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Biochemical and Biophysical Research Communications 293 (2002) 993–999

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Subgenomic replicon derived from a cell line infected with the hepatitis C virus

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Received 8 April 2002

Abstract

Recently, cell culture systems have been established, where a hepatitis C virus (HCV) subgenomic replicon was efficiently replicated and maintained for a long period. To see whether a HCV sequence derived from HCV-infected cultured cell sequence can be used for the construction of a functional replicon, a HCV subgenomic RNA carrying a neomycin-resistant gene was constructed using the HCV genome RNA obtained from cultured cells infected with HCV. After transfection, G418-resistant Huh-7 cells were selected and subcloned. Finally, the production of HCV proteins and de novo synthesis of subgenomic RNA were confirmed in the selected cell clone, indicating that this subgenomic RNA replicated in cultured cells and functioned as a replicon. These results suggest that the HCV genome obtained from an in vitro HCV infection system with cultured cells can be used to develop a subgenomic replicon system with diverse HCV sequences.

Keywords: Hepatitis C virus; Huh-7; Replication; Replicon

Hepatitis C virus (HCV) infections frequently cause chronic hepatitis, followed by the development of the cirrhosis of liver and hepatocellular carcinoma [1–5]. A highly effective anti-HCV drug, however, is yet to be developed, possibly due to the lack of detailed information about the life cycle of this virus. A recent report indicated that a synthetic HCV subgenomic RNA including the neomycin phosphotransferase gene (*neo*^{r}), instead of the structural protein-encoding region, replicated efficiently in the cell line Huh-7 [6]. The neomycin-resistant cells, where this subgenomic RNA persistently replicated, have been used as the starting point for a model system to investigate the HCV genomic replication as well as HCV–cell interaction [7–15].

All the HCV subgenomic replicons, but one exception, used in the experiments reported thus far, were derivatives of a single genomic sequence of HCV. Genomic sequences, however, are well known to be highly diverse among the HCV isolates [16,17]. Most of these sequences are derived from tissue or plasma, rather than from HCV particles which have been proven to be infectious, suggesting that cloned sequences show some defective characteristics. Therefore to generalize the results obtained from this experimental replicon system and to clarify the clonal diversity, an analysis of subgenomic RNA with sequences different from the above-described clones was necessary. Until now, replicons have been constructed using the HCV genome isolated from human liver tissues chronically infected with HCV [6] or through the use of a HCV RNA clone in which case the infectivity was confirmed by direct intrahepatic inoculation using chimpanzees [18]. It may be difficult to apply these strategies to the preparation of new HCV replicons bearing different HCV sequences due to the high complexity of the HCV genome sequence in liver tissues and the rarity of experimental systems using chimpanzees. We previously reported that some cultured cell lines are susceptible to HCV infection and that

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0006-291X/02/$ - see front matter
PII: S0006-291X(02)00342-X

the limited sequences of HCV genome among those in the inocula become predominant in those cells after the passage [19–23], leading us to hypothesize that the HCV genome persisting in long cultured cells may be a good candidate for the replicon.

In this paper, we report a subgenomic replicon, which has a HCV sequence obtained from the cultured human T cell line MT-2C infected with HCV in vitro [19–21], with the acquisition of some amino acid mutations during replication in Huh-7 cells.

Materials and methods

Cell cultures. Huh-7 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS) (complete DMEM), as described [13].

Plasmid construction. The cDNA for the full-length HCV genome is prepared as follows. The total RNA was extracted from MT-2C cells, which were infected with HCV (genotype 1b) in vitro at day 8 post-inoculation [21]. The cDNA fragments for the HCV genome were obtained by the reverse transcription-polymerase chain reaction (RT-PCR) using total RNA as a template. The primers used in this RT-PCR are listed in Table 1. To obtain cDNA fragments covering the entire HCV genome, these fragments were assembled by ligation using appropriate restriction enzyme sites in the overlapping regions and subcloned into pBR322MC, which was derived from pBR322 and contained the multicloning site of pUC19. The HCV sequence in that plasmid was modified by PCR-based site-directed mutagenesis to exclude indeterminate amino acids in the open reading frame of the HCV genome [24], resulting in pM1LE (GenBank Accession No. AB080299). The region from nucleotides 378 to 3419 of the HCV genomic sequence in pM1LE was replaced with a fragment including the sequences for the *neo*^{r} gene from pEGFP-N1 (CLONTECH) and encephalomyocarditis virus (EMCV) internal ribosomal entry site (IRES) from pCXbsr (GenBank Accession No. AB041927) [25] by PCR as described above, followed by insertion of the fragment corresponding to hepatitis D virus (HDV) ribozyme [26] at the 3′ terminal end of the HCV subgenomic region to obtain pNNRZ2. After insertion of the sequence for the hammerhead ribozyme [27], which was obtained from pCALN/HCV RBZ02 (provided by Dr. M. Kohara, Tokyo Metropolitan Institute of Medical Science) at the site between the T7 promoter and the 5′ terminal end of the HCV 5′ untranslated region (UTR) of pNNRZ2, pNNRZ2RU was finally constructed. These ribozyme sequences were used for the formation of accurate terminal structures of the synthesized RNA. We replaced the *MluI–BglII* fragment of pNNRZ2 with the *MuI–BglII* fragment of plasmid containing an inactive NS5B sequence [28], so that pNNRZ2GHD containing the defective genome by mutation in the active site of NS5B (Asp^{2737} to His substitution) was obtained.

RNA synthesis. HCV subgenomic RNAs were prepared by in vitro transcription using MEGAscript T7 kit (Ambion) according to manufacturer's instruction. After DNase treatment, the synthesized RNA was purified using conventional molecular biological techniques.

RNA transfection and selection of G418-resistant cells. RNA transfection was performed by electroporation, as described by Lohmann et al. [13]. Selection of cells was performed in complete DMEM with 300 μg/ml of G418 (GENETICINE, Invitrogen).

PCR. Genomic DNA was purified from G418-resistant clones as described [29]. DNA fragment of the *neo*^{r} gene integrated into the genomic DNA was amplified by PCR using AmpliTaq DNA polymerase (Applied Biosystems) and oligonucleotides 5′-CGCGCCAT GATTGACAAGA-3′ and 5′-CGTCAAGAAGGCGATAGAAG-3′ as primers.

Northern blot analysis. The total RNA was isolated from G418-resistant cell clones using Sepasol RNA I reagent (Nacalai Tesque, Japan) according to manufacturer's protocol. Northern blot analysis was performed, as described previously [30]. The probe used in this experiment was complementary to the NS5B sequence and synthesized in the presence of [α-^{32}P]dCTP by asymmetrical PCR using *Eco*T22I-digested pNNRZ2RU and the oligonucleotide 5′-CTATTGATCT CACCTGGAGAG-3′, as a template and primer, respectively.

Sequencing analysis. The cDNA fragments for the HCV subgenomic replicon RNA were amplified from the total RNA of G418-resistant cell clones by RT-PCR. The amplified fragments were sequenced directly or after subcloning into pGEM-T-Easy vector (Promega).

Detection of RNA synthesized de novo from the cells bearing HCV subgenomic replicon. Following the preculture in phosphate-free DMEM (Invitrogen) containing 2% dialyzed FBS, 200 μg/ml G418 and 5 μg/ml actinomycin D (Sigma) for 2 h, 5×10^5 cells of #50-1, which is a G418-resistant clone, were cultured overnight in the same medium with the addition of 200 μCi of ^{32}P-orthophosphate as

Table 1
Primers used in the RT-PCR for construction of the full length HCV genome cDNA

Region	Primer	Sequence (5′–3′)	Reaction
5′UTR-structure	491R	TACTGATTCGGCCGAAGTCGACTGTCTGAGTGAC	RT
	420	GGCGACACTCCACCATAGATCACTC	First PCR
	290LR	CCCTCAACCTGGTTCTTGTCCCG	First PCR
	*Eco*T7	CTTCGAGAATTCTAATACGACTCACTATAGC	Second PCR
	−420	CAGCCCCCGATTGGGGGCGACACTCCACCATAGATCACTC	
	424R	GTCTGTTGGGAGTAAGCCGTGAT	Second PCR
NS	386R	AATGGCCTATTGGCCTGGAG	RT
	415	CTTGTGGATGATGCTGCTGATAGC	First PCR
	391R	CCTATTGGCCTGGAGTGTTTAG	First PCR
	416	TGCTGATAGCCCAGGCTGAGGCC	Second PCR
	419R	GAGTGTTTAGCTCCCCGTTCACCGGTTGG	Second PCR
3′UTR	R7	TCTGCAGAGAGGCCAGTATC	RT, first PCR
	9348[a]	GGCATCTACCTGCTCCCCAA	First PCR
	428	CCCAACCGGTGAACGGGGAG	Second PCR
	R3[a]	CGGCTCACGGACCTTTCACA	Second PCR

[a] Ref. [44].

described above [6,31]. Total RNA was then purified from these cells with an RNeasy kit (Qiagen) according to manufacturer's instruction. The HCV subgenomic RNA was isolated from the total RNA using an mRNA isolation kit (Roche Diagnostics), in which the HCV genome-specific biotin-labeled probe (5′-TAGGATTCGTGCTCATGGTGCA CGGTCTACGAGAC-3′) was used instead of the biotin-labeled oligo(dT)$_{20}$ probe. The isolated RNA was separated by formaldehyde containing agarose gel electrophoresis. The electrophoretic pattern of the radiolabeled RNA was analyzed by BAS-5000 (Fuji Photo Film).

Immunoblotting analysis. Preparation of the cell lysate, sodium dodecyl sulfate–polyacrylamide gel electrophoresis, and immunoblotting were performed, as described previously [32]. The HCV positive plasma and the antibodies against HCV NS3 (α-p70) [33], NS4A (α-NS4), NS5A (α-NS5A) [32], NS4B (NS4B-52), NS5B (NS5B-14), and actin (ANTI-ACTIN, Sigma) were used in this experiment. NS4B-52 and NS5B-14 were a gift from Dr. M. Kohara (Tokyo Metropolitan Institute of Medical Science).

Results

Establishment of G418-resistant Huh-7 cells transfected with HCV subgenomic RNA

We previously demonstrated that several cultured cell lines were susceptible to HCV infection and supported the replication of the viral genome [19–23]. The HCV genome was isolated from cultured cells to test its ability to establish a HCV subgenomic replicon system. We constructed a HCV subgenomic RNA expression plasmid using cDNA from HCV RNA replicated in MT-2C cells [21]. The structure of the HCV subgenomic RNA conformed to that originally reported by Lohmann et al. [6], so as to contain the 5′UTR, the first 36 nucleotides of the core region, a non-structural (NS) protein region from NS3 to NS5B and the 3′UTR of the HCV genome and the *neo*^{r} gene and EMCV IRES to be inserted into the region downstream of the short stretch of the core region as shown in Fig. 1. The NS protein region was constructed from the cDNA fragment obtained from HCV RNA multiplying in the MT-2C cells. After transcription in vitro using the constructs pNNRZ2RU and pNNRZ2GHD as templates (see Materials and methods), structures of the 5′ and 3′ ends of the synthesized RNAs were verified by a rapid amplification of the cDNA ends and sequencing (data not shown).

The HCV subgenomic RNA was transfected into Huh-7 cells. Following 21–30 days of culturing in the presence of G418, six colonies were obtained from cells transfected with the RNA NN2 from pNNRRZ2RU and subcloned. Finally, two G418-resistant clones, #24-1 and #50-1, were established. In contrast, no colonies were obtained from cells transfected with the RNA from pNNRZ2GHD.

The presence of a *neo*^{r} gene fragment in the genomic DNA from clones #24-1 and #50-1 was examined by PCR. A DNA fragment of the expected size was only amplified from the genomic DNA of #24-1 cells [5] (Fig. 2A, lane 3), but not from #50-1 cells or naive Huh-7 cells (Fig. 2, lanes 1 and 2), indicating that a DNA fragment containing the *neo*^{r} gene was integrated into the chromosomal DNA of #24-1 cells. This suggested that the G418 resistance of #50-1 cells was due to RNA-based expression of the *neo*^{r} gene product.

Detection of the HCV subgenomic RNA maintained in #50-1 cell clone

The presence of the HCV subgenomic RNA was examined by Northern blotting using an antisense strand sequence of the NS5B region in the HCV genome as a probe. As shown in Fig. 2B, an 8.1 kb band, which was equivalent in size to the synthetic HCV subgenomic RNA used for RNA transfection (Fig. 2B, lane 1) was detected in the total RNA sample extracted from #50-1 cells (Fig. 2B, lane 3), but not in RNA from naive Huh-7 cells or #24-1 cells (Fig. 2B, lanes 2 and 4). These results indicated that HCV subgenomic RNA was maintained in #50-1 cells even after a long period of G418 selection. In comparison with the signal of the probe hybridized with 0.5 μg of in vitro transcribed RNA used as a size marker, the amount of subgenomic RNA in 4.3×10^5 cells of #50-1 was estimated to be approximately 13.6 ng. Therefore in the calculation the average number of copies of the subgenomic RNA in a #50-1 cell was approximately 7.9×10^3.

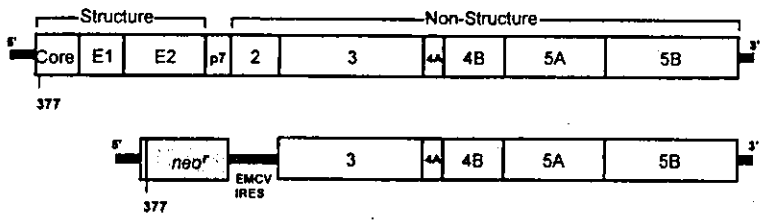

Fig. 1. Schematic representations of the HCV genome and subgenomic replicon. Top, physical map of the HCV genome. Bottom, structure of the subgenomic replicon. Open boxes, the shaded box, thick lines, and the striped thick line represent HCV protein encoding regions, the *neo*^{r} gene, UTRs, and EMCV IRES, respectively. The region from nucleotide position 1 to 377 in the HCV genome was used as the 5′-terminal structure including the HCV IRES region in this replicon.

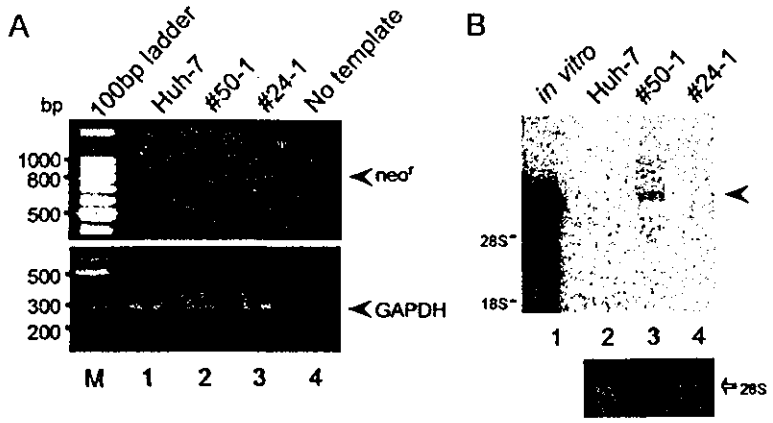

Fig. 2. Detection of the integrated *neo*^{r} gene in the genomic DNA of G418-resistant cells by PCR and the HCV subgenomic RNA in G418-resistant cells. (A) Amplified fragments from genomic DNA of naive Huh-7 (lane 1), #50-1 (lane 2), and #24-1 cells (lane 3) were analyzed by agarose gel electrophoresis. PCR was performed without the template DNA as a negative control (lane 4). The positions of PCR fragments derived from neomycin phosphotransferase (*neo*^{r}) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) genes are indicated by arrowheads. (B) Total RNA from Huh-7 (lane 2), #50-1 (lane 3), and #24-1 (lane 4) cells was analyzed by Northern blotting using a positive-strand HCV genome-specific probe (upper panel) and equalized for 28S rRNA stained with ethidium bromide (lower panel). HCV subgenomic RNA transcribed and radiolabeled with [α-^{32}P]UTP in vitro was used as a size marker (lane 1). The positions of HCV subgenomic RNA and ribosomal RNAs (28S and 18S) are indicated by an arrowhead and bars, respectively (upper panel). The 28S ribosomal RNA is indicated by an open arrow in the lower panel.

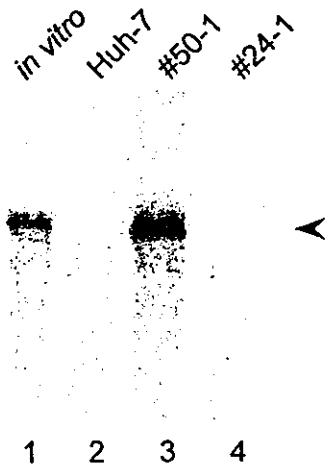

Fig. 3. Detection of de novo synthesis of the HCV subgenomic RNA in G418-resistant cells. After isolation of the subgenomic RNA-specific probe, metabolically radiolabeled RNAs from naive Huh-7, #50-1, and #24-1 cells were fractionated in lanes 2, 3, and 4, respectively, in a denaturing agarose gel. The HCV subgenomic RNA transcribed in vitro and radiolabeled with [α-^{32}P]UTP was used for the size marker (lane 1). The position of the HCV subgenomic RNA is indicated by an arrowhead.

De novo synthesis of the HCV subgenomic RNA in #50-1 cell clone

To see whether the HCV subgenomic RNA replicated in #50-1 cells or not, de novo synthesis of the HCV subgenomic RNA in the cells was investigated using a metabolic radiolabeling procedure as described (see Materials and methods). A radiolabeled RNA molecule, migrating at the same size as the in vitro transcribed and radiolabeled RNAs (Fig. 3, lane 1), was detected in the RNA sample from #50-1 cells (Fig. 3, lane 3), but not from naive Huh-7 or #24-1 cells (Fig. 3, lanes 2 and 4). These results suggested that the subgenomic RNA in #50-1 cells self-replicated and acted as a HCV subgenomic replicon.

Production of HCV proteins from the HCV subgenomic RNA in #50-1 cell clone

The production of HCV proteins in #50-1 cells was examined by immunoblotting using antibodies against HCV NS proteins and HCV-positive plasma. As shown in Fig. 4, all the HCV proteins (NS3, NS4A, NS4B, NS5A, and NS5B) encoded in the subgenomic RNA were only detected in the cell lysate of #50-1 cells (lanes 3, 7, 11, 15, and 19) and migrated in a manner similar to each protein transiently produced in the cells upon transfection of the relevant expression plasmid (lanes 1, 2, 6, 10, 14, and 18). No HCV proteins, however, were detected in clone #24-1 or in naive Huh-7 cells (Fig. 4, lanes 4, 5, 8, 9, 12, 13, 16, 17, 20, and 21).

Acquisition of mutations in the HCV subgenomic RNA of the replicon replicating in #50-1 cell clone

The nucleotide sequence of the HCV subgenomic replicon RNA in #50-1 cells, designated as NNU50-1, was compared to that of the parental subgenomic RNA, NN2. Two mutations were found to be introduced in NNU50-1. One mutation was an adenine to guanine transition at nucleotide position (nt) 5166 (the number corresponds to the nucleotide number of the HCV

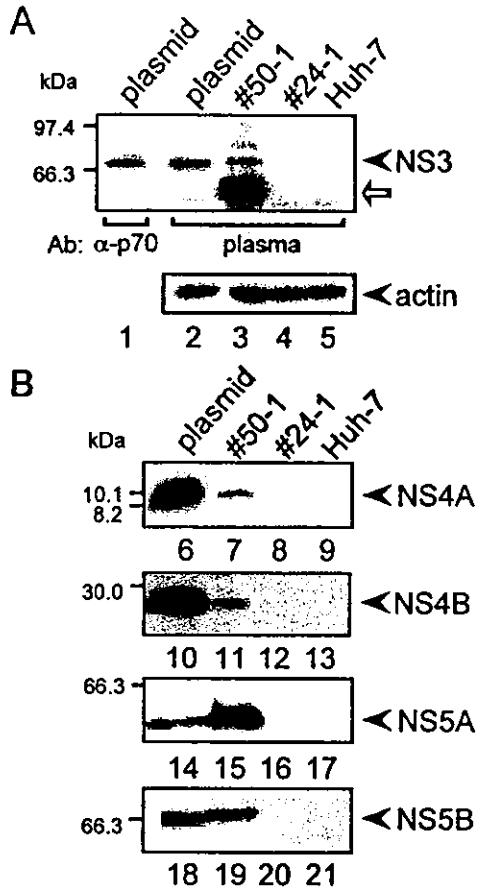

Fig. 4. Detection of HCV NS proteins in G418-resistant cells. Production of NS3 (panel A, upper panel), NS4A, NS4B, NS5A, and NS5B (panel B) in G418-resistant cells was analyzed by immunoblotting using the HCV-positive plasma, anti-NS4A, anti-NS4B, anti-NS5A, and anti-NS5B antibodies, respectively. The actin in the lower panel A was used as a control for the amount of protein loaded per lane. The band corresponding to each NS protein is indicated by an arrowhead. Cell lysates of Huh-7 cells transfected with the expression plasmid pCMV/N1027-1657 encoding NS3 (HCV-JT, genotype 1b) [41] were used for the positive control of NS3 production (lanes 1 and 2). Similarly, pCMV/NS1658-1711 (HCV-JT) [42], pCMV-4B (HCV-JT), pcDNA3/N1973-2419 (pCV-J4L6S, genotype 1b) [43], and pcDNA3-NS5B (pCV-J4L6S) [43] were used for production of NS4A (lane 6), NS4B (lane 10), NS5A (lane 14), and NS5B (lane 18), respectively. Results for #50-1 cells are shown in lanes 3, 7, 11, 15, and 19. Results for #24-1 cells are shown in lanes 4, 8, 12, 16, and 20. Exogenously expressed NS3 protein detected by anti-NS3 antibody (α-p70) was used as the size marker for NS3 (lane 1). The open arrow in panel A indicates NS5A detected in HCV-positive plasma.

genotype 1b genome), resulting in the substitution of glutamic acid for lysine at amino acid position aa 1609 in the NS3 protein. Another mutation was a guanine to uridine transition at nt 6027, resulting in the substitution of phenylalanine for valine at aa 1896 in the NS4B protein. The substitution at aa 1609 was also reported by Lohmann et al. [13], but the substitution at aa 1896 was newly identified in this paper.

Discussion

In this paper, we report the construction of a HCV subgenomic replicon containing a unique HCV sequence. We investigated the diversity of the nucleotide and amino acid sequences of the HCV regions in these three HCV subgenomic RNAs, I_{377}/NS3-3′ [6], HCV1bneo [9], and NN2. The nucleotide sequences originating from HCV genomes in these constructs were compared, without taking into account the 12 nucleotides in the NS5A region of HCV1bneo, which did not exist in the other two clones, and the poly(U) stretch in the 3′UTR of all the clones. The overall homology and the homology for each UTR and NS encoding region ranged from 89.6% to 100%. Similarly, the percentage of the homology of amino acid sequences in these clones, with the exception of the additional four amino acids in the NS5A region of HCV1bneo, was calculated. The overall homology and the homology for each NS region ranged from 89.5% to 98.1%. The homologies of nucleotide and amino acid sequences were in the same range as those found in isolates of the same genotype [16,17,34,35]. This implies that a subgenomic replication system can be constructed by using not only particular HCV nucleotide and amino acid sequences, but also sequences with a diversity found within the same genotype of HCV.

The first reported HCV subgenomic replicon was constructed with a HCV genome from the total liver RNA isolated from a chronically infected patient who had undergone liver transplantation [6]. The recently reported HCV sequence in the second subgenomic replicon originated from HCV RNA sequence infectious to chimpanzee [9,10,18]. Due to the diversity found among the genomic sequences of HCV isolates [16,17], the use of a sequence from the HCV genome, which actually replicates in cells or whose capacity to replicate has been verified, is crucial for the development of a successful replicon system. The two procedures described above, however, did not seem to be easily applicable to the preparation of a HCV replicon system for many HCV isolates and genotypes, due to the high complexity of HCV genome sequences in the total liver RNA from chronically infected patients and the rarity of experimental systems using chimpanzees. We previously reported that limited HCV populations in the inocula of HCV-infected plasma are able to replicate in MT-2C cells [21]. In this study, we showed that the sequence of the HCV genome obtained from the HCV-infected cultured cells has the potential to self-replicate. Taken together, we suggest an advantage for the use of an in vitro HCV infection and proliferation systems using cultured cells to select the sequence for the construction of the replicon from serum or plasma of HCV carriers in which many defective HCV genome sequences are present.

All HCV genomic sequences in the subgenomic replication systems reported to date, including this study, have been only from the genotype 1b. There are, however, six major genotypes of HCV in the world [36]. Although interferon (IFN) has been used for the treatment of hepatitis C as an anti-HCV drug, sensitivity to IFN treatment has been reported to vary among the genotypes of HCV [37] and even among different clones of the same genotype [38]. The translational activity of the IRES was also reported to differ among the genotypes of HCV [39]. Therefore the establishment of a replication system representing all the HCV genotypes and many of the HCV isolates is extremely important to develop an effective anti-HCV drug and the analysis of the general mechanism of viral replication. With several in vitro infection and proliferation systems for HCV using culture cells being available [40], it may be possible to construct subgenomic replicons using sequences from various HCV isolates from these in vitro systems.

Acknowledgments

The first two authors contributed equally to this work. We thank Dr. Naito for the discussion portion. This work was 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, through grants-in-aid for scientific research from the Ministry of Education, Culture, Sports, Science and Technology, grants-in-aid of research for the future from the Japanese Society for the Promotion of Science, and by the Program for Promotion of Fundamental Studies in Health Science of the Organization for Pharmaceutical Safety and Research (OPSR) of Japan.

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Acta Med. Okayama, 2002
Vol. 56, No. 2, pp. 107-110

Acta Medica
Okayama
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Short Communication

Quantitative Method of Intracellular Hepatitis C Virus RNA using LightCycler PCR

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Based on recent LightCycler techniques developed for the quantitation of serum HCV RNA, we have developed a quantitative method for the intracellular hepatitis C virus (HCV) RNA using LightCycler PCR. A simple real-time PCR assay, based on the SYBR Green I dye and LightCycler fluorimeter and with no probe requirement, is described. In the presence of 0.5 μg of cellular RNA, it was demonstrated that as few as 25 copies of HCV RNA could be specifically detected with a set of primers that amplify a 144-base pair sequence unique to the 5'-noncoding region of HCV RNA. We demonstrated that this method was useful for the evaluation of antiviral reagents using HCV-infected human cultured cells.

Key words: hepatitis C virus, real-time PCR, LightCycler

Hepatitis C virus (HCV) is the main causative agent of chronic viral hepatitis. Most patients progress to liver cirrhosis and then to hepatocellular carcinoma. HCV is an enveloped virus belonging to the *Flaviviridae*, whose genome contains a 9.6 kilobase positive-stranded RNA encoding at least 10 viral proteins. While a number of studies have investigated the genetics of HCV, the mechanism of HCV replication in infected cells is poorly understood [for review, see 1]. We previously reported that the human MT-2C T-cell line [2] and human hepatocyte-derived PH5CH8 cell line [3] could support HCV replication. These cell culture systems were useful for evaluation of antiviral reagents, due to the fact that bovine lactoferrin (LF), a milk glycoprotein belonging to the iron transporter family, markedly inhibited HCV infection in the cells [4].

To monitor replication of HCV in these HCV-infected cell culture systems, we measured HCV RNA semi-quantitatively by a conventional reverse transcription-nested polymerase chain reaction (RT-nested PCR) method, checking the amplification level of the 5'-noncoding (NC) region in every fifth cycle of the second PCR [2]. Although we could roughly estimate the level of intracellular HCV RNA (10^2-10^3 copies/μg RNA), this method was labor intensive and time consuming because it required sampling at every fifth cycle and agarose gel electrophoresis for the detection of PCR products.

Recently, a new PCR method that can finish within 30 min, using real-time PCR technology and a LightCycler, was adapted to the quantitative detection of HCV RNA in clinical serum samples [5, 6]. This method does not require a probe for the detection, and the PCR product is monitored continuously by SYBR Green I dye binding to double stranded DNA during one-step PCR. We confirmed that 10 copies of HCV RNA from human serum could be detected, according to the protocol using the primer sets reported [5, 6] (data not shown). Using

Received September 25, 2001; accepted November 13, 2001.
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this method, we tried to detect the HCV RNA from *in vitro* HCV-infected PH5CH8 cells. However, we failed to detect the intracellular HCV RNA (10^2–10^3 copies/μg RNA), because cellular RNA (0.5 μg) lowered the specificity and sensitivity of this one-step PCR method. This result led us to modify the protocol of one-step PCR using the LightCycler, and we subsequently applied LightCycler PCR instead of a second PCR in a previous report on our semiquantitative method [2]. Amplification was performed in 20 μl of LightCycler FastStart DNA Master SYBR Green I mix containing 2.5 mM $MgCl_2$ by using 1 μl of the first PCR product obtained by the method described previously [2] and primers 104 and 197R [2], resulting in amplification of the 144-base pair of the 5'-NC region. LightCycler PCR was performed in 35 cycles of 15 sec at 95 °C (denaturation), 5 sec at 57 °C (annealing), and 8 sec at 72 °C (extension) with fluorescence detection at 88 °C after each cycle. After the final cycle, melting-point analysis of the samples was performed within the range of 65 to 95 °C. In this condition, we confirmed the successful detection of about 100 copies of HCV RNA in the RNA specimen (0.5 μg) from *in vitro* HCV-infected PH5CH8 cells (approximately 5×10^4 cells).

Using HCV RNA synthesized *in vitro* as described previously [2], we next examined the sensitivity and specificity of LightCycler PCR under this condition. RNA (0.5 μg) derived from PH5CH8 cells was added to each reaction tube so as to be equivalent to the amount of RNA in the actual experimental specimens. Standard

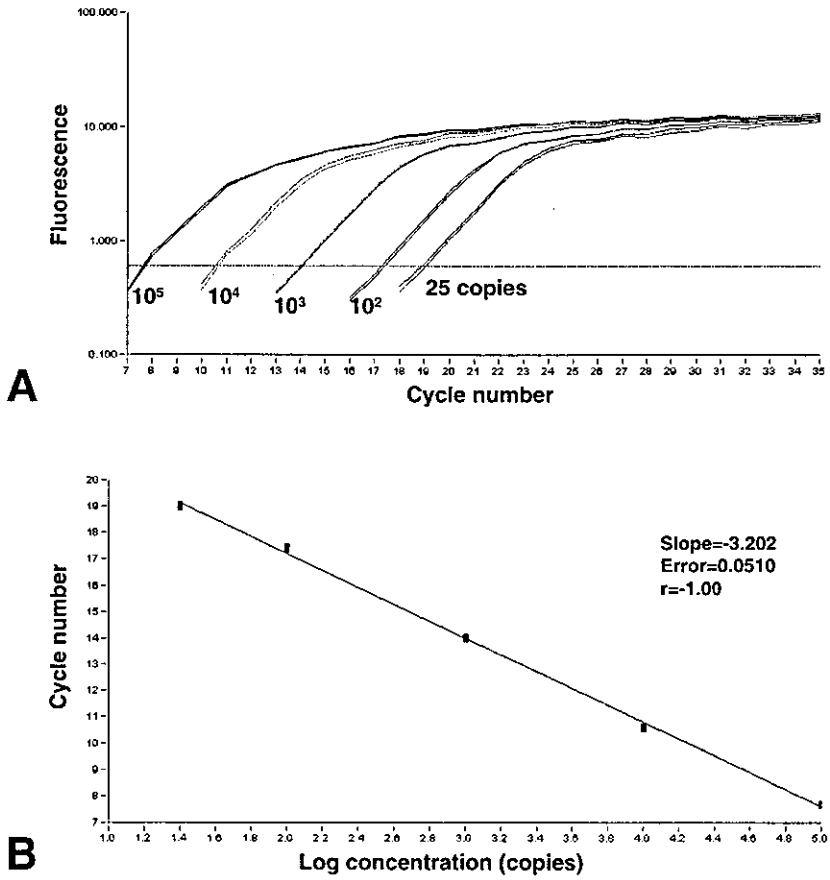

Fig. 1 Standard curve obtained by LightCycler PCR using *in vitro* synthesized HCV RNA. (A) Logarithmic curve of fluorescence versus cycle numbers for each sample. Cellular RNA (0.5 μg) from PH5CH8 cells was added to each reaction tube before RT so as to be equivalent to the amount of RNA in the actual experimental specimens. The horizontal line corresponds to the baseline as described in the text. The results of duplicate samples are indicated. (B) Crossing points (cycle numbers) plotted against the logarithmic concentration of the initial copies of HCV RNA.

curves were constructed from serial 10-fold dilutions (10^5 to 10^1 copies) and 25 copies of synthetic HCV RNA. After real-time PCR was completed, logarithmic values of fluorescence for each dilution were plotted against the cycle number. A baseline was set above the fluorescence background and a crossing point was determined using the amplification curves obtained during the exponential phase of amplification. As shown in Fig. 1A, as few as 25 copies of HCV RNA could be detected, although 10 copies of HCV RNA were not detected. There was a good relationship between the cycle number corresponding to the crossing point and the log concentration of initial copies of HCV RNA used, as shown in Fig. 1B. Good linearity was obtained in the range of 10^5 to 25 copies of HCV RNA. As shown in Fig. 1, high reproducibility of our quantitative method was obtained. The specificity of the amplified product was determined by melting curve analysis. Melting curve acquisitions were performed immediately after PCR was performed within the range of 65 to 95 °C. The melting temperature (Tm) of the PCR product appeared to be 89 °C, although the primer dimer had a Tm of 81 °C (Fig. 2). The other merit of our method is that a reproducible standard curve is obtained in each LightCycler PCR, using the stable first PCR products, which were amplified from *in vitro* synthesized HCV RNA and stored at −80 °C as the templates. Using this PCR system, we actually performed the quantitation of HCV RNA in RNA specimens derived from *in vitro* HCV-infected PH5CH8 cells. The results showed that the amount of HCV RNA in HCV-infected cells (7–14 days postinoculation) was 10^2–10^3 copies per μg RNA. These values were comparable to those obtained in a previous semi-quantitative analysis [2]. These results indicate that our PCR method using the LightCycler is useful for quantitation of intracellular HCV RNA. In contrast, the intracellular HCV RNA (10^2–10^3 copies/μg RNA) was not detected by the usual PCR methods using LightCycler PCR [5, 6].

To demonstrate the usefulness of LightCycler PCR, we examined whether this method could be applied as the evaluating system of anti-HCV activities in bovine and human LFs, which prevent HCV infection in PH5CH8 cells [4]. Using the same assay conditions [4], excepting the shifts to small scale (5×10^4 cells in 96-wells plate) and LightCycler PCR technology, we attempted to determine the IC_{50} dose of LF against HCV infection. As shown in Fig. 3, the quantification of intracellular HCV RNA by LightCycler PCR demonstrated that the prevention of HCV infection with bovine LF occurred in a dose-dependent manner. From this result, we determined that the IC_{50} dose of bovine LF was 0.12 mg/ml. Using the same assay method, the IC_{50} dose of human LF was also determined to be 0.4 mg/ml (data not shown). These results suggest that the HCV-inhibiting activity of bovine LF was relatively stronger than that of human LF. These results indicate that our quantitative

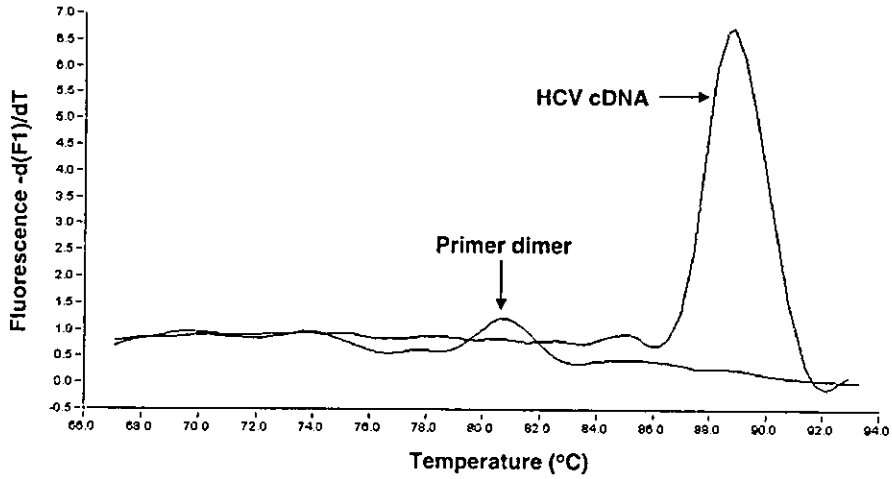

Fig. 2 Schematic diagram of a Tm analysis as accomplished at the end of PCR. HCV PCR product has a Tm of 89 °C and is easily distinguishable from primer dimer with a Tm of 81 °C. The graph displays the negative first derivative of the melting curve data (-dF/dT) versus temperature.

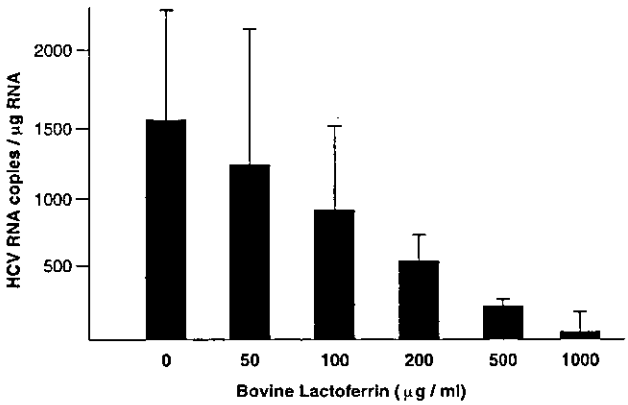

Fig. 3 HCV-inhibiting activity of bovine LF in PH5CH8 cells. PH5CH8 cells and inoculum 1B-2 were used for the HCV-inhibiting assay of LF as described previously [4]. Quantitative RT-nested PCR using LightCycler PCR was performed using RNA samples in greater than triplicate. The number in the axis of the ordinate indicates the copies of HCV RNA per μg RNA.

method using LightCycler PCR is highly useful for the evaluation of anti-HCV reagents. Furthermore, this method may be useful for the quantitative analysis not only of HCV genomes but also of other RNA viral genomes derived from infected cells.

Acknowledgements. We thank A. Naganuma for helpful suggestions. This work was supported by grants from the Japanese Ministry of Health, Labor and Welfare, for the Second-Term Comprehensive 10-Year Strategy for Cancer Control, and from the Organization for Pharmaceutical Safety and Research (OPSR).

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Acta Med. Okayama, 2002
Vol. 56, No. 3, pp. 141-147

Acta Medica Okayama
http://www.lib.okayama-u.ac.jp/www/acta/

Original Article

Hepatitis C Virus Quasispecies in Cancerous and Noncancerous Hepatic Lesions: The Core Protein-encoding Region

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We have shown that highly proofreading DNA polymerase is required for the polymerase chain reaction in the genetic analysis of hepatitis C virus (HCV). To clarify the status of HCV quasispecies in hepatic tissue using proofreading DNA polymerase, we performed a genetic analysis of the HCV core protein-encoding region in cancerous and noncancerous lesions derived from 4 patients with hepatocellular carcinoma. In contrast to the previously published data, we observed neither deletions nor stop codons in the analyzed region and no significant difference in the complexity of HCV quasispecies between cancerous and noncancerous lesions. This result suggests that the HCV core gene is never structurally defective in hepatic tissues, including cancerous lesions. However, in 3 of the patients, the consensus HCV species differed between cancerous and noncancerous lesions, suggesting that the predominant replicating HCV species differs between these 2 types of lesions. Moreover, during the course of the study, we obtained several interesting variants possessing a substitution at codon 9 of the core gene, whose substitution has been shown to induce the production of the F protein synthesized by a −2/+1 ribosomal frameshift.

Key words: hepatitis C virus, core gene, hepatocellular carcinoma, quasispecies, proofreading DNA polymerase

Hepatitis C virus (HCV) infection frequently causes chronic hepatitis (CH) [1, 2] and progresses to liver cirrhosis (LC) and hepatocellular carcinoma (HCC) [3, 4]. HCV is an enveloped positive single-stranded RNA (9.6-kilobases) virus belonging to the family *Flaviviridae* [5, 6]. To date, a large number of genetic analyses of HCV have been reported, and the viral genome structure has been elucidated [for review, see 7, 8]. The HCV genome encodes a large polyprotein precursor of approximately 3,000 amino acid residues, and this precursor protein is cleaved by the host and viral proteinases to generate at least 10 proteins in the following order: NH2-core-envelope (E1)-E2-p7-nonstructural protein 2 (NS2)-NS3-NS4A-NS4B-NS5A-NS5B-COOH. The most characteristic feature of the HCV genome is its remarkable genetic heterogeneity. To date, at least 6 major HCV genotypes have been identified, and these have been further grouped into more than 50 subtypes. Furthermore, HCV may show quasispecies distribution in an infected individual. These genetic features of HCV are considered to be one of the most important factors regarding maintenance of the chronic viremic state and have hampered the development of vaccines [for review, see 7, 8].

The HCV core protein is a 21-kDa protein produced from the N-terminal portion of the precursor protein by

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host-cell signal peptidase(s) [9], and resides primarily in the cytoplasm [10]. To date, it has appeared that the HCV core protein not only functions in viral replication as a viral capsid protein, but also affects a variety of cellular functions, including gene expression, signal transduction, and apoptosis [11]. The core protein is thus a multifunctional protein and may play an important role in hepatocellular carcinogenesis. To understand the relationship between the progression of hepatic disease and the genomic characteristics of the HCV core protein-encoding region, genetic analyses of HCV derived from patients with acute hepatitis, CH, LC and HCC have been performed [12, 13]. In addition, several groups have carried out comparative sequence analyses of the HCV core gene in cancerous and noncancerous hepatic lesions [14-16]. The results obtained from these studies have shown that the intra-patient variation in nucleotides increases with the progression of liver disease, and that deletions and mutations in the core gene occur more frequently in cancerous than in noncancerous lesions. However, because nonproofreading DNA polymerase was used for the polymerase chain reaction (PCR) in these studies, the possibility remains that some of these observations were experimental artifacts. Therefore, to accurately elucidate the status of quasispecies of the HCV core gene, we consider the use of highly proofreading DNA polymerase for amplification of the HCV genomes to be indispensable. In the present study, we have shown that nonproofreading DNA polymerases give artifactural quasispecies representations, and that there appears to be no difference in core-gene genetic diversity between cancerous and noncancerous hepatic lesions, although different sequences are clearly evident. In addition, during the course of the study, we obtained several interesting variants possessing a substitution at codon 9 of the core gene.

Materials and Methods

Patients. Four Japanese patients with HCC were selected for this study. Patient 1 was a 68-year-old man with CH and HCC, and a serum alanine aminotransferase (ALT) level was 49 IU/l. Patient 2 was a 69-year-old man with LC and HCC and a serum ALT level of 120 IU/l. Patient 3 was a 67-year-old female with LC and HCC and a serum ALT level of 22 IU/l. Patient 4 was a 58-year-old man with LC and HCC and a serum ALT level of 48 IU/l. The results of the pathogenic examination of these HCCs were as follows: patient 1, highly differentiated HCC; patient 2, a mixture of highly and moderately differentiated HCC; patient 3, moderately differentiated HCC; patient 4, undifferentiated HCC. All patients were positive for antibodies against HCV but negative for hepatitis B surface antigen. The HCV genotype in these patients was determined to be genotype 1b by a previously described method [17]. Informed consent was obtained from each patient before the study. Fresh tissue samples (including a cancerous and noncancerous portion) were collected intraoperatively from the resected specimens, immediately frozen, and stored overnight at -80 °C.

RNA extraction and reverse transcription (RT)-nested PCR. RNAs from hepatic tissues were prepared using the ISOGEN extraction kit (Nippon Gene Co., Toyama, Japan). These RNA samples (1 μg of RNA) were used as templates for the RT-nested PCR to amplify the core gene. An antisense primer, 968R, 5'-GTTGGAGCAGTCGTTCGTGAC-3' (corresponding to position 948-968 of HCV-JS [18]), was used to prime cDNA synthesis by SuperScript II (Life Technologies, Rockville, MD, USA). One-tenth of the synthesized cDNA was used for the first round of PCR. Primers 201A, 5'-GCCTGATAGGGTGCTTGCGA-3' (corresponding to position 291-310 of HCV-JS [18]), and 968R were employed in the first round of PCR (35 cycles in 50 μl). Two microliters of the first reaction mixture was used for the second round of PCR. An internal primer pair, 338(B), 5'-ATTATGGATCCGGAGGTCTCGTAGACCGTGC-3' (corresponding to position 319-338 of HCV-JS [18] and containing a *Bam*HI recognition site (underlined) in the 5' region), and 948R(H), 5'-ATTATAAGCTTCATGGTATATCCCGGACGCGTT-3' (corresponding to position 927-948 of HCV-JS [18] and containing a *Hind*III recognition site (underlined) in the 5' region) was used for the second round of PCR (30 cycles in 50 μl). KOD-plus DNA polymerase (Toyobo, Osaka, Japan) was used as a proofreading enzyme for PCR, and each PCR cycle consisted of annealing at 55 °C for 45 sec, primer extension at 74 °C for 1 min and denaturation at 94 °C for 30 sec. Taq DNA polymerase (Sawady, Tokyo, Japan) was

Footnotes: The nucleotide-sequence data reported in this paper will appear in the DDBJ, EMBL, and GenBank nucleotide-sequence databases under accession numbers AB061927 to AB062026 (pTL1~pTL4 series), AB062173 to AB062272 (pTH1~pTH4 series).

used as a nonproofreading enzyme for PCR under the same conditions as those used with the KOD-plus DNA polymerase. The second PCR reaction of the HCV core gene yielded a 652-bp amplified product.

cDNA cloning and sequencing. PCR products containing the HCV core protein-encoding region were cloned into the *Bam*HI and *Hin*dIII sites of pTZ19R, as described previously [19]. Plasmid inserts were sequenced in both the sense and antisense directions using Big Dye terminator-cycle sequencing on an Applied Biosystems 310 automated sequencer (Applied Biosystems, Inc., Norwalk, CT, USA).

Results

To examine the influence of using the different kinds of DNA polymerase in RT-nested PCR, we used KOD-plus DNA polymerase and Taq DNA polymerase as highly proofreading and nonproofreading enzymes, respectively. A RNA specimen derived from a cancerous lesion in patient 1 with HCC was used for cDNA synthesis with SuperScript II. The obtained cDNA was used as a template for amplification of the core gene by KOD-plus or Taq DNA polymerase. We compared the nucleotide sequences of each of 20 clones (pTH1 and pTH1S series) obtained from the PCR products amplified by the KOD-plus and Taq DNA polymerases, respectively. We obtained the same consensus sequence from both series. However, the level of nucleotide substitution in clones of the pTH1 series was drastically lower than that of clones of the pTH1S series, as shown in Table 1. In particular, the number of nonsynonymous substitutions with a transition in the pTH1 series (3 positions) was 7 times lower than that in the pTH1S series (21 positions), although the number of synonymous substitutions with transition was 1.5 times lower in the pTH1 series than in the pTH1S series (Table 1). The number of synonymous and nonsynonymous substitutions with transversion in the pTH1 series was also 7 times lower than that in the pTH1S series (Table 1). Furthermore, we obtained 6 clones possessing one nucleotide deletion at different positions in the pTH1S series, but no such clones were obtained in the pTH1 series. The distribution pattern of clones diverging from the consensus sequences was also rather different between series at both the nucleotide and amino acid-sequence levels. In the pTH1 series, 6 and 17 out of 20 clones were the same as the consensus sequences at the nucleotide and amino acid levels, respectively, but in the pTH1S series, only 1 and 5 out of 20 clones were the same as the consensus clones at the nucleotide and amino acid levels, respectively. These results suggest that the genetic differences detected between the pTH1 and pTH1S series were due to the differences in proofreading activity between the DNA polymerases used. Therefore, we judged that use of a proofreading DNA polymerase is required for the PCR in genetic analysis of HCV.

To elucidate the status of HCV quasispecies in cancerous and noncancerous lesions, RNA specimens derived from 4 HCV-positive patients with HCC were used for amplification of the core gene by RT-nested PCR using KOD-plus polymerase. We determined the nucleotide sequences of 20 independent clones obtained from each of two specimens of patients 1-3 (n = 120 clones in total). In the case of patient 4, we analyzed 40 clones from the cancerous lesion and 40 from the noncancerous lesion, as several interesting clones were obtained (these will be described later in this report). Based on these sequence data, we analyzed the status of the sequence diversity of the core gene in cancerous (pTH series) and noncancerous (pTL series) lesions. As shown in Table 2, the consensus sequences obtained in each of the specimens from the cancerous and noncancerous lesions showed 1.4-2.6%

Table 1 Sequence diversity of PCR products amplified with proofreading and nonproofreading DNA polymerases

Series	No. of clones sequenced	No. of nt substituted from consensus sequences						No. of deleted nt
		Transition			Transversion			
		Synonymous	Nonsynonymous	Average/clone	Synonymous	Nonsynonymous	Average/clone	
pTH1	20	23	3	1.30	1	0	0.05	0
pTH1S	20	36	21	2.85	5	2	0.35	6

The pTH1 and pTH1S series were obtained by amplification using proofreading (KOD-plus) and nonproofreading (Taq) DNA polymerases, respectively.

nucleotide-sequence diversity compared with the consensus sequence of genotype 1b. The amino acid sequences deduced from the consensus sequences also differed from the consensus sequences of genotype 1b by 1-6 amino acids (Table 2), although these sequences did not differ in the pTH2 series. These values were within the sequence variation in genotype 1b. However, we found that the consensus sequences differed between the cancerous and noncancerous lesions in 3 of 4 patients. The consensus sequences of the pTH4 and pTL4 series differed by 9 nucleotides and 1 amino acid. 5 nucleotides and 2 amino acids were different between the consensus sequences of the pTH2 and pTL2 series (Table 2). We further found that, except in the case of patient 3, the distribution of HCV species substituted from each of the consensus sequences also differed between cancerous and noncancerous lesions, particularly at the nucleotide level (Table 3). Fifty percent of clones in the pTH4 series (cancerous lesion) were identical to the consensus sequence, but none of the clones in the pTL4 series (noncancerous lesion) were the same. Forty percent of clones in the pTL2 series (noncancerous lesion) showed sequences identical to the consensus sequences; however, there were no clones possessing the consensus sequences in the pTH2 series

Table 2 Comparison of the consensus sequences obtained from cancerous and noncancerous lesions

Consensus sequences	Differences from consensus sequence of genotype 1b		Differences between consensus sequences of pTH and pTL series	
	nt/573 nts	aa/191 aa	nts/573 nts	aa/191 aa
pTH1	14	6	0	0
pTL1	14	6		
pTH2	12	0	5	2
pTL2	15	2		
pTH3	12	2	1	0
pTL3	11	2		
pTH4	11	2	9	1
pTL4	8	1		

Table 3 Distribution of HCV clones diverged by substitution from each consensus sequence

No. of substitutions differing from each consensus sequence	No. of clones															
	pTH1		pTL1		pTH2		pTL2		pTH3		pTL3		pTH4		pTL4	
	nt	aa	nt	aa	nt	aa	nt	aa	nt	aa	nt	aa	nt	aa	nt	aa
0	6	17	1	13		4	8	12	4	14	4	17	20	22		10
1	8	3	7	4	1	11	1	6	2	5	6	2	1	3		15
2	2		8	2		5	4	1	7	1	5		1	7		8
3	2		2	1	2		3		3		2	1		6	2	4
4	1		2		1		3	1	2		2			2	1	3
5	1				11				2		1				1	
6					2								1		11	
7					2								3		6	
8															8	
9					1										6	
10													2		3	
11							1								2	
12													2			
13													2			
14													3			
15													1			
16													4			

(cancerous lesion). In the case of patient 1, who also showed identical consensus sequences between the cancerous and noncancerous lesions, only 1 clone from the noncancerous lesion was identical to the consensus sequence, while 6 clones possessing the consensus sequence were obtained from the cancerous lesion (Table 3). These results suggest that the HCV quasispecies proliferating in cancerous and noncancerous lesions are rather different in each case.

Table 4 summarizes the numbers of nucleotides deviating from each of the consensus sequences. In both patients 2 and 3, the frequencies of substitutions with transition or transversion were higher in cancerous than in noncancerous lesions, while in patients 1 and 4, noncancerous lesions showed higher frequencies of substitutions than the cancerous lesion. These results do not support the conclusion in previous reports [14-16] that the complexity of quasispecies on the core gene in cancerous lesions is generally higher than that in noncancerous lesions. Furthermore, in contrast to previous reports [14-16], no clones possessing the deletion or stop codon were obtained in the core gene. Nonsynonymous substitutions were 2.4 to 8 times less frequent than synonymous ones, and transitions were 7.8 to 40 times more frequent than transversion, particularly at the third position of the codon.

During the course of this study, we obtained 6 interesitng clones from the noncancerous lesion of patient 4 (pTL4 series); all of these had AGA (Arg) to AAA (Lys) substitutions at codon 9, resulting in an A stretch (A_8 to A_{10}) at codons 8-11 (Fig. 1). Although the 4 major clones possessed an A_9 stretch, one possessed an A_{10} stretch due to an additional substitution (ACC to AAC) at codon 11, while another possessed an A_8 stretch due to an additional substitution (ACC to CCC) at codon 11. To date, only the prototype HCV-1 strain has been reported to possess the same A_{10} stretch at codons 8-11 [20]. It has also been reported that the HCV-1 genome produces a small 17-kDa protein with a 21-kDa core protein in *in vitro* transcription and translation experiments [21]. Furthermore, codon 9 (AAA) has been shown to play an important role in determining production of the 17-kDa protein [21]. Recently, this

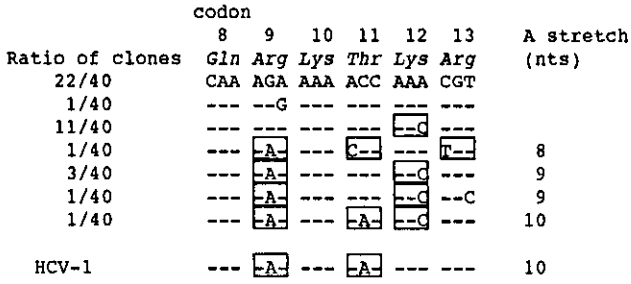

Fig. 1 Nucleotide sequences of codons 8-13 in the pTL4 series. The status of the quasispecies on codons 8-13 is shown. Boxed codons indicate the nonsynonymous substitutions. The known HCV-1 strain possessing an AAA sequence at codon 9 is also shown.

Table 4 Sequence diversities of the HCV core protein-encoding region derived from cancerous and noncancerous lesions

Series	No. of clones sequenced	No. of nt substituted from each consensus sequences						Synonymous/ nonsynonymous	Transition/ transversion
		Transition			Transversion				
		Synonymous	Nonsynonymous	Average	Synoymous	Nonsynonymous	Average		
pTH1	20	23	3	1.30	1	0	0.05	8.0	26.0
pTL1	20	26	7	1.65	0	4	0.20	2.4	8.3
pTH2	20	80	16	4.80	0	5	0.25	3.8	19.2
pTL2	20	28	12	2.00	1	0	0.05	2.4	40.0
pTH3	20	33	6	1.95	3	1	0.20	5.1	9.8
pTL3	20	27	4	1.55	3	1	0.20	6.0	7.8
pTH4	40	173	25	4.95	0	19	0.48	3.9	10.3
pTL4	40	233	34	6.67	5	20	0.62	4.4	10.8
cancerous lesion*		223	37.5	3.25	4.0	15.5	0.24	4.2	13.3
noncancerous lesion**		198	40.0	2.97	6.5	15.0	0.27	3.9	11.0

*One half of the total number of clones from the pTH4 series was added to the calculation. **One half of the total number of clones from the pTL4 series was added to the calculation.

17-kDa protein, named the F protein, has been clarified to be produced by a ribosomal frameshift into the −2/+1 reading frame [22]. The obtained 6 mutants possessing a substitution at codon 9 may also produce the F protein.

Discussion

In this study, we have shown the importance of using proofreading DNA polymerase in the genetic analysis of HCV. Regarding this point, it has recently been shown that the use of nonproofreading Taq DNA polymerase to assess viral diversity could yield an incorrect quasispecies spectrum [23]. In this previous study, sequence analysis of hypervariable region 1 of the E2 envelope gene and the interferon sensitivity-determining region of the NS5A gene revealed a great number of minor variants in the PCR products amplified by nonproofreading DNA polymerase, 80% of which were not observed in the PCR products amplified by proofreading DNA polymerase [23]. These findings thus support our present results. Our assertion of the importance of using a proofreading DNA polymerase also seems reasonable in light of the finding that the fidelity of proofreading DNA polymerase is more than 10 times greater than that of Taq DNA polymerase [24, 25].

A number of sequence analyses have been carried out on quasispecies of 5' UTR and the envelope gene [for review, see 7, 8]; however, there is much less information on quasispecies of the core gene. In this study, we compared the status of quasispecies of the core gene between cancerous and noncancerous lesions by sequence analysis of the PCR products amplified with highly proofreading thermostable DNA polymerase. Previous studies [13-16] have revealed the appearance of in-frame stop codons and deletions leading to frame-shifts within the core gene, especially in the case of RNA specimens from cancerous lesions. In contrast to these published data, neither stop codons nor deletions were observed among the 200 HCV clones derived from cancerous and noncancerous lesions in the present study. Because we also observed several one-nucleotide deletions with a frequency of 30% when nonproofreading Taq DNA polymerase was used for the PCR, it may be concluded that such discrepancy depends on the fidelities of thermostable DNA polymerases used for PCR.

Although previous studies [13-16] have shown a significantly higher genetic variability of HCV in cancerous than in noncancerous lesions, our results demonstrated that the quasispecies diversity of HCV is not dependent on the status of hepatic diseases. This discrepancy is also probably due to differences in the DNA polymerases used for PCR. To obtain a conclusion regarding this point, analysis of several additional specimens will be required.

In this study, we obtained several core variants possessing an AAA sequence at codon 9, resulting in an A stretch (A_8 to A_{10}) at codon 8-11. It is unlikely that detection of the A stretch was due to an error of DNA polymerase or reverse-transcriptase, as such an A stretch was reproducibly obtained from other RT-PCR products derived from patient 4. Although the frequency of clones possessing an A stretch of more than 8 nucleotides was 15% in the noncancerous lesion of patient 4, no such clones were obtained from the cancerous lesion of this patient, indicating that the frequency of such clones in cancerous lesion is less than 2.5%. However, it will be necessary to clarify the prevalence of such core variants, which may produce an F protein synthesized by a −2/+1 ribosomal frameshift, because it has been estimated that the F protein plays an important role in the HCV life cycle [22].

Finally, the present data obtained using a proofreading DNA polymerase showed that none of the HCV species obtained from hepatic tissues - including cancerous lesions - were structurally defective at least in the core protein-encoding region. Genetic analyses of other HCV protein-encoding regions will be needed to confirm this observation.

Acknowledgements. We would like to thank Dr. T. Tsuji of Okayama University Graduate School of Medicine and Dentistry for his encouragement and support. We would like to thank S. Nobukiyo and Y. Inoue for their helpful assistance. This work was supported by grants from Grants-in-Aid for Scientific Research from the Japanese Ministry of Education, Culture, Sports, Science, and Technology, and by Grants-in-Aid for Cancer Research and for the Second-Term Comprehensive 10-Year Strategy for Cancer Control from the Japanese Ministry of Health, Labor, and Welfare, and from the Organization for Pharmaceutical Safety and Research (OPSR).

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Hepatology Research 24 (2002) 228–235

Hepatology Research

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Lactoferrin inhibits hepatitis B virus infection in cultured human hepatocytes

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Received 19 November 2001; received in revised form 19 March 2002; accepted 5 April 2002

Abstract

We recently reported that lactoferrin (LF), a milk protein belonging to the iron transporter family, inhibits hepatitis C virus (HCV) infection in cultured human hepatocytes (PH5CH8) and that the interaction of LF with HCV is responsible for this inhibitory effect. As PH5CH8 cells were found to be a human hepatocyte line susceptible to hepatitis B virus (HBV) infection, we therefore examined if LF could effectively prevent HBV infection in PH5CH8 cells. Preincubation of the cell with bovine LF (bLF) or human LF (hLF) was required to prevent HBV infection of cells, and preincubation of HBV with bLF or hLF had no inhibitory effect on HBV infection. We further found that bovine transferrin, casein, and lactoalbumin had no anti-HBV activity. Our findings suggest that the interaction of LF with cells was important for its inhibitory effect, and that LF may well be among the candidates for an anti-HBV reagent that could prove effective in the treatment of patients with chronic hepatitis.

Keywords: Lactoferrin; Hepatitis B virus; Infection; Anti-viral effect; Hepatocytes; Nested PCR

1. Introduction

A close association between hepatitis B virus (HBV) and hepatocellular carcinoma (HCC) has been reported in many countries, especially in Asia and Africa [1,2]. Chronic HBV infection progresses to liver cirrhosis and HCC [3]. HBV is made up of a circular DNA molecule, which is

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1386-6346/02/$ - see front matter © 2002 Published by Elsevier Science B.V.
PII: S1386-6346(02)00088-8

partially single-stranded and about 3200 base-pairs long [4,5]. HBV DNA encodes four sets of viral products and has a complex, multiparticle structure. HBV achieves its genomic economy by relying on an efficient strategy of encoding proteins from four overlapping genes; surface, core, polymerase, and X protein [6]. Lamivudine reportedly has anti-HBV effects in vitro and in vivo [7], but some problems have arisen such as drug resistance and amino acid substitution in the YMDD motif during treatment with lamivudine [8].

Lactoferrin (LF), a milk protein belonging to the iron transporter family, is found at especially high levels in colostrum, at a maximum level of 3% [9]. It is also contained in human milk, tears, saliva, vaginal secretions, semen, bronchoalveolar lavage fluid [10–12] and other epithelial secretions [13] and is present in the secondary granules of neutrophils [14]. LF is thought to be responsible for primary defense against pathogenic microorganisms [15], including *Candida albicans* [16], human immunodeficiency virus (HIV) -1, human cytomegalovirus (HCMV) [17], and human herpes simplex virus (HSV) -1 [9]. Many other functions have been attributed to LF, including immunomodulation and cell growth regulation. Some of these functions are independent of the iron-binding activity of LF.

Recently, we reported that LF inhibited hepatitis C virus (HCV) infection in cultured human hepatocytes (PH5CH8) [18]. PH5CH8 is a non-neoplastic human hepatocyte line, which was immortalized with SV40 large T antigen [19], and cloned PH5CH8 cells are susceptible to HCV infection [20,21]. This cultured cell system allows us to evaluate the antiviral activities of several reagents, such as LF. Previous studies on HSV-1, HCMV, HIV-1 and HCV showed LF inhibited adsorption and/or internalization of the virus into host cells [9,17,18]. However, no studies have examined whether LF inhibits HBV infection *in vivo* and *in vitro*. Recently, we found that the PH5CH8 cell line was susceptible to HBV infection (unpublished observation), we therefore, focused on whether LF similarly protects cultured hepatocytes from HBV infection. This is the first report describing the anti-HBV activity of LF.

2. Materials and methods

2.1. Cells and inoculum

The PH5CH8 cell line was cloned from non-neoplastic human hepatocyes, PH5CH cells, and maintained as described previously [19]. Serum from a HBV carrier with acute exacerbation of hepatitis was used as the inoculum.

2.2. Nested polymerase chain reaction (PCR)

Samples of DNA from cells were prepared using an ISOGEN extension kit (Nippon Gene Co., Tokyo, Japan). In this study, the pRL-TK plasmid vector (Promga) encoding *Renilla* luciferase (Rluc) was used as an internal control to monitor the efficiency of the nested PCR [22–24]. We added 10 ng of the pRL-TK plasmid vector to the DNA samples. One fifth of these DNA samples were used for detection of the S-region of HBV DNA by nested PCR. PCR amplification with Taq DNA polymerase (Sawady Technology, Tokyo, Japan) was performed for 35 cycles using an HBV outer primer set (HB0: ACTCGTGGTGGACTTC-TCTC, HB0R: GCACTAGTAAACTGAGCCA-GG) and an Rluc primer set (RlucI: TGTGCCA-CATATTGAGCCA and RlucIR: ATGGCAA-CATGGTTTCCACG). For the second amplification, 2 μl of the first reaction mixture was removed and further amplified for 35 cycles using an HBV inner primer set (HB1: TCATCCT-GCTGCTATGCCTC, HB1R: TGAGGCCCA-CTCCCATAGG), and an Rluc set (RlucI and RlucIR). PCR products (278 bps for HBV and 374 bps for Rluc) were detected by staining with ethidium bromide after separation by electrophoresis on a 3% agarose gel.

2.3. Infection of HBV to cells

PH5CH8 cells (1×10^5) were plated in a 24-well microtiter plate and cultured for 2 days before viral inoculation. After the addition of 500 μl of medium containing 10 μl of HBV serum, the cells were incubated for 210 min at 32 °C. After the incubation, free HBV was removed by washing twice with 1 ml of PBS, and the cells were