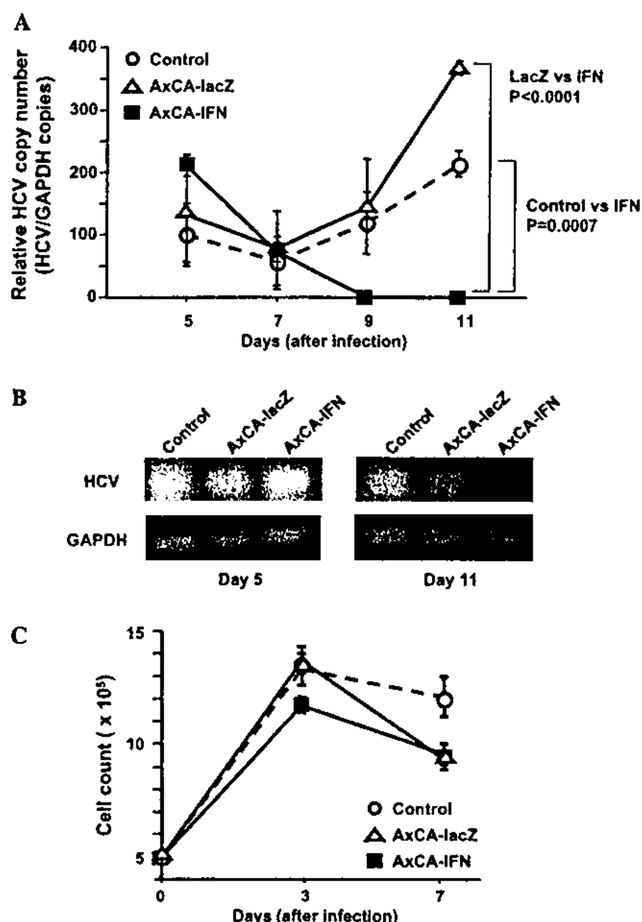


Fig. 3. Inhibition of HCV by IFN-α gene transfer. (A) Time course of HCV RNA measured by real-time detection (RTD)-PCR. Twenty-four hours after inoculation of HCV (8×10^5 HCV) in PH5CH8 cells, the cells were infected with either AxCA-IFN or AxCA-lacZ at an moi of 30. Relative HCV copy number is the copy number of HCV RNA divided by copy number of GAPDH mRNA. (B) Double stranded DNA products of RTD-PCR (53 cycles) are shown in an agarose gel stained with ethidium bromide. The strand specificity was confirmed by sequencing analysis. (C) Growth of the cells. PH5CH8 cells were seeded at 5×10^5 cells in 6 cm dishes in triplicate, inoculated with serum 1B-2, and 1 day later infected with AxCA-IFN at an moi of 30. The cell number of infected PH5CH8 cells was counted by the dye exclusion method 3 and 7 days after the infection of AxCA-IFN. The assays were repeated a minimum of two times and the mean ± standard deviations were plotted.

phology between AxCA-IFN- and AxCA-lacZ-infected hepatocytes (Fig. 3C) and both cells reached a confluence at day 7 and remained confluent up to day 11, indicating that cytotoxicity of IFN-α could not explain the inhibition of HCV replication.

Discussion

IFNs play a central role in the antiviral defense of the host [26]. IFNs exert their biological functions by binding to specific cell-surface receptors. In turn, this triggers the intracellular IFN signaling pathway, mainly the JAK-STAT pathway, and then eventually induces the expression of a large number of IFN-stimulated genes including double stranded RNA dependent protein kinase, 2′–5′AS and Mx, which are involved in a non-virus-specific antiviral state [26]. Specific activation of IFN-induced genes leading to the establishment of an antiviral state in hepatocytes has not yet been documented.

Chronic HCV infections have been cured only in a fraction of patients treated with the current IFN therapy. The mechanisms for anti-HCV action of IFN are still poorly understood. Neuman et al. [27] reported that the major initial effect of IFN is to block virion production or release by mathematical modeling of HCV dynamics during clinical IFN-α therapy. Recently, the inhibition of HCV replication by IFN-α protein has been reported in a human lymphocytic cell line supporting HCV genome replication, in hepatoma cell lines, and in primary cultures of normal hepatocytes [8,9,28,29]. Our study also showed that IFN-α protein produced by gene transfer significantly inhibited the increase of HCV copy numbers in an in vivo culture system after day 7, suggesting that one of the major anti-HCV effects of IFN-α is the inhibition of HCV replication.

On the other hand, IFN-α protein is a cytokine with multiple biological activities in vivo that include antiviral activity, regulation of cell proliferation and differentiation, and immunomodulation. IFN-α-induced humoral responses (e.g., production of neutralizing antibodies) effectively prevent HCV entry into hepatocytes [30]. The clearance of the host HCV-infected cells by IFN-modulated immune response may also be an important factor in the anti-HCV effect of IFN-α [27], which could not be evaluated in a hepatocyte culture system. The anti-HCV effect and safety of IFN gene therapy should be evaluated in a series of next-step preclinical studies using animal models such as chimpanzees.

In addition to the antiviral effect, IFN-α inhibits the expression of profibrotic cytokines such as TGF-β and the activity of collagen synthesis of hepatic stellate cells, which resulted in curtailing liver cirrhosis in experimental animal models and clinical studies [5,31–37]. Our recent study also showed that a single injection of an adenoviral vector expressing rat IFN-α achieved an efficient confinement of IFN-α production in the liver, significantly prevented the progression of liver injury and fibrosis without apparent systemic toxicity, and improved the survival rate in a dimethylnitrosamine-induced rat LC model [6]. This anti-fibrotic effect should add an advantage to IFN gene therapy for HCV-induced LC. IFN-α is therefore expected to be particularly powerful in preventing the development of HCV-related diseases.

We employed an adenovirus vector in our strategy of HCV gene therapy, since the adenovirus vector could more efficiently express transgenes in the HCV target organ, the liver, than the other viral vectors and polycation-based non-viral vectors [6]. Adenovirus vectors can develop hepatotoxicity apparently in a dose-dependent manner. However, our preclinical study suggested that a high dose of adenovirus vector may not be required for this treatment strategy [6], because a cytokine such as IFN is effective in a small dose. Moreover, the intrahepatic arterial infusion, instead of a systemic administration, could further reduce the amount of the adenovirus vector. Development of less immunogenic adenoviral vectors such as the 'gutted' vector and/or targeting vectors such as the fiber-mutant adenovirus vector may further enhance the efficacy and safety of the cytokine gene therapy.

The cost of a standard IFN regimen in Japan is about 2 million Yen (approximately 15,000 USD) per patient for six months under the current national health insurance program, and thus the treatment is officially justified only to patients with chronic active hepatitis. However, if we can afford the IFN therapy at an earlier stage of HCV infection, where the virus load is lower, the success rate for virus eradication will be increased. IFN gene therapy is expected to offer a better cost–benefit alternative to conventional IFN protein therapy.

Acknowledgments

This work was supported in part by a grant-in-aid for the Second Term Comprehensive 10-year Strategy for Cancer Research from the Ministry of Health, Labour and Welfare of Japan, and by grants-in-aid for Cancer Research from the Ministry of Health, Labour, and Welfare of Japan.

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[CANCER RESEARCH 64, 1307–1314, February 15, 2004]

Promotion of Microsatellite Instability by Hepatitis C Virus Core Protein in Human Non-neoplastic Hepatocyte Cells

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ABSTRACT

Hepatitis C virus proteins exert an effect on a variety of cellular functions, including gene expression, signal transduction, and apoptosis, and because they possess oncogenic potentials, they have also been suggested to play an important role in hepatocarcinogenesis. Although the mechanisms of hepatocarcinogenesis remain poorly understood, we hypothesized that the disease may arise because of a disturbance of the DNA repair system by hepatitis C virus proteins. To test this hypothesis, we developed a reproducible microsatellite instability assay system for mismatch-repair using human-cultured cells transducted with pCXpur retrovirus expression vector, in which the puromycin resistance gene was rendered out-of-frame by insertion of a $(CA)_{17}$ dinucleotide repeat tract immediately following the ATG start codon. Using several human cancer cell lines known to be replication error positive or negative, we demonstrated that this assay system was useful for monitoring the propensity for mismatch-repair in the cells. This assay system was applicable to non-neoplastic human PH5CH8 hepatocytes, which could support hepatitis C virus replication. Using PH5CH8 cells, in which hepatitis C virus proteins were stably expressed by the retrovirus-mediated gene transfer, we found that the core protein promoted microsatellite instability in PH5CH8 cells. Interestingly, such promotion by the core protein only occurred in cells having the core protein belonging to genotype 1b or 2a and did not occur in cells having the core protein belonging to genotype 1a, 2b, or 3a. This is the first report to demonstrate that the core protein may disturb the DNA repair system.

INTRODUCTION

Hepatitis C virus (HCV), discovered in 1989, is the major causative agent of parenteral non-A, non-B hepatitis worldwide (1). Following the development of a method of diagnosing HCV infection (2), it became apparent that HCV infection frequently causes chronic hepatitis, and the persistent infection with HCV is implicated in liver cirrhosis and hepatocellular carcinoma (HCC; 1–4). HCV is an enveloped positive single-stranded RNA (9.6 kb) virus belonging to the family *Flaviviridae* (5, 6). The HCV genome shows remarkable genetic heterogeneity and at least six major HCV genotypes, further grouped into >50 subtypes, have been identified to date (7, 8). The HCV genome encodes a large polyprotein precursor of approximately 3,000 amino acid (aa) residues, and this precursor protein is cleaved by the host and viral proteinases to generate at least 10 proteins in the following order: NH_2-core-envelope 1 (E1); E2; p7; nonstructural protein 2 (NS2); NS3; NS4A; NS4B; NS5A; NS5B; and COOH (9–11). These HCV proteins not only play a role in viral replication but also affect a variety of cellular functions, including gene expression, signal transduction, and apoptosis (12, 13).

HCV replication and the viral protein expression have been observed in HCCs, but the molecular mechanism of HCV-associated hepatocarcinogenesis remains poorly understood. One major reason for this is the lack of reproducible and efficient HCV proliferation in cell culture (14). In HCV-related hepatocarcinogenesis, it has been speculated that repeated hepatocytic regeneration processes also occur in HCV-infected individuals to offset the damage caused by HCV multiplication and maintain sufficient liver function. Such a process of damage and regeneration probably enhances the likelihood of genetic alteration (15). In addition, it has also been reported that no significant differences were found in the number and type of chromosomal imbalances between hepatitis B virus- and HCV-infected HCCs (16). This finding is consistent with models suggesting that hepatitis B virus and HCV cause cancer through nonspecific inflammatory and regenerative processes (17). On the other hand, it has been demonstrated that HCV proteins significantly influence a variety of oncogenic processes. For example, the HCV core protein may cooperate with H-ras in the process of transforming the cells into malignant phenotypes (18), and the constitutive expression of core protein in transgenic mice has been shown to induce HCC (19). Furthermore, it has been reported that the HCV NS3, NS4B, and NS5A proteins also have oncogenic potential (20–22). Therefore, it is likely that HCV proteins contribute to the initiation or development of HCC.

We reported previously that PH5CH8 cells cloned from PH5CH cell line (23) could support HCV replication (24), although the level of HCV proliferation was fairly low. PH5CH cell line was established by immortalization with SV40 large T antigen using non-neoplastic liver tissue from a patient with HCV-related HCC (23). PH5CH8 cells are considered to be useful in examining the role of HCV proteins during the process of hepatocarcinogenesis. In addition, PH5CH8 cells possess wild type of p53 and Rb protein and show nonmalignant phenotype (23), although SV40 large T antigen would partially repress the function of p53. Then, we speculated that the DNA repair system of host cells may be one of the target sites of HCV proteins, because the constant operation of this system is crucial to the process of inflammation and regeneration of hepatic lesions in patients with chronic hepatitis C. Although DNA damages caused by such damaging agents as X-rays, UV light, and alkylating agents are repaired by base excision, nucleotide excision, recombinational repair, and so forth, the mismatch-repair (MMR) system is used to repair A-G or T-C mismatches, insertion, and deletion caused by the replication errors (RER) during the regenerative process (25). In addition, studies on genetic instability using clinical specimens from patients with HCC have revealed that microsatellite instability (MSI) was found in approximately 20% of the patients examined (26, 27), whereas no MSI was found in the histologically normal liver (26). In this study, we focused on the MMR system to examine the effects of HCV proteins. For this purpose, we developed a novel MSI assay system in human cultured cells using the retrovirus expression vector containing the (CA) repeat sequence. Our results indicate that the core protein may promote MSI in PH5CH8 cells.

Received 10/1/03; revised 11/19/03; accepted 12/12/03.

Grant support: Grants-in-aid from the Second-Term Comprehensive 10-Year Strategy for Cancer Control; from the Ministry of Health, Labor, and Welfare of Japan for research on hepatitis and BSE; and from the Organization for Pharmaceutical Safety and Research for scientific research.

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MATERIALS AND METHODS

Cell Lines. The human colon cancer cell lines LS174T, LoVo, and SW480 were provided by the Cell Resource Center of the Biomedical Research Institute of Development, Aging, and Cancer (Tohoku University). LS174T, LoVo, and SW480 cells were maintained in DMEM supplemented with 10% fetal bovine serum (FBS), F12 supplemented with 20% FBS, and RPMI 1640 supplemented with 10% FBS, respectively. The additional human colon cancer cell line HCT116 was a kind gift of K. Shimizu (Okayama University) and was maintained in DMEM supplemented with 10% FBS. Cells of the human cervical carcinoma cell line HeLa were maintained in DMEM supplemented with 10% FBS. PH5CH8 hepatocytes were maintained as described previously (23). Bosc 23 cells were also maintained in DMEM supplemented with 10% FBS.

Construction of Expression Vectors. pCX retroviral vector pCXpur (28), a kind gift of T. Akagi (Osaka Bioscience Institute), which contains the resistance gene for puromycin, was used for the construction of pCXpur containing 17 CA repeats [pCXpur/$(CA)_{17}$/out-of-frame and pCXpur/$(CA)_{17}$/in-frame]. pCXpur/$(CA)_{17}$/out-of-frame was generated by inserting a fragment that was ligated with *Sal*I sites of the PCR products of two primer sets (primers PU-NotI5′ and FCA3′; primers TA5′ and PU-StuI3′; Table 1) using pCXpur as a template, into the *Not*I-*Stu*I sites of pCXpur. A schematic of the construction of the pCXpur/$(CA)_{17}$/out-of-frame retrovirus vector is shown in Fig. 1. pCXpur/$(CA)_{17}$/in-frame was also generated by inserting a fragment that was ligated with *Sal*I sites of the PCR products of two primer sets (primers PU-NotI5′ and FCA3′; primers PU-5′ and PU-StuI3′; Table 1) using pCXpur as a template, into the *Not*I-*Stu*I sites of pCXpur. The nucleotide sequences of the obtained pCXpur/$(CA)_{17}$/out-of-frame and pCXpur/$(CA)_{17}$/in-frame retrovirus vector were confirmed by Big Dye termination cycle sequencing using an Applied Biosystem 310 automated sequencer (Applied Biosystems, Norwalk, CT).

Retrovirus Infection of the Recipient Cell Lines. Transient transfections were performed with 2 μg of DNA and 6 μl of FuGENE6 (Boehringer Mannheim, Mannheim, Germany) in 6-well plates according to the manufacturer's instructions. Retrovirus infection was performed by a method described previously (29, 30). At 3 h postinfection, the cells were cultured with fresh medium until reaching confluence (approximately 3 days), and then the cells were used for the selection with puromycin (5 μg/ml) or blasticidin (20 μg/ml).

MSI Assay Using Human Cultured Cells. Various human-cultured cells (2×10^5 cells) were plated on a 6-well plate for 24 h before retrovirus infection and were infected with the retrovirus pCXpur/$(CA)_{17}$/out-of-frame or pCXpur/$(CA)_{17}$/in-frame. Mock infection was also performed as a control. At 3 days postinfection, cells were passaged to a 10-cm dish and further cultured for 2 days, and then aliquots of 1×10^5 cells were placed in 10-cm dishes for selection by puromycin (5 or 10 μg/ml) and grown for 14 days with a medium change every 3–4 days. In some experiments, the culture period from retrovirus infection to addition of puromycin was changed to 9–21 days from 5 days. The obtained puromycin-resistant (pur^R) colonies were stained with Coomassie Brilliant Blue (0.6g/liter in 50% methanol-10% acetic acid) and were automatically counted using a ChemiImager 4000 (α Innotech Corp.). Triplicate infection experiments were repeated at least three times to verify the reproducibility of the results. To examine the efficiency of retrovirus infection, the retrovirus pCLMFG-LacZ (31) was used, and at 2 days postinfection, the cells were fixed and stained with 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside.

Preparation of PH5CH8 Cells Stably Expressing HCV Proteins. PH5CH8 cells were infected with retrovirus pCXbsr encoding various HCV proteins, as described above. Because the HCV core protein constructs, pCXbsr/core(1b-M) (32), pCXbsr/core(1b-P) (32), pCXbsr/core(1b-HCC) (33), pCXbsr/core(1a) (30), pCXbsr/core(2a) (30), pCXbsr/core(2b) (30), and pCXbsr/core(3a) (30), which are able to express core(1b-M), core(1b-P), core(1b-HCC), core(1a), core(2a), core(2b), and core(3a) proteins, respectively (the numbers and letters in parentheses indicate the HCV genotype), were used to obtain the PH5CH8 cells stably expressing the core protein. Because the other HCV protein constructs, pCXbsr/E1(1b-P), pCXbsr/E2(1b-P), and pCXbsr/NS5A(1b-P), which are able to express E1(1b-P), E2(1b-P), and NS5A(1b-P) proteins, respectively, were also used to obtain the PH5CH8 cells stably expressing E1(1b-P), E2(1b-P), and NS5A(1b-P) proteins, respectively. pCXbsr was used as a control. At 2 days postinfection, the PH5CH8 cells were changed with fresh medium containing blasticidin (20 μg/ml), and the culture was continued for 7 days to select the cells expressing HCV proteins.

MSI Assay Using PH5CH8 Cells Stably Expressing HCV Proteins. The PH5CH8 cells (2×10^5 cells) stably expressing HCV protein [core, E1, E2, or NS5A] were used for the MSI assay as described above. The PH5CH8 cells, which became resistant to blasticidin after infection of the retrovirus pCXbsr, were used as a control. These cells were further infected with the retrovirus pCXpur/$(CA)_{17}$/out-of-frame, and then an MSI assay was performed according to the method described above, with the exception that double-selections were performed using both blasticidin (20 μg/ml) and puromycin (5 or 10 μg/ml).

Sequence Analysis of the (CA) Repeat-Surrounding Region Recovered from pur^R Colonies. The pur^R colonies (7–10 independent colonies) obtained in the MSI assay were randomly selected and grown to confluence in 6-well plates, and their genomic DNAs were prepared using an ISOGEN extraction kit (Nippon Gene Co., Toyama, Japan) as indicated by the manufacturer. These DNAs (each 1 μg) were used for the amplification of the (CA) repeat-surrounding region by PCR using a primer set (primers MIB5′ and MIE3′; Table 1). Thirty-five PCR cycles were performed, and each cycle consisted of annealing at 63°C for 45 s, primer extension at 68°C for 1 min, and denatur-

Table 1 *Oligonucleotides used in this study*

Oligonucleotide	Sequence	Direction
PU-NotI5′	aattaaGCGGCCGCGGTAACAATTGTTAACTAAC	Forward
FCA3′	attatGTCGACTGTGTGTGTGTGTGTGTGTGTGTGTGTG GTGGTCATGGTATTATCGTGTTTTTCAAAGGAAAACC ACGTCCCGTGGTTCGGGGGGCCTAGAC	Reverse
TA5′	attatGTCGACTAACCGAGTACAAGCCCACGGTG	Forward
PU-StuI3′	attatAGGCCTTCCATCTGTTGCTGCGCGGCCAG	Reverse
PU-5′	attatGTCGACACCGAGTACAAGCCCCACGGTG	Forward
MIB5′	attatGGATCCGCACATGCTTTACATGTGTTTAGTCG	Forward
MIE3′	attatGAATTCAGGCGCACCGTGGGCTTGTACTC	Reverse
hMLH1F	TTCTCAGGTTATCGGAGCCAGCAC	Forward
hMLH1R	CTTCGTCCCAATTCACCTCAGTGG	Reverse
hMSH2F	TACATGTCACAGCACTCACCACTG	Forward
hMSH2R	CTCCTCAAGTTCCAGGGCTTTCTG	Reverse
hMSH6F	ACAAGGGGCTGGGTTAGCAAAAG	Forward
hMSH6R	AGTCACCATTCTCTTCCGCTTTCG	Reverse
hPMS2F	CAGGTTTCATTTCACAATGCACGC	Forward
hPMS2R	TTACCTTCAACATCCAGCAGTGGC	Reverse
hMSH3F	GATGGCATTTTCACAAGGATGGG	Forward
hMSH3R	CTGGCGGATAATGGGTGACAAAC	Reverse
hPMS1F	GGCAACAGTTCGACTCCTTTCAAG	Forward
hPMS1R	TTGATACCCTCCCCGTTATCTCGC	Reverse
GAPDHF	GATGCTGGCGCTGAGTACGTCG	Forward
GAPDHR	GAGGAGACCACCTGGTGCTCAG	Reverse

The recognition sites of restriction enzymes are underlined.

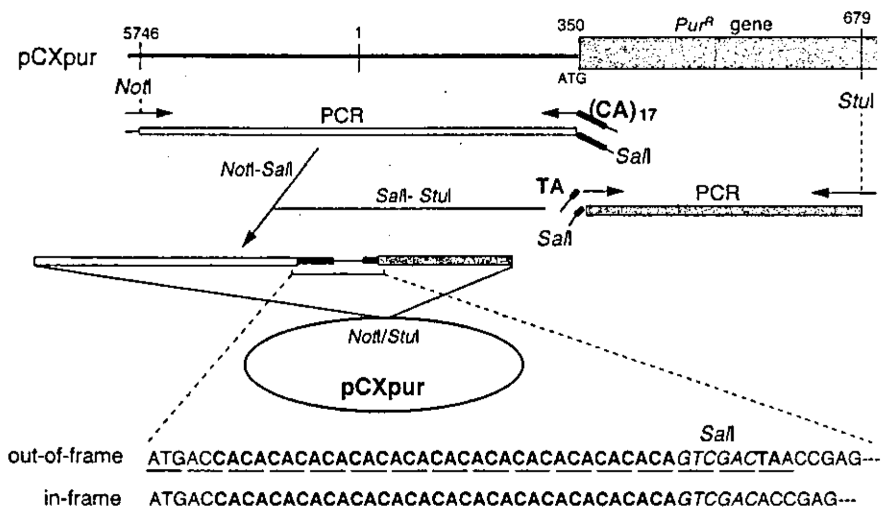

Fig. 1. Construction of pCXpur retrovirus vector containing the (CA) repeat sequence. The outline for the construction of the pCXbsr/$(CA)_{17}$/out-of-frame retrovirus vector is presented schematically. The nucleotide sequences of the (CA) repeat unit of pCXbsr/$(CA)_{17}$/out-of-frame and pCXbsr/$(CA)_{17}$/in-frame are shown (*bottom*), and each codon is *underlined*. pur^R, puromycin-resistant.

ation at 97°C for 45 s using proofreading KOD-Plus DNA polymerase (Toyobo, Osaka, Japan). PCR products (186 bp) containing the (CA) repeat sequence were cloned into the *Bam*HI and *Eco*RI sites of pCRII-TOPO (Invitrogen, Carlsbad, CA). Plasmid inserts were sequenced in both the sense and antisense.

Western Blot Analysis. The preparation of cell lysates, SDS-PAGE, and immunoblotting analysis with a polyvinylidene difluoride membrane were performed as described previously (10). Anti-core monoclonal antibody (2ZCP9; Institute of Immunology Co., Tokyo, Japan), anti-E1 monoclonal antibody (a generous gift from M. Kohara, Tokyo Metropolitan Institute of Medical Science), anti-E2 monoclonal antibody (34), and anti-NS5A antibody (a generous gift from A. Takamizawa, Osaka University) were used for the detection of core, E1, E2, and NS5A proteins, respectively. Anti-β-actin antibody (AC-15; Sigma) was also used for the detection of β-actin as an internal control. Immunocomplexes on the filters were detected by enhanced chemiluminescence assay (Renaissance; NEN, Boston, MA).

Reverse Transcription (RT)-PCR. Total cellular RNA was extracted using an ISOGEN extraction kit for the RT-PCR analysis. RT-PCR was performed by a method described previously (30). The sequences of hMLH1 (accession number U07418), hMSH2 (accession number U03911), hMSH6 (accession number U54777), hPMS1 (accession number U13695), hPMS2 (accession number U14658), hMSH3 (accession number U61981), and glyceraldehyde-3-phosphate dehydrogenase (accession number NM 002046) were used to design the primers listed in Table 1. Twenty-five cycles of PCR (20 cycles for glyceraldehyde-3-phosphate dehydrogenase only) were performed, and the amplified DNA was detected by staining with ethidium bromide after separation by 3% agarose gel electrophoresis.

RESULTS

Construction of the Retrovirus Vectors Containing the Microsatellite (CA) Repeat Sequence. The retrovirus expression vector pCXpur (28) contains a pur^R gene to select for transduced cells. Initially, we made a pCXpur/$(CA)_{17}$/in-frame, in which 42 nucleotides [AC +17 CA repeats + GTCGAC (*Sal*I site)] were inserted immediately following the ATG initiation codon of the pur^R gene, and examined the influence of this insert on the pur^R activity. We confirmed that the human colon cancer SW480 cells (35), which were known to possess RER− (MMR proficient) phenotype, infected with the retrovirus pCXpur/$(CA)_{17}$/in-frame were able to proliferate in the presence of puromycin (10 μg/ml; data not shown), indicating that the pur^R gene product from pCXpur/$(CA)_{17}$/in-frame is functional in the cells. We next constructed pCXpur/$(CA)_{17}$/out-of-frame, in which the pur^R gene was rendered out-of-frame by the insertion of 44 nucleotides [AC +17 CA repeats + GTCGAC (*Sal*I site) + TA (to make a TAA stop codon)] immediately following the ATG initiation codon, as shown in Fig. 1. By this modification, the pur^R gene product should not be produced from pCXpur/$(CA)_{17}$/out-of-frame. Using the SW480 cells (RER−), we confirmed that cells infected with the retrovirus pCXpur/$(CA)_{17}$/out-of-frame were also unable to survive in the presence of puromycin (10 μg/ml; data not shown), indicating that the pur^R gene product is not produced from pCXpur/$(CA)_{17}$/out-of-frame, as we expected (Fig. 1). With regard to the plasmid vector for MSI assay at the cell-culture level, to date, several similar vector systems using the neomycin resistance gene, hygromycin B phosphotransferase gene, or β-galactosidase gene have been reported (36–39), but there has been no system using the pur^R gene. Puromycin has an advantage for the fast (within a few days) and keen-edged selection of the cells. In the present study, none of the cells lines examined were able to survive in the presence of 1 μg/ml of puromycin.

Establishment of the MSI Assay System. In this assay, after the transduction of pCXpur/$(CA)_{17}$/out-of-frame [pCXpur/$(CA)_{17}$/in-frame as a positive control], the recipient cells were cultured for 5 days, and then the cells were selected with puromycin (5 or 10 μg/ml). In theory, although the cells transducted with pCXpur/$(CA)_{17}$/in-frame are able to proliferate in the presence of puromycin, the cells transducted with pCXpur/$(CA)_{17}$/out-of-frame should not be able to survive in the presence of puromycin, as we confirmed in RER− cells. However, if some frameshift mutations do occur in the vicinity of the $(CA)_{17}$ sequence during the 5 days of culture before addition of puromycin, such cells would become pur^R cells and grow up even in the presence of puromycin. As a consequence, we therefore considered the colonies to be pur^R colonies at about 2 weeks after addition of puromycin. Because the microsatellite insert puts the pur^R gene in the −1 reading frame, detectable dinucleotide frameshift mutations include the deletions of 2, 8, 14, 20, 26, or 32 bp and insertions of 4, 10, or 16 bp, and so forth. As the method of gene transduction, we used retrovirus infection because of its highly efficient gene transfer into cells. Recently, Zienolddiny *et al.*(38) also used a retrovirus infection system for MSI assay.

We initially verified our method using several human cell lines. It has been reported that HCT116 and LoVo cells exhibited marked dinucleotide repeat instability, because HCT116 cells possessed a nonsense mutation in exon 9 in *hMLH1* gene, and LoVo cells were *hMSH2*-deficient (deletion of exons 4–8; 40). LS174T cells have been also reported to possess RER+ (MMR deficient) phenotype by the analysis of 32 microsatellite loci (41). On the other hand, HeLa and SW480 cells are known to possess RER− phenotype because of accurately replication of repetitive DNA and correction of mismatches

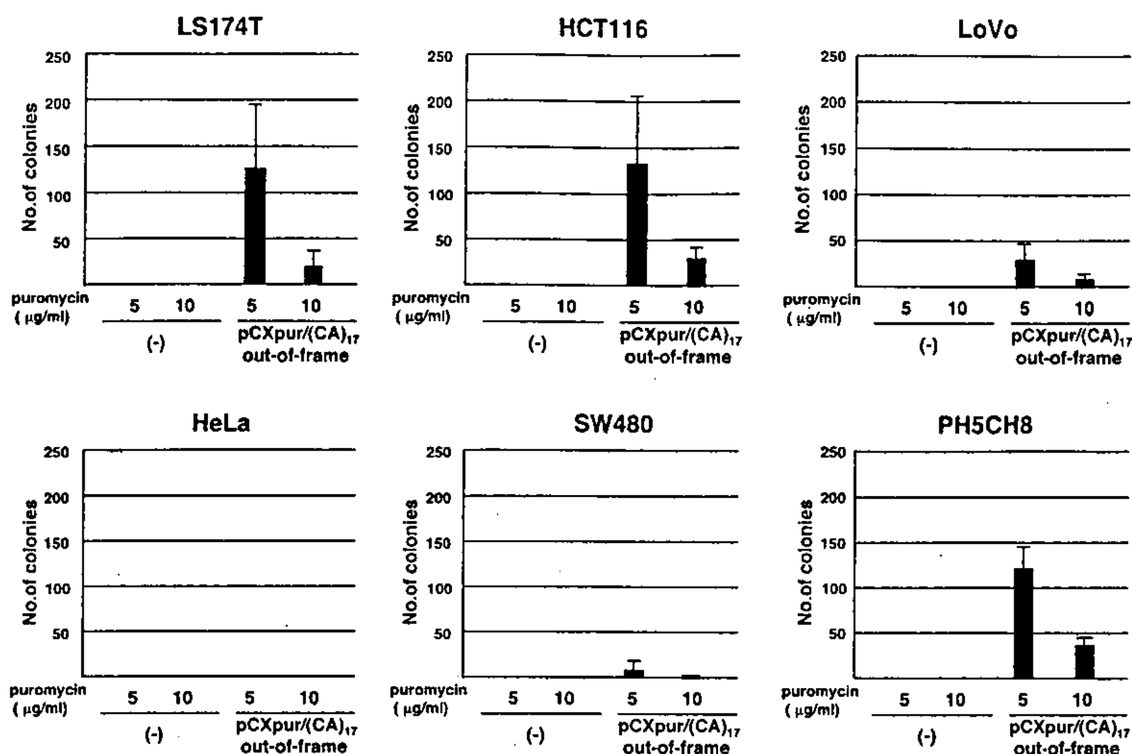

Fig. 2. Microsatellite instability assay using pCXbsr/$(CA)_{17}$/out-of-frame in various cell lines. The puromycin-resistant colonies stained with Coomassie Brilliant Blue were automatically counted by a ChemiImager 4000. (-), mock infection.

(35). Therefore, HCT116, LoVo and LS174T were used as the RER+ cell lines, and HeLa and SW480 were used as the RER− cell lines. PH5CH8 cells were also used for the analysis using our method, although the state of MMR system has not yet been determined by the analysis of microsatellite loci.

All cell lines examined at 2 days postinfection with the retrovirus pCLMFG-LacZ were efficiently stained with 5-bromo-4-chloro-3-indolyl-b-D-galactropyranoside, although the level of staining and the percentage of stained cells in the LoVo and HeLa cell lines were somewhat lower than in the other cell lines (data not shown). At 2 weeks after the selection with puromycin, pur^R colonies were counted after staining with Coomassie Brilliant Blue. As shown in Fig. 2, a substantial number of colonies were obtained in the RER+ cell lines (LS174T, HCT116, and LoVo), whereas no or only a few colonies were obtained in RER− cell lines (HeLa and SW480). Because the growth rate of LoVo cells was rather lower than those of LS174T and HCT116 cells, it might cause the low number of pur^R colonies in LoVo cell line despite RER+ phenotype. In all cases, the number of pur^R colonies obtained in the presence of puromycin (10 μg/ml) was lower than that obtained in the presence of puromycin (5 μg/ml), suggesting that the colonies expressing *pur*^{R} gene at low level were not able to survive in the presence of puromycin (10 μg/ml). This phenomenon may be explained by the reason that the expression level of *pur*^{R} gene depends on the integration site of the retrovirus. All cell lines infected with the retrovirus pCXpur/$(CA)_{17}$/in-frame became fully confluent up to 2 weeks after the selection with puromycin (data not shown), and no colonies were obtained from any of the mock-infected cell lines (Fig. 2). These results revealed that the number of pur^R colonies obtained indicated a good correlation with the RER phenotypes. Interestingly, however, nonmalignant PH5CH8 cells showed the RER+ phenotype, because the number of pur^R colonies obtained in PH5CH8 cells was similar to that obtained in LS174T and HCT 116 cells showing the RER+ phenotype. In addition, the modification of the culture period (from 5 days to 14 or 21 days) before addition of puromycin in the MSI assay using LS174T cells revealed that the number of pur^R colonies increased in a time-dependent manner at both of two different concentrations (5 and 10 μg/ml) of puromycin (data not shown).

Sequence Analysis of the Integrated (CA) Repeat Unit in the pur^R Colonies. To further evaluate the reliability of our method, 7–10 independent pur^R colonies derived from LS174T, HCT116, and PH5CH8 cells were isolated and expanded. Using the pCXpur/$(CA)_{17}$/out-of-frame vector DNA, we initially confirmed that KOD-plus DNA polymerase was superior to nonproofreading *Taq*DNA polymerases, as described previously (33), because 3 of 10 clones obtained by *Taq*DNA polymerases showed deletions of 1–3 nucleotides, whereas all 10 clones obtained by KOD-plus DNA polymerase showed the exact $(CA)_{17}$ sequence. Therefore, using the genomic DNA from each colony, a fragment of 186 bp containing the CA repeat unit was amplified by proofreading KOD-plus DNA polymerase and was cloned into pCRII-TOPO for sequencing analysis. In most cases, four-independent clones were obtained from each pur^R colony and sequenced. Table 2 provides a summary of all of the sequenced clones. As can be seen, at least one clone, which became in-frame by the deletion of 2 bp (CA) from $(CA)_{17}$, was obtained from all pur^R colonies examined. In addition to $(CA)_{16}$, $(CA)_{13}$ resulting in in-frame was obtained from one colony in LS174T cells, and $(CA)_{19}$, $(CA)_{10}$, and $(CA)_7$ resulting in in-frame were obtained from four colonies in HCT116 cells. One interesting additional sequence, $(CA)_{17}A$, which resulted in in-frame was also obtained from one colony in HCT116 cells. Although all of the clones obtained from HCT116-derived colonies showed the expected pattern of frameshift mutation resulting in in-frame, a single clone possessing the original $(CA)_{17}$ without mutation was also obtained from 4 LS174T-derived colonies. Because each of the remaining three clones from these four colonies possessed $(CA)_{16}$ resulting in in-frame, it is suggested that more than two copies including the retrovirus possessing $(CA)_{17}$ sequence were infected and integrated in a single target cell. Com-

Table 2 *Sequence analysis of (CA) repeat region obtained from the pur[R] colonies*

The numbers in the table indicate the actual number of plasmid clones obtained and sequenced.

PCR product		Colony no. 1	2	3	4	5	6	7	8	9	10
LS174T (resistant to puromycin 10 μg/ml)											
$(CA)_{17}$	out-of-frame	1	1			1					1
$(CA)_{16}$	in-frame	3	3	4	4	3	4	3	4	4	3
$(CA)_{13}$	in-frame							1			
HCT116 (resistant to puromycin 10 μg/ml)											
$(CA)_{19}$	in-frame				3						
$(CA)_{17}A$	in-frame					3					
$(CA)_{16}$	in-frame	3	4	3	1	1	4	2			
$(CA)_{10}$	in-frame							2			
$(CA)_{7}$	in-frame	1		1							
PH5CH8 (resistant to puromycin 10 μg/ml)											
$(CA)_{17}$	out-of-frame							1	1	1	
$(CA)_{16}$	in-frame	4	4	3	2	2	4	3	2	1	2
$(CA)_{15}$	out-of-frame								1		
$(CA)_{14}$	out-of-frame									2	
$(CA)_{13}$	in-frame			1	2						
$(CA)_{9}$+CC	in-frame										2
$(CA)_{7}$	in-frame					2					

pared with the results from LS174T and HCT116 cells, PH5CH8-derived colonies showed a variety of mutation patterns. Although the $(CA)_{16}$ sequence was obtained from all colonies, $(CA)_{13}$ and $(CA)_{7}$ resulting in in-frame were obtained from two colonies and one colony, respectively, and $(CA)_{9}$CC resulting in in-frame was also obtained from one additional colony. In addition, $(CA)_{15}$ and $(CA)_{14}$ resulting in out-of-frame were obtained from a single colony, respectively, and the original $(CA)_{17}$ without mutation was also obtained from the three colonies. These results suggest that at least three copies of retrovirus were initially infected and integrated in a single target cell. In summary, sequence data on the (CA) repeat region indicated that the pur[R] colonies possessed the frameshift mutation (2-bp deletion) resulting in in-frame in the open reading frame of pur[R] gene. Taken together with these results, we concluded that our method can be used as an MSI assay at the cell-culture level.

HCV Core Protein Promoted MSI in PH5CH8 Cells. Because PH5CH8 cells did not show any tumorigenic potential when inoculated s.c. into thymic nude mice (23), we were surprised by the result that PH5CH8 cells showed the RER+ phenotype, as did the human colon cancer cell lines. Although the mechanism responsible for this finding is unclear, we speculate that HCV proteins may have further promoted MSI in PH5CH8 cells. Therefore, to evaluate this possibility, we initially prepared PH5CH8 cells stably expressing HCV protein [core(1b-P), E1(1b-P), E2(1b-P), or NS5A(1b-P)] as the recipient cells for the pCXpur/$(CA)_{17}$/out-of-frame retrovirus infection, by the pCXbsr/core(1b-P), pCXbsr/E1(1b-P), pCXbsr/E2(1b-P), or pCXbsr/NS5A(1b-P) retrovirus infection and following selection with blasticidin. As control recipient cells, we prepared PH5CH8 cells infected with retrovirus pCXbsr and selected with blasticidin. After retrovirus infection and following selection with blasticidin for 7 days, we monitored the growth curve of these blasticidin-resistant PH5CH8 cells, and we observed that the growth rates of these cells were almost the same (data not shown). We also confirmed by Western blot analysis the stable expression of core(1b-P), NS5A(1b-P), E1(1b-P), and E2(1b-P) proteins in PH5CH8 cells at day 10 and day 19 post-infection with retrovirus pCXbsr encoding HCV proteins (data not shown). Using these PH5CH8 cells, we performed an MSI assay, and found that the number of pur[R] colonies obtained from the cells expressing the core(1b-P) protein was approximately 1.5-fold (selection with 5 μg/ml of puromycin) and approximately 2.5-fold (selection with 10 μg/ml of puromycin) higher than that from the control cells, as shown in Fig. 3. As compared with the core(1b-P) protein, the E1(1b-P), E2(1b-P), and NS5A(1b-P) proteins did not increase the number of pur[R] colonies, although NS5A(1b-P) protein slightly decreased the number of pur[R] colonies. Because the increase of pur[R] colonies in PH5CH8 cells expressing the core(1b-P) protein was reproducibly observed, it was suggested that core(1b-P) protein was able to further promote the MSI in PH5CH8 cells.

Promotion of MSI by the Core Protein Depends on HCV Genotype or Strain. Because the core protein is known to show some aa sequence heterogeneity among HCV genotypes (7, 8), we examined whether or not HCV core proteins other than the core(1b-P) protein are able to promote the MSI, using pCXbsr/core(1a), pCXbsr/core(2a), pCXbsr/core(2b), and pCXbsr/core(3a) retrovirus vectors

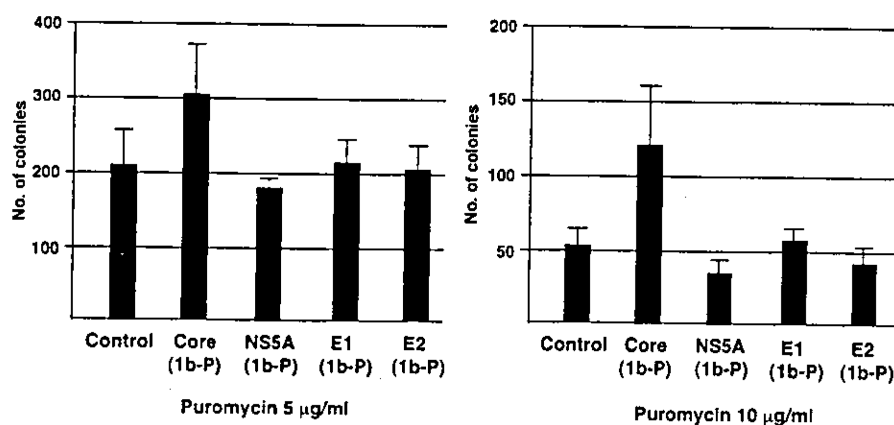

Fig. 3. Hepatitis C virus core protein promoted microsatellite instability in PH5CH8 cells. Microsatellite instability assay using pCXbsr/$(CA)_{17}$/out-of-frame was carried out in PH5CH8 cells stably expressing core(1b-P), NS5A(1b-P), E1(1b-P), or E2(1b-P) protein. The culture period from retrovirus infection to addition of puromycin was 9 days. The puromycin-resistant colonies were counted by the method described in Fig. 2. *Control*, PH5CH8 cells infected with retrovirus pCXbsr.

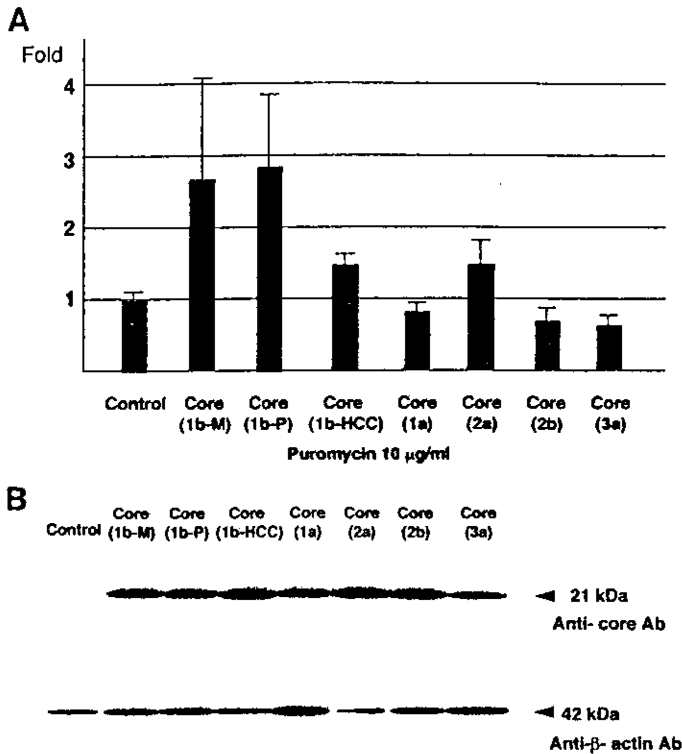

Fig. 4. *A*, promotion of microsatellite instability by the core protein depends on hepatitis C virus genotype. Microsatellite instability assay using pCXbsr/$(CA)_{17}$/out-of-frame was carried out in PH5CH8 cells stably expressing the core protein derived from various hepatitis C virus genotypes. The culture period from retrovirus infection to addition of puromycin was 9 days. The puromycin-resistant colonies were counted by the method described in Fig. 2. *Control*, PH5CH8 cells infected with retrovirus pCXbsr. *B*, stable expression of the core protein in PH5CH8 cells. PH5CH8 cells were infected with retrovirus pCXbsr encoding the core protein belonging to various genotypes, and at 19 days postinfection, the lysate of cells was used for the detection of core protein and β-actin by Western blot analysis. *Control*, PH5CH8 cells infected with retrovirus pCXbsr.

encoding the core(1a), core(2a), core(2b), and core(3a) protein, respectively. In addition, pCXbsr/core(1b-HCC) was also used as a retrovirus vector encoding the core(1b-HCC) protein, which was derived from a cancerous HCC lesion. The pCXbsr/core(1b-M) retrovirus vector (32) encoding core(1b-M) protein, which possessed the consensus sequence of genotype 1b, was also used for the MSI assay. The core(1b-P), core(1b-HCC), core (1a), core(2a), core(2b), and core(3a) proteins differed by 1, 6, 3, 14, 22, and 17 aa from the core(1b-M) protein, respectively (30). Using these retrovirus vectors, including pCXbsr as a control vector, we initially prepared PH5CH8 cells stably expressing the core(1b-M), core(1b-P), core(1b-HCC), core(1a), core(2a), core(2b), and core(3a) proteins, respectively. We performed an MSI assay using these core protein-expressing cells. As shown in Fig. 4*A*, the results revealed that the number of pur^R colonies obtained from the cells expressing the core(1b-M), core(1b-P), core(1b-HCC), or core(2a) protein was 1.5- to 2.8-fold higher than that obtained in the control, whereas the number of pur^R colonies obtained from the cells expressing the core(1a), core(2b), or core(3a) protein was similar to that obtained in the control. Western blot analysis confirmed that these core proteins were stably and equally expressed in PH5CH8 cells at day 19 postinfection with the retrovirus pCXbsr expressing the core protein (Fig. 4*B*). These results suggest that the effectiveness of the core protein in promoting MSI, that is, in down-regulating MMR, is dependent on the HCV genotype or strain.

Expression Level of MMR-Related Genes in PH5CH8 Cells Expressing the Core Protein. To investigate the possibility that the core protein represses the expression of genes functioning in MMR, we examined the effect of the core protein on the expression level of MMR-related genes, including *hMLH1* and *hMSH2*, the frequent genetic mutations of which have been observed in the hereditary nonpolyposis colorectal cancer and a variety of sporadic cancers (25). As shown in Fig. 5, we were not able to find any significant differences in the expression level of *hMLH1*, *hMSH2*, *hMSH6*, *hPMS2*, *hMSH3*, and *hPMS1* genes between PH5CH8 cells expressing the core(1b-P) or NS5A(1b-P) protein, and PH5CH8 cells infected with retrovirus pCXbsr. This result suggests that the down-regulation of MMR by the core protein occurs by an as yet unknown mechanism other than the repression of MMR-related genes.

DISCUSSION

In this study, we first demonstrated that HCV core proteins were able to further repress the down-regulation of MMR activity in cultured human non-neoplastic hepatocytes, by a newly developed MSI assay system using a microsatellite sequence consisting of $(CA)_{17}$.

Regarding the MSI assay system developed in this study, we used retrovirus infection as a method for transduction of a microsatellite (CA) repeat sequence to the cells. However, it remains possible that the RER of pCXpur/$(CA)_{17}$/out-of-frame occurs in the packaging of Bosc23 cells and results in the production of the retrovirus possessing the (CA) repeat sequence altered in-frame. Although we cannot absolutely exclude this possibility, it is unlikely that such an event occurs in Bosc23 cells, because we observed a good correlation between the RER+ and RER− phenotypes of the examined cell lines with respect to the number of pur^R colonies obtained. In addition, we observed that the number of pur^R colonies increased in a culture-time-dependent manner. Therefore, the MSI assay developed in this study will be a useful method at the cell culture level.

The fact that non-neoplastic PH5CH8 cells showed remarkable RER+ phenotype was an unexpected result. Although the PH5CH8 cell line was cloned from PH5CH cells as an HCV-susceptible clone (24), we observed that not only the PH5CH8 cells but also the parental PH5CH cells showed the RER+ phenotype (data not shown). PH5CH cells were established from the non-neoplastic liver as a SV40 large T antigen-immortalized cell line and express hepatocyte characteristics (23). Therefore, the activity of p53 and pRb, two tumor suppressor

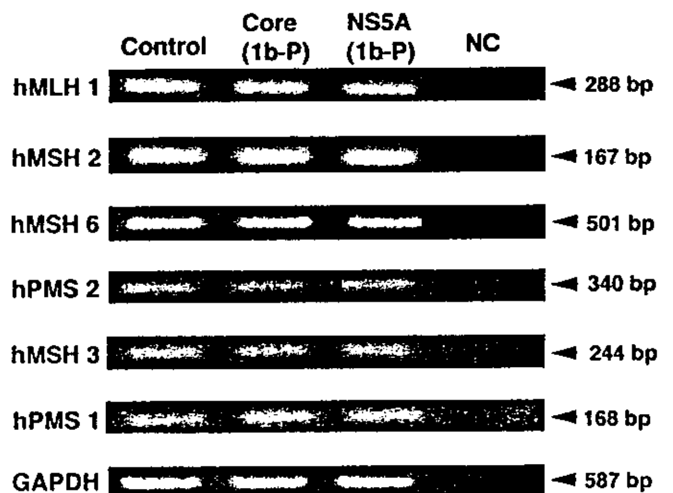

Fig. 5. Effect of the core protein on the expression level of mismatch-repair-related genes in PH5CH8 cells. PH5CH8 cells were infected with retrovirus pCXbsr/core(1b-P) or pCXbsr/NS5A(1b-P), and the cells were used for reverse transcription-PCR analysis of mismatch-repair-related genes. As a control, PH5CH8 cells infected with retrovirus pCXbsr were also used for the analysis. *Control*, PH5CH8 cells infected with retrovirus pCXbsr; *Core(1b-P)*, PH5CH8 cells stably expressing core(1b-P) protein; *NS5A(1b-P)*, PH5CH8 cells stably expressing NS5A(1b-P) protein; *NC*, no RNA.

proteins, in PH5CH cells should be partially repressed by the physical binding of the SV40 large T antigen (42). By complex with p53, the SV40 large T antigen blocks the apoptotic function of p53 and allows proliferation (43), and by binding pRb, the SV40 large T antigen induces the release of the E2F transcription factor, which activates the promoters of genes required for the S-phase transition (44). The functional repression of p53 or pRb may be involved in the repression of MMR activity, although no data suggesting such a relation has yet been reported. As an alternative possibility, the SV40 large T antigen may bind and repress some proteins that function in the MMR system, because it was reported recently that the SV40 large T antigen bound MRE11-NBS1-RAD50 complex, which was involved in homologous recombination, and, as a consequence, perturbed the double-strand break repair (45). Preliminary experiments using NKNT-3 cells (SV40-large T antigen immortalized non-neoplastic human hepatocytes) derived from primary normal human hepatocytes (46) and Saos-2 cells (derived from p53-deficient human osteogenic sarcoma; Ref. 47) revealed that NKNT-3 cells, like PH5CH8 cells, also showed the RER+ phenotype, but Saos-2 cells showed the RER− phenotype in our MSI assay. These results suggest that the activity of MMR is influenced by the SV40 large T antigen but not by p53; however, in addition to PH5CH8 cells, the analysis of cell lines derived from HCV-related HCC cases will be necessary to clarify the reason that PH5CH8 cells show the RER+ phenotype.

Although we found that the core protein promoted MSI in PH5CH8 cells, it is difficult to prove our findings in an HCV replication system because of the lack of a sufficiently reproducible and efficient HCV proliferation system (14). Alternatively, several HCV subgenomic replicons containing NS3-NS5B regions have been established using a human hepatoma cell line Huh-7 (48–50). These subgenomic replicon systems may be useful for the functional evaluation of the core protein. However, our preliminary results revealed that these subgenomic replicon cells showed the RER− phenotype and that no pur^R colonies were obtained from these subgenomic replicon cells stably expressing the core(1b-P) protein. These results suggest that these replicon cells have an intact MMR system that is not influenced by the core protein. To reproduce the promotion of MSI by the core protein in cells in which the HCV genome is replicated, we are currently establishing an HCV subgenomic replicon using PH5CH8 cells.

Our observation that the core proteins belonging to genotypes 1b and 2a, but not those belonging to genotypes 1a, 2b, and 3a, may promote MSI in human hepatocytes is interesting. Although it is not yet defined which region of the core protein is responsible for the promotion of MSI, comparison of aa sequences among these core proteins revealed that aa position 91 was a Cys residue in the core(1a), core(2b), and core(3a) proteins, whereas this position was a Leu residue in the core(1b-M), core(1b-P), and core(2a) proteins and a Met residue in the core(1b-HCC) protein. Only this aa position showed good correlation with the effect of the core proteins in the MSI assay. To clarify whether or not aa position 91 is important to promote MSI, further analysis using chimeric core proteins will be necessary. On the other hand, several studies have described an increased risk of HCC in patients infected with HCV genotype 1b (51, 52), although the contradictory result has also been reported (53). The fact that the core protein belonging to genotype 1b was most effective at promoting the MSI in hepatocyte cells may be related to the increased risk of HCC in patients infected with HCV genotype 1b. To examine this possibility, further MSI analysis using various core proteins derived from many HCV strains belonging to different genotypes will be needed. In addition, our preliminary experiment showed that the number of pur^R colonies in PH5CH8 cells increased approximately 1.5-fold in the presence of $FeSO_4$ (100 μM), suggesting that the Fe(II) compound promotes microsatellite mutations. Although the mechanism of this phenomenon has not yet been clarified, it has been reported that Nickel(II) also induces microsatellite mutations in human lung cancer cell lines (39). Future studies on the relationship between the core protein and these cation compounds will also be important to clarify their roles during the process of hepatocarcinogenesis.

Because we could find no effect of the core protein on the expression level of MMR-related gene, the mechanism by which the core protein promotes MSI in human hepatocytes is still unclear. However, it remains possible that the core protein directly interacts with these components involved in MMR and then suppresses their functions. An alternative possibility—that the core protein affects the functions of the other proteins involved in MMR, including DNA polymerase δ/ϵ, exonuclease 1, and endonuclease FEN1—remains to be examined. Future analyses to evaluate these possibilities may clarify the mechanism of the down-regulation of the MMR system by the core protein.

ACKNOWLEDGMENTS

We thank Y. Inoue for helpful assistance.

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Cyclosporin A Suppresses Replication of Hepatitis C Virus Genome in Cultured Hepatocytes

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Persistent infection of hepatitis C virus (HCV) is a major cause of liver diseases such as chronic hepatitis, liver cirrhosis, and hepatocellular carcinoma. Searching for a substance with anti-HCV potential, we examined the effects of a variety of compounds on HCV replication using a HCV subgenomic replicon cell culture system. Consequently, the immunosuppressant cyclosporin A (CsA) was found to have a suppressive effect on the HCV replicon RNA level and HCV protein expression in these cells. CsA also inhibited multiplication of the HCV genome in a cultured human hepatocyte cell line infected with HCV using HCV-positive plasma. This anti-HCV activity of CsA appeared to be independent of its immunosuppressive function. In conclusion, our results suggest that CsA may represent a new approach for the development of anti-HCV therapy. (HEPATOLOGY 2003;38:1282-1288.)

Persistent infection with the hepatitis C virus (HCV), identified as the major causative agent of non-A, non-B hepatitis,[1,2] has been closely related to liver diseases such as chronic hepatitis, liver cirrhosis, and hepatocellular carcinoma.[3] The development of these liver diseases from HCV carriers, an estimated 170 million people throughout the world, is a major public health problem. Effective anti-HCV therapy has been restricted mainly to therapy with interferon (IFN) and a combination of IFN and ribavirin. However, because the virus is not eliminated from approximately one half of HCV-infected patients treated with these agents,[4] alternative approaches to the treatment of HCV infection are needed.

Recently, an HCV subgenomic replicon cell culture system has been established in which an HCV subgenomic replicon autonomously replicated in Huh-7 cells (HCV replicon cells).[5] This replicon is composed of the HCV 5′-untranslated region containing an internal ribosomal entry site, the neomycin phosphotransferase gene, the encephalomyocarditis virus internal ribosomal entry site, HCV nonstructural proteins (NS) 3 through NS5B; and the HCV 3′-untranslated region (Fig. 1A). This system provides a unique tool for studying the mechanisms of HCV replication and screening as well as evaluating anti-HCV compounds. Taking advantage of this feature, we examined the effects of various types of compounds on the replication of HCV using HCV replicon cells established in our laboratory[6] (Miyanari et al., manuscript accepted for publication). Consequently, we found that a well-known immunosuppressant, cyclosporin A (CsA),[7] had a strong suppressive effect on HCV replication in these cells. Moreover, we found suppressive activity of CsA for multiplication of the HCV genome in cultured human hepatocytes infected with HCV. The mechanism of the anti-HCV activity of CsA was also studied.

Abbreviations: HCV, hepatitis C virus; IFN, interferon; NS, nonstructural protein; CsA, cyclosporin A; RT-PCR, reverse-transcription polymerase chain reaction; CyP, cyclophilin; CN, calcineurin.

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Received May 6, 2003; accepted August 10, 2003.

Supported by grants-in-aid for cancer research and for the second-term comprehensive 10-year strategy for cancer control from the Ministry of Health, Labor, and Welfare; grants-in-aid for scientific research from the Ministry of Education, Culture, Sports, Science and Technology; grants-in-aid for Research for the Future from the Japanese Society for the Promotion of Science; and the Program for Promotion of Fundamental Studies in Health Science of the Organization for Pharmaceutical Safety and Research of Japan.

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0270-9139/03/3805-0027$30.00/0

doi:10.1053/jhep.2003.50449

Materials and Methods

Cell Culture. Huh-7 and MH-14 cells, HCV replicon cells, were cultured in Dulbecco's modified Eagle medium with 10% fetal bovine serum. PH5CH8 cells were cultured in a 1:1 mixture of Dulbecco's modified Eagle medium and F12 medium supplemented with 100 ng/mL epidermal growth factor, 10 μg/mL insulin, 0.36 μg/mL hydrocortisone, 5 μg/mL transferrin, 5 μg/mL linoleic acid, 20 ng/mL selenium, 4 μg/mL glucagon, 10 ng/mL prolactin, 10 μg/mL gentamicin, 200 μg/mL kanamycin, and 2% fetal bovine serum.