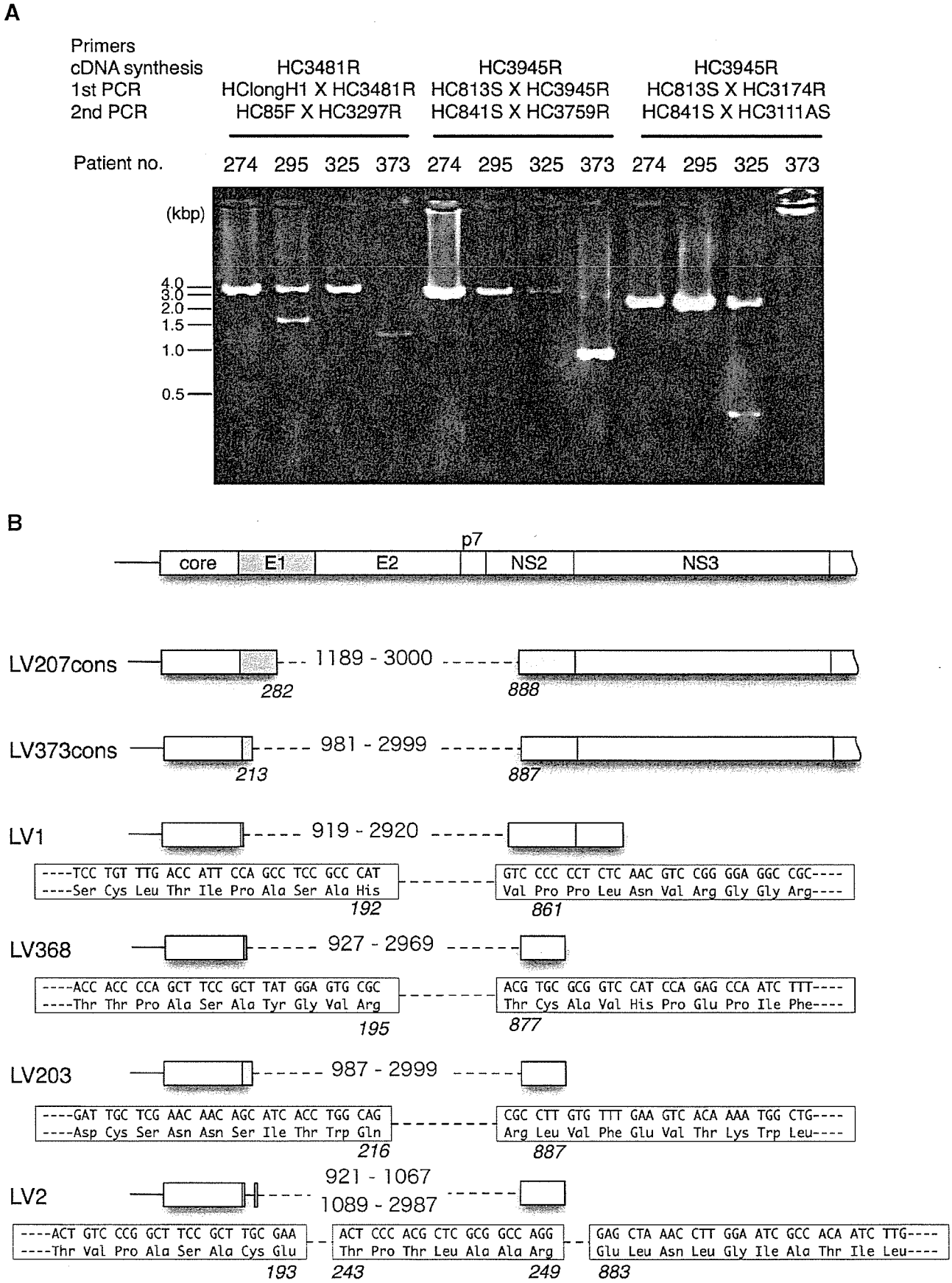

A
Primers
cDNA synthesis
1st PCR
2nd PCR
HC3481R
HClongH1 X HC3481R
HC85F X HC3297R
HC3945R
HC813S X HC3945R
HC841S X HC3759R
HC3945R
HC813S X HC3174R
HC841S X HC3111AS
Patient no.
274 295 325 373 274 295 325 373 274 295 325 373
(kbp)
4.0
3.0
2.0
1.5
1.0
0.5
B
p7
core
E1
E2
NS2
NS3
LV207cons
1189 - 3000
282
888
LV373cons
981 - 2999
213
887
LV1
919 - 2920
----TCC TGT TTG ACC ATT CCA GCC TCC GCC CAT
----Ser Cys Leu Thr Ile Pro Ala Ser Ala His
192
GTC CCC CCT CTC AAC GTC CGG GGA GGC CGC----
Val Pro Pro Leu Asn Val Arg Gly Gly Arg----
861
LV368
927 - 2969
----ACC ACC CCA GCT TCC GCT TAT GGA GTG CGC
----Thr Thr Pro Ala Ser Ala Tyr Gly Val Arg
195
ACG TGC GCG GTC CAT CCA GAG CCA ATC TTT----
Thr Cys Ala Val His Pro Glu Pro Ile Phe----
877
LV203
987 - 2999
----GAT TGC TCG AAC AAC AGC ATC ACC TGG CAG
----Asp Cys Ser Asn Asn Ser Ile Thr Trp Gln
216
CGC CTT GTG TTT GAA GTC ACA AAA TGG CTG----
Arg Leu Val Phe Glu Val Thr Lys Trp Leu----
887
LV2
921 - 1067
1089 - 2987
----ACT GTC CCG GCT TCC GCT TGC GAA
----Thr Val Pro Ala Ser Ala Cys Glu
193
ACT CCC ACG CTC GCG GCC AGG
Thr Pro Thr Leu Ala Ala Arg
243
249
GAG CTA AAC CTT GGA ATC GCC ACA ATC TTG----
Glu Leu Asn Leu Gly Ile Ala Thr Ile Leu----
883

Fig. 3.

junction site. The expected length of the PCR fragment was 112 bp.

Analysis of HCV Proteins by Western Blotting

HCV cDNA expression plasmids were transfected into HEK293 cells with Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. After 24 hr, cells were recovered and lysed in RIPA buffer containing protease inhibitor cocktails. After removing debris by centrifugation, the lysate was subjected to SDS-polyacrylamide gel electrophoresis (10–20% gradient gel, Daiichi Chemical, Tokyo, Japan), and proteins were transferred to a PVDF membrane (Millipore, Tokyo, Japan) under semi-dry conditions. The membrane was probed with the relevant antibodies. Bound antibodies were detected with anti-mouse or rabbit antibodies conjugated with HRP, and visualized by detecting the chemiluminescent signals developed using SuperSignal West Pico reagent (Pierce) with the LAS-1000 image analysis system (Fujifilm K.K., Tokyo, Japan) or exposure to Kodak Bio Max film. The membrane was rinsed in Restore reagent (Pierce) in order to remove bound antibodies, and was then probed with other antibodies.

RESULTS

Quantitation of HCV in Clinical Specimens

Table I summarizes viral parameters in 23 chronic active hepatitis C patients subsequently treated with interferon. All patients responded to IFN administration, exhibiting reduced virus titers and alanine transaminase (ALT) levels in serum; however, IFN efficacy varied (not shown). Serum and liver specimens were taken before IFN administration, and HCV viral loads were then determined by quantitation of HCV RNA by real-time RT-PCR, and by measuring HCV core antigen levels using core antigen ELISA (Table I). Correlations among viral loads were analyzed. The amount of HCV core antigen and RNA in serum and liver were well correlated ($R = 0.968$ and $R = 0.728$, respectively). In contrast, the correlation between HCV titers in serum and liver was significant but poor ($R = 0.575$ stand for core antigen and $R = 0.373$ for RNA). No obvious correlation was observed between efficacy and viral load in liver biopsy specimens.

Truncated Subgenomic HCV RNA in the Liver

In order to investigate the structure of the HCV genome in patient liver, HCV cDNAs were cloned from two cases with high viral load (207 and 373). Figure 1A shows the strategy of LD-RT-PCR for cloning of the HCV genome. The length of LD-RT-PCR products varied between the patients. Comparison with the control cDNA, which was previously cloned from a blood donor (G14) plasma sample, indicated a 2-kb deletion occurred in the 5′-half of the HCV RNA from these patients (Fig. 1B).

The nucleotide sequences of cDNA clones from Patient 207 liver (LV207-0193-1, -3, -6 and -15) were analyzed and the deduced amino acid sequences were compared (Fig. 2A). All clones had almost identical sequences. While the open reading frames (ORFs) of two isolates were discontinued (by two stop codons in LV207-0193-6 and a one-base deletion in LV207-0193-15), LV207-0193-1 and -3 encoded a continuous 2,383 amino acid polypeptide. In the case of Patient 373, LV373-0193-10 encoded a 2,314 amino acid polypeptide. Two cDNA clones, LV373-0153-6 and -5, overlapped with LV373-0193-10, and identity in the nucleotide sequence of the overlapped region was 98%. With the exception of LV373-0153-5, which contained an in-frame stop codon, each cDNA had a contiguous ORF. The consensus HCV cDNA sequence for each patient was generated from these cDNA isolates (Fig. 2B).

Sequence comparison with authentic genotype 1b HCV (J1) [Aizaki et al., 1998] is illustrated in Figure 2B. Both consensus sequences from patient livers lacked sequences between E1 and NS2; the regions corresponded to the nucleotides 1189–3000 (amino acids 283–887), and 981–2999 (amino acids 214–886) of HCV-J1.

To confirm that the deletion was not produced during LD-RT-PCR (due to effects such as strong RNA secondary structure), non-truncated RNA transcripts were reverse-transcribed and amplified by the same protocol to give the truncated genome in Patient 207. While a deleted DNA fragment of about 7 kbp was amplified from truncated RNA transcripts of Patient 207, a full-length DNA fragment of about 9 kbp was amplified from non-truncated RNA transcripts of Donor G14, (Fig. 2C).

Deletions Found in Livers of Other Patients

In order to examine the incidence of HCV subgenomes with E1–E2 deletions, HCV genomes in other liver specimens were examined by RT-PCR with the primers listed in Table II. Two surgical samples (Patient 1 and 2) from hepatic cirrhosis patients with hepatocellular carcinoma were also subjected to this analysis. While the patterns of PCR fragments depended on the primer sets (Fig. 3A, Table II), they were classified into four

Fig. 3. Demonstration of deletions in the structural protein coding sequences of HCV genomes from patient livers. **A**: RNA from patient biopsies was analyzed by RT-PCR with primers for 5′-UTR to NS2 or core to NS2. Primer sets and Patient numbers are shown above the agarose electrophoresis image, and positions of the DNA markers are indicated on the left side of the image. **B**: Illustration shows schematic views of in-frame deletions and sequences of PCR fragments obtained by RT-PCR with the 5′-UTR and NS2 primers. cDNA isolates were designated as described in legend of Figure 2. The clones (LV1, LV368, LV203, and LV2) of Patient 1, 368, 203, and 2 were obtained by RT-PCR using primer sets g, j, b, and f (Table II), respectively. Only truncated fragments were amplified in these cases after RT-PCR for 5′-UTR to NS2 (Table I). Structures of the truncated HCV genomes, LV207cons and LV373cons, are shown for comparison. Numbers between dot-tagged graphs represent deleted regions as they correspond to HCV-J1. Bottom lines show the corresponding amino acid positions of the deletion boundaries.

types (Table I): no amplified fragments (five cases); fragments corresponding to the non-truncated HCV genome (eight cases); fragments corresponding to both the truncated and non-truncated genomes (six cases); and fragments corresponding only to the truncated genome (four cases, including Patient 207 and 373).

RT-PCR of HCV RNA in Patient 1, 368, and 203 yielded only fragments corresponding to the truncated HCV genome. The isolated molecular clones contained in-frame deletions of amino acids extending from E1 to NS2, similarly to those isolated from Patient 207 and 373. Although cDNA from Patient 2 contained two in-frame deletions in E1 and E1–NS2, it shared the characteristics of the truncated genome; entire core, partial E1, and NS2 (Fig. 3B).

In cases with both truncated and non-truncated HCV cDNA, the predominant PCR product was non-truncated (Table I, Fig. 3A). Figure 4 shows the structures of the cDNA isolates. Deletions similar to those described above were found only in Patient 325. Patient 295, 288, 274, and 331 had out-of-frame deletions, and except for a clone from Patient 331 [LV331-(i)], sequence comparison between truncated and non-truncated HCV cDNA indicated sequence identity (3–8 nucleotides in length) in these out-of-frame deletions between the deletion donor and acceptor regions.

In Patient 207, Both Truncated and Non-Truncated HCV RNA Co-Existed for Years

E2 primers for PCR were designed to avoid PCR bias under competitive PCR conditions in order to confirm the presence of the non-truncated HCV genome in Patient 207 [Alvarez et al., 2000]. Two overlapping HCV cDNA sequences (LV0922 and LV2030) were isolated from a liver biopsy specimen from Patient 207 (Fig. 5A). The overlapping regions of the consensus sequences (LV0922cons and LV2030cons in Fig. 5B) were identical; however, the amino acid sequence identity to the truncated HCV genome (LV207cons: consensus sequence determined with isolates) was 92.1% in the E1 region, and 82.1% in the NS2 region (Fig. 5B).

Both the truncated and non-truncated genomes were detected by RT-PCR in serum from Patient 207 (Fig. 5A). The sequence of an isolate (S831) showed 99.4% nucleotide and amino acid sequence identity with LV207cons and the same in-frame deletions (Fig. 5A). The cDNA for the non-truncated genome (S2531) was also isolated from the serum by PCR with primers for E2 sequences. This cDNA was nearly identical (99.8% in nucleotide sequence) to that from the liver (LV2030cons), but differed from the truncated HCV genome identified in the liver (LV207cons) and serum (S831) (Fig. 5B).

From the RNA extracted from Patient 207 serum, a PCR fragment of the expected length was amplified using anti-sense primer for junction site at the deleted position between E1 and NS2 of the truncated genome of Patient 207. However, the PCR fragment was not amplified from the RNA of Donor G14 plasma, in which only the full-length HCV genome was detected. Similarly, the PCR fragment was amplified from truncated RNA transcripts, but not amplified from non-truncated RNA transcripts (Fig. 5C).

In order to examine the persistence of both HCV genomes, a series of serum specimens consisting of samples obtained from Patient 207 were examined. Only the truncated RNA was detected by RT-PCR with primers for the core and NS2 in all samples. The sequences of PCR fragment clones were conserved at both the nucleotide and amino acid level (92–99% identity, Fig. 6A). All clones had the same deletions as observed in biopsy samples (Fig. 6A) at the E1–NS2 junction. Novel quasispecies with amino acid deletions at 13 amino acids upstream of the junction were found in March 1998 (designated as 9803). Another quasispecies was identified in March 1999. HCV E2 cDNAs of the non-truncated genome were isolated by RT-PCR from all serum samples, and were found to have conserved sequences (Fig. 6B).

Significant Difference in the Ratio of Truncated to Non-Truncated Genome Between Serum and Liver

HCV RNA from Patient 207 was measured by real-time RT-PCR for the 5′-UTR and E2 sequences. The quantity of 5′-UTR is indicative of the entire HCV genome, while that of E2 is only indicative of the non-truncated genome. The ratio of HCV E2 RNA to 5′-UTR RNA in serum was nearly constant throughout the 15-months monitoring period (Table III). Significant differences in the ratio of truncated to non-truncated genome were observed between liver and serum RNA samples; the ratio of HCV 5′-UTR to E2 RNA in liver specimens was about a hundred times of those in serum.

Expression of Truncated HCV cDNA In Vitro

HCV proteins processed from subgenomic HCV cDNA were investigated in a transient cDNA expression experiment. Chimeric cDNA for the truncated genome, LV207cont, consisted of cDNA fragments selected from 4 cDNA isolates from Patient 207 and the 3′-end of NS5B to the 3′-UTR sequence isolated from another patient (Fig. 7A). For expression of full-length HCV cDNA, chimeric cDNAs having the core–NS2 sequence of LV207cont instead of the corresponding region of J1 HCV cDNA (J1NLV) were used.

Figure 7B shows the Western blots of lysates from cells transfected with cDNA expression vectors. Anti-core monoclonal antibody detected a 19-kDa protein, thus indicating that the truncated polyprotein was correctly processed at the core-E1 junction. Each 35-kDa protein expressed from HCV cDNA, which was reactive to anti-E1 monoclonal antibody, was susceptible to endoglycosidase (Endo H), and converted to different molecular masses by enzyme treatment. The migration of deglycosylated E1 reactive peptide from the truncated HCV cDNA corresponded to the predicted molecular mass of the E1–NS2 fusion protein, 24 kDa, which differed from that of the non truncated E1 (19 kDa). The

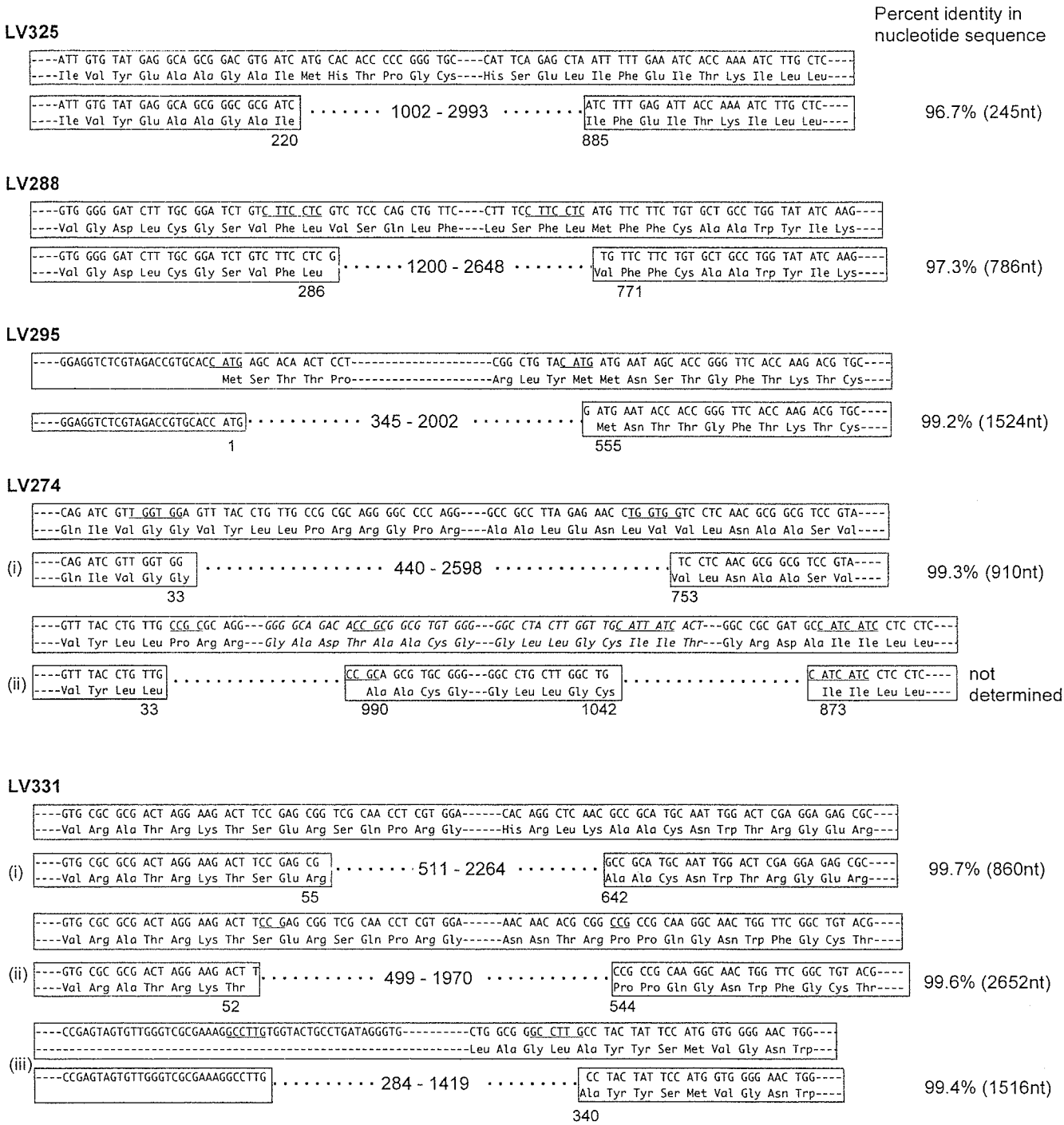
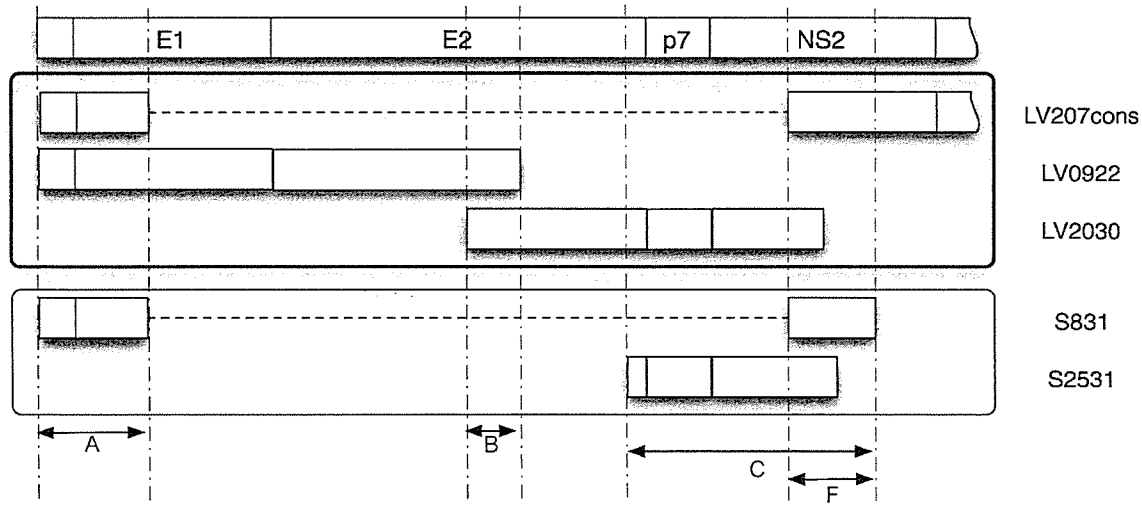


Fig. 4. Sequence comparisons between HCV genomes with and without deletions isolated from the same patient. Partial sequences of RT-PCR fragments isolated from liver biopsies are shown. cDNA fragments are labeled as described for previous figures. Nucleotide and amino acid positions are represented as the corresponding positions in HCV-J1. In these cases, both non-truncated and truncated HCV RNA were amplified from each biopsy specimen (Table I). The non-truncated and truncated RT-PCR fragments from Patient 325, 288, 295, 274, and 331 were obtained with primer sets d and d, d and d, b and b, b and e, and a and b (Table II), respectively. Numbers between tagged graphs represent deleted regions as they correspond to HCV-J1. Bottom lines show the corresponding amino acid positions of the boundaries. The right side of the sequences shows the identities of overlapping nucleotide sequences in the truncated and non-truncated genome, and the length of the overlap is given in parentheses. The LV274-(ii) had a translocation of the NS3 sequence inserted between the core and NS2 sequences. Because we did not clone the NS3 sequence from this case, the corresponding region of HCV-J1 is italicized in the figure and the sequence identity was not determined. Sequences that coincided between the boundaries of the deletions are underlined.

A



B

			Serum cDNA		
			S831		S2531
Liver cDNA	LV207cons	Region	A	F	F
		Nucleotide	99.7% (315)	98.9% (180)	91.8% (98)
		Amino acid	100% (105)	98.3% (60)	81.8% (33)
	LV0922cons	Region	A		
		Nucleotide	95.0% (303)		
		Amino acid	92.1% (101)		
	LV2030cons	Region		F	C
		Nucleotide		90.2% (82)	99.8% (513)
		Amino acid		81.4% (27)	99.4% (171)

			Liver cDNA	
			LV0922cons	LV2030cons
Liver cDNA	LV207cons	Region	A	F
		Nucleotide	95.0% (303)	91.6% (98)
		Amino acid	92.1% (101)	82.1% (28)
	LV2030cons	Region	B	
		Nucleotide	100% (76)	–
		Amino acid	100% (25)	

Number in parentheses expresses
The length of the overlapped sequences

C

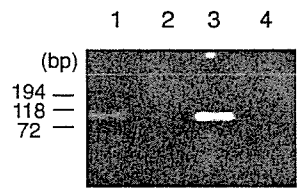


Fig. 5. Cloning of the non-truncated HCV RNA from patient 207. **A**: Schematic views of RT-PCR fragments for non-truncated HCV RNA in liver (LV0922 and LV2030) and in serum (S2531) from Patient 207, and for the truncated sequence in serum (S831). LV0922 fragment was obtained by RT-PCR with HC1b9405R for cDNA synthesis, HC841S and HC2199AS for 1st PCR, and HC948S and HC2199AS for 2nd PCR. LV2030 fragment was obtained by RT-PCR with XR58R for cDNA synthesis, HC2048SLV and LVC1392AS for 1st PCR, and HC2069S and LVC1280AS for 2nd PCR. S2531 fragment was obtained by RT-PCR with HC3174AS for cDNA synthesis, HC2430S and HC3174AS for 1st PCR, and HC2546S and HC3111AS for 2nd PCR. Arrows indicate regions being compared in the Tables (**B**), which show sequence identities between the non-truncated and truncated sequences in serum (**upper**), and those between sequences isolated from liver and liver (**lower**). **C**: Images of agarose gel electrophoresis of RT-PCR products amplified using junction site primer, from extracted RNA from Patient 207 serum (**lane 1**) and Donor G14 plasma (**lane 2**), truncated in vitro RNA transcripts of Patient 207 (**lane 3**) and non-truncated in vitro RNA transcripts of Donor G14 (**lane 4**).

protein at the 70-kDa position, which was the predicted mass of NS3, was detected by anti-NS3 polyclonal antibody. These data suggested that processing of the truncated HCV polyprotein is same as that of the full-length HCV polyprotein at the core–E1 and NS2–NS3 junctions.

DISCUSSION

Characteristics of HCV Subgenome With In-Frame Deletion

Novel truncated HCV genomes with in-frame deletions from E1 to NS2 were identified in the livers of two

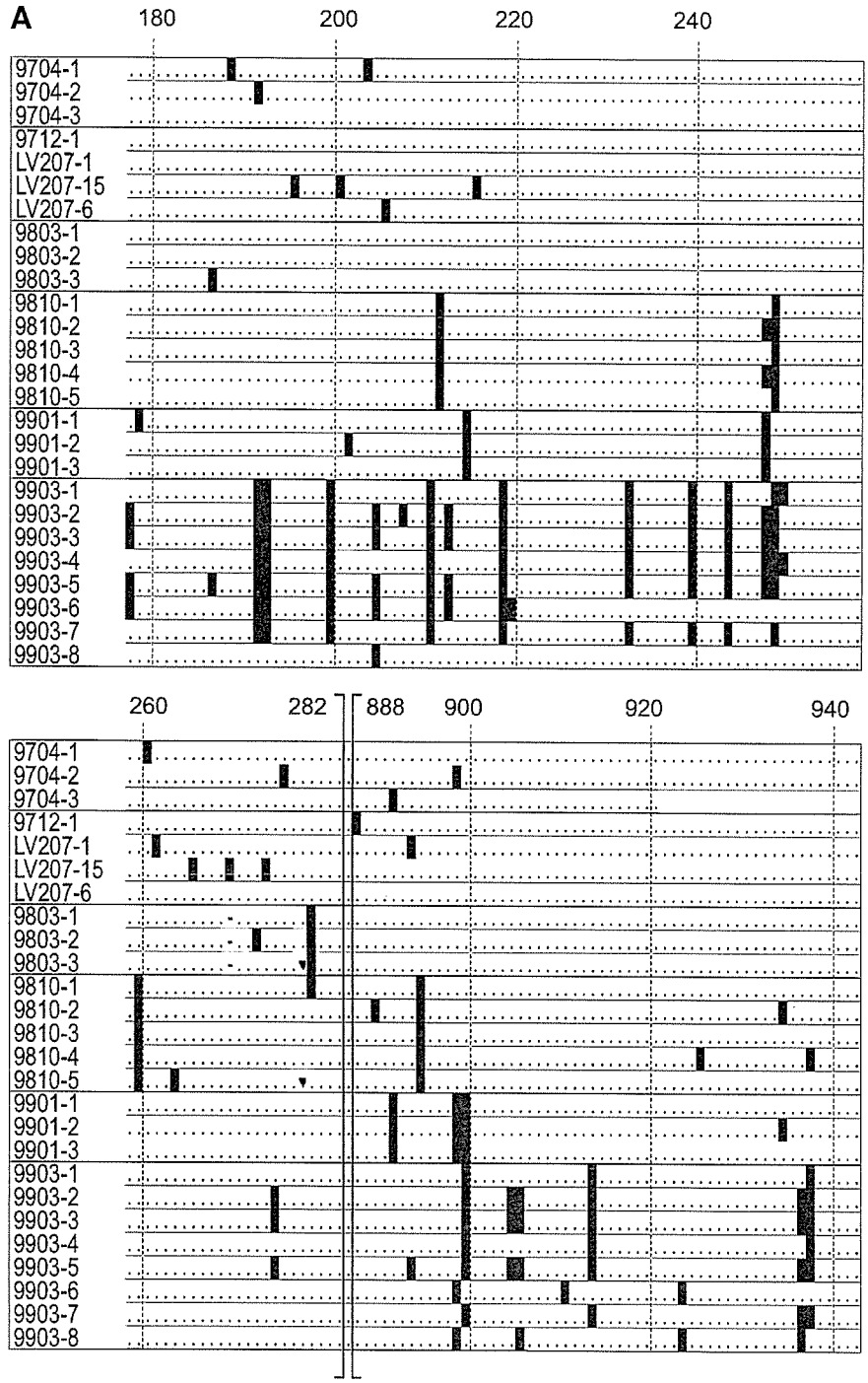


Fig. 6. Alignment of amino acid sequences of cDNA isolates from a series of serum samples from Patient 207. Comparison of nucleotide sequences of truncated genome (**A**) and non-truncated genome (**B**) was carried out using Clustal W algorithm. Numbers on top of the alignment show the corresponding amino acid positions of HCV J1. Names of the sequences indicate the date the serum was taken; 9712, for example, refers to December 1997. Bold bar represents amino acid position that differs from consensus sequence. Coinciding amino acid positions are represented by dots. All cDNA isolates from March 1998 (9803) had an amino acid deletion at the position marked by the horizontal bar. Bold vertical bars in A show the boundaries of the deletion. Inverted triangles indicate positions of nucleotide deletions in cDNA isolates. Deduced amino acid sequences with nucleotide deletions were obtained by inserting a nucleotide at the position. Fragments of the truncated genome (A) were obtained by RT-PCR with HC3297R for cDNA synthesis, HC813S and HC3297R for 1st PCR, and HC841S and HC3174AS for 2nd PCR. Fragments of the non-truncated genome (B) were obtained by RT-PCR with HC2378R for cDNA synthesis, HC1979S and HC2378R for 1st PCR, and HC1979S and HC2300R for 2nd PCR.

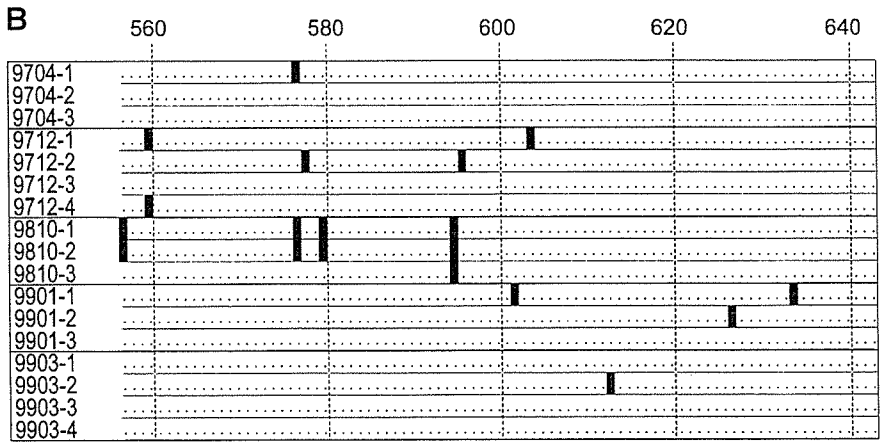


Fig. 6. (*Continued*)

chronic active hepatitis C patients. These HCV subgenomes encoded a single polyprotein for the entire core, five NS proteins (from NS3 to NS5B), and E1–NS2 fusion protein. This type of HCV subgenome was found in 4 of 23 chronic active hepatitis patients (16 cases with positive results by RT-PCR with primer sets tested), and in 2 hepatocellular carcinoma patients. These data suggested that HCV subgenomic RNA is generated in a certain number of chronic active hepatitis C patients.

HCV polypeptide expressed in cells with the subgenomic cDNA was processed in the same way as the authentic HCV polyprotein. The E1–NS2 fusion protein from subgenomic HCV cDNA was glycosylated and was susceptible to Endo H treatment, thus suggesting that it was located in the ER. NS2 is a membrane protein located in the ER region [Santolini et al., 1995; Kim et al., 1999]. On the other hand, localization and modification of E1 protein was governed by E2 [Cocquerel et al., 1998; Duvet et al., 1998]. These data indicate that ER retention in the E1–NS2 fusion protein is determined by NS2.

TABLE III. Quantitation of HCV RNA in a Series of Serum Samples Taken From Patient 207

Date	5′-UTR (copies/ml)	Percentage of E2/UTR
December 8, 1997	7.75×10^4	19.5%
	(5.75×10^3	0.2%)
	Numbers in parenthesis represents HCV quantity in liver biopsy specimen	
March 6, 1998	2.93×10^3	36.2%
March 9, 1998	1.19×10^4	22.1%
	IFN treatment From March 9, 1998 to September 2, 1998	
September 29, 1998	4.17×10^4	23.8%
October 28, 1998	5.25×10^4	21.8%
December 8, 1998	2.24×10^4	26.1%
January 12, 1999	5.81×10^4	28.5%
February 10, 1999	2.57×10^4	20.3%
March 31, 1999	5.30×10^4	23.1%

Do HCV Subgenomes Replicate Autonomously in Patients?

Viral subgenomes have been isolated from viruses closely related to HCV, such as flavivirus (Murray Valley encephalitis virus) [Lancaster et al., 1998], pestivirus (classical swine fever virus; SFV [Aoki et al., 2001], and bovine viral diarrhea virus; BVDV [Tautz et al., 1994; Kupfermann et al., 1996]. The HCV subgenomes shared common structural features with these subgenomic RNAs.

The HCV subgenomes fulfilled the minimal requirements for autonomous RNA replication; the 5′-UTR, nonstructural proteins (NS3–NS5B), and the 3′-UTR, as demonstrated using artificial HCV subgenomic replicons [Lohmann et al., 1999; Blight et al., 2000]. In addition, defective genomes of DI autonomously replicate their RNA [Behrens et al., 1998]. Furthermore, sequence comparisons of the truncated and non-truncated HCV genome sequences, which were isolated from a serum series obtained from a single patient, suggested that both genomes have been replicating independently for years. These data suggest that HCV subgenomes with in-frame deletions in structural proteins replicate themselves. However, it is possible that the full-length genome is required for the replication of the subgenome, as both genomes were present for years despite the dominance of the subgenome.

The dominance of the subgenome over the full-length genome (approximately 500-fold in the liver) was indicated by real-time RT-PCR analysis for the HCV 5′-UTR and E2. The dominance of HCV subgenome suggests an advantage in RNA replication. The length of the genome is probably a key factor in viral replication. If processing velocities in translation and transcription are equal over the HCV genome, the HCV subgenome would be replicated about 20% faster than the non-truncated genome. However, other mechanisms affecting efficiency are likely present.

Heterogeneous molecular clones with out-of-frame deletions, which shared sequences with the full-length

genome, indicated that the HCV subgenome frequently arises from its full-length genome by such mechanisms found in other RNA viruses [Nagy and Simon, 1997]. However, the fact only one type of subgenome with an in-frame deletion persisted suggested that a competent subgenome for replication is selected. The NS2 in all HCV subgenomes, which preserved their NS2–NS3 protease domains [Grakoui et al., 1993a; Hijikata et al., 1993], indicated protease activity is involved in the persistence of the HCV subgenome. Furthermore, we believe that core protein is required for virus replication in vivo, because the core sequence in the HCV subgenomes was preserved among the dominant HCV subgenomes.

Comparison With HCV Subgenomes or Recombinants Described Previously

Quadri and Negro [2001] identified recently a positive-strand subgenomic RNA starting from the 5′-UTR without the 3′-UTR, and a negative-strand subgenomic RNA with the 3′-UTR lacking the 5′-UTR. Although we did not clone the 3′-UTR from the Patient 207 sample, an HCV subgenome with same deletion was isolated from cDNA with a primer corresponding to the X-region in the 3′-UTR(data not shown), and we isolated the 3′-UTR from Patient 373 liver RNA. Based on these observations, it is considered that the subgenomic HCV RNA contains the entire 3′-UTR, rather than their proposed RNA populations.

Intergenotypic recombination has been described between genotype 2k and genotype 1b HCV at between nucleotides 3175 and 3176, about 200 nucleotides from the recombination region of the HCV subgenomes [Kalinina et al., 2002]. We did not examine the possibility of this type of recombination because the number of HCV cDNAs covering this region was too few in the present study.

HCV Subgenome and Pathogenesis

The question whether the HCV subgenome involved in the mechanism of viral persistence and pathogenesis in a similar manner as the DI particles of other viruses

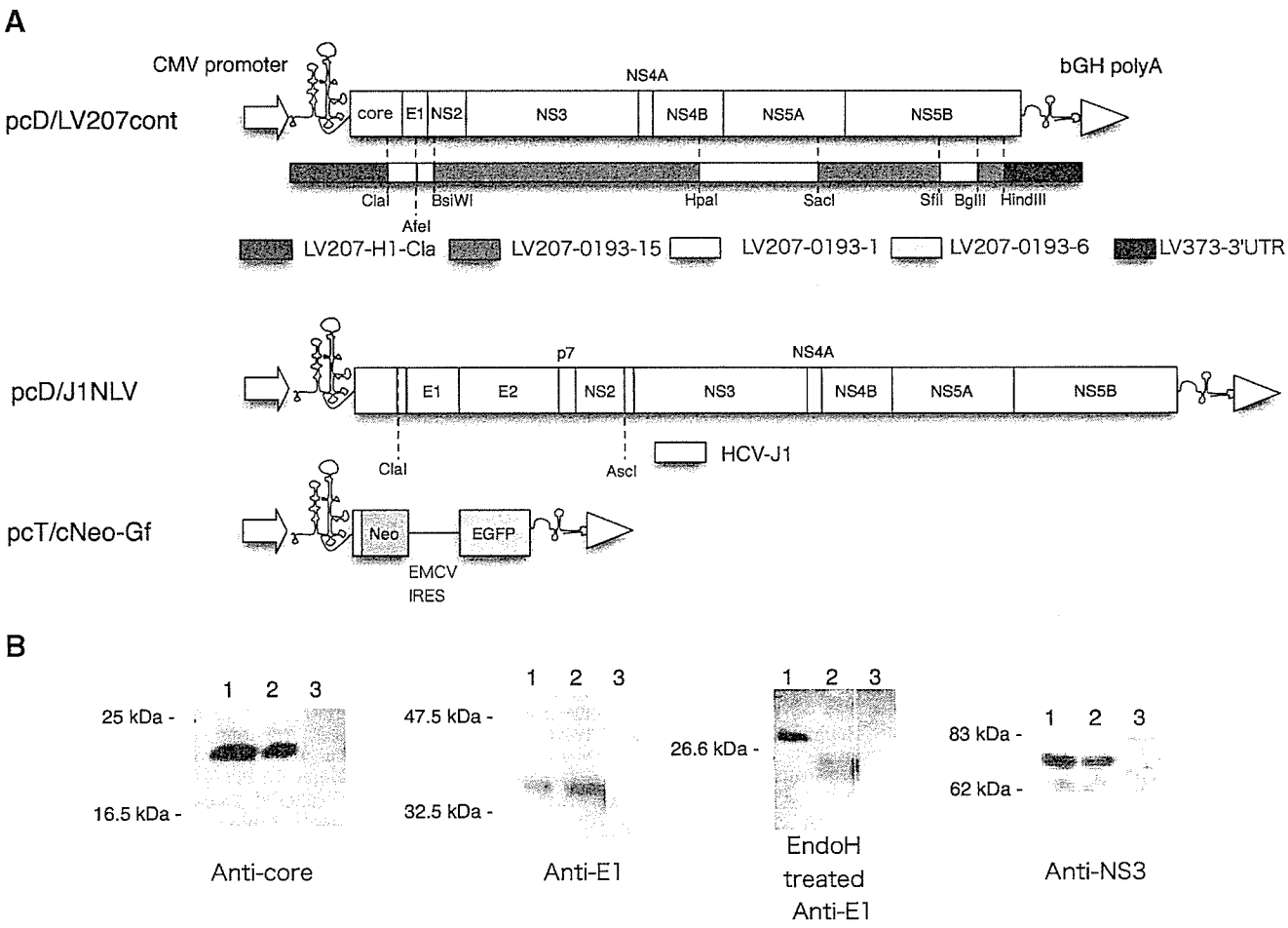


Fig. 7. Expression of HCV proteins from truncated and non-truncated HCV cDNA in mammalian cells. **A**: Chimeric HCV subgenomic cDNA, LV207cont, was composed of fragments from four cDNAs from patient liver (LV207-H1-Cla, 0193-15, 0193-1, and 0193-6) and a fragment from Patient 373 (LV373-3′-UTR) by using restriction sites depicted. Other chimeric cDNAs consisted of LV207cont and a fragment of HCV-J1 (J1NLV), as indicated. Dicistronic DNA constructs of HCV core-Neo resistant fusion and EGFP genes, cNeo-EGFP, was used as a negative control for HCV protein expression. All DNA constructs were transiently expressed in HEK293 cells under the control of the CMV promoter in pcDNA3.1. **B**: Western blotting analysis results for core, E1 and NS3 in transfected cells are shown. The positions of pre-stained molecular weight markers are indicated on the left side of the images.

[Tautz et al., 1994; Kupfermann et al., 1996] is remained. The amounts of core protein in patients with the HCV subgenome were larger than in patients without the subgenome (not statically significant). Transgenic mice expressing core protein in liver developed steatosis and later cancer, indicating that the core protein is a potent carcinogen in mice [Moriya et al., 1997, 1998]. It was found that two HCC patients had this subgenome. These data suggested that the involvement of the truncated genome in pathogenesis; however, we must examine more cases in order to elucidate any correlations between HCV subgenome and disease, particularly for progression of the disease to HCC.

The heterogeneous nature of the HCV genomes in patients may contribute to the persistence of HCV in escaping the host defense system. Particularly, the deletion of E1/E2 proteins may have a great impact on host immune response to the virus; E1/E2 is believed to be a target molecule for neutralizing antibodies, which block the binding of virions to virus receptor [Beyene et al., 2002]. The function of the truncated HCV genome in the life cycle of HCV is uncertain, but we believe the presence of this subgenomic RNA in both the liver and serum is important for illustrating that much about the nature of HCV remains unknown.

Added in Proof

Wakita et al. recently reported that a man-made HCV subgenomic RNA lacking E1/E2 replicated in vitro. [Wakita et al., 2005, NatureMedicine, published online 12 June]

ACKNOWLEDGMENTS

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Age-specific antibody to hepatitis E virus has remained constant during the past 20 years in Japan

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SUMMARY. We investigated the presence of antibodies to hepatitis E virus (anti-HEV) and hepatitis A virus (anti-HAV) by enzyme immunoassays in sera from 1015 individuals collected in 1974, 1984 and 1994. Age-specific profiles of anti-HEV remained unchanged with a peak at 40–49 years, while those of anti-HAV started to increase in individuals aged 20–29 years in 1974, 30–39 years in 1984 and 40–49 years in 1994. These results suggest that a silent HEV infection has been taking place in the last 20 years or so in Japan, while HAV infection has been terminated at least since 1974.

Keywords: hepatitis A virus, hepatitis A virus antibody, hepatitis E virus, hepatitis E virus antibody, seroepidemiology.

INTRODUCTION

Hepatitis E virus (HEV) is transmitted mainly by the faecal–oral route, and causes waterborne outbreaks and sporadic cases of acute hepatitis in developing countries with poor sanitary conditions [1]. Outbreaks of HEV have been primarily noted in developing countries, whereas cases in developed countries were considered to have been exposed in foreign countries. However, cases of acute hepatitis because of indigenous HEV strains were reported in patients in the United States, Europe and Japan who had never travelled abroad [2–4]. Recently, HEV strains have been isolated from pigs in developed countries, which are closely related to local human HEV strains, suggesting zoonotic infection [5–7]. Because of these lines of evidence, HEV has attracted increasing attention even in developed countries where HEV is not endemic. For example, in Japan, more than 20% of acute, sporadic hepatitis cases are non-A, non-B, non-C [8].

The impact of HEV infection in developed countries, however, has not been fully explored. Past exposure to HEV can be examined by enzyme-linked immunosorbent assay (ELISA) by detecting antibody to HEV (anti-HEV). In the present study, we surveyed the extent and changes of HEV infection in Japan by testing sex- and age-specific prevalence of anti-HEV in serum samples collected in 1974, 1984 and 1994. Using the same samples, we also tested for antibodies to hepatitis A virus (anti-HAV) for comparison.

Abbreviations: ELISA, enzyme-linked immunosorbent assay; HAV, hepatitis A virus; HEV, hepatitis E virus; VLP, virus-like particles.

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MATERIALS AND METHODS

Serum samples

A total of 1015 samples were selected at random from the Serum Reference Bank of the National Institute of Infectious Diseases, Tokyo. They were obtained from healthy volunteers aged from 0 to 89 years (median 35.6 years) living in seven prefectures in the central part of Japan. Of them, 349 were collected in 1974, 324 in 1984 and 342 in 1994. The present study, was reviewed by the ethical committee of the National Institute of Infectious Diseases.

Hepatitis viral markers

Anti-HAV (total antibody) was determined by radioimmunoassay using a commercial kit (HAV-AB RIA kit; Dainabot Co., Ltd, Tokyo, Japan). Positive and negative results were judged according to the manufacturer's instructions with intermediate results recorded as negative. Anti-HEV was determined by ELISA using the method of Li *et al.* [9]. Briefly, wells of microtitre plates were coated with purified virus-like particles (VLP) of HEV expressed by a recombinant baculovirus. One hundred microlitres of test serum was then added to each well and the plate was incubated at 37 °C for 1 h. The plate was washed six times with 10 mM phosphate

buffered saline containing 0.05% Tween 20, and then 100 μL of buffer containing goat anti-human IgG conjugated with horseradish peroxidase was delivered to each well. The plate was incubated for a further 1-h period at 37 °C, washed six times, and thereafter, each well received 100 μL of buffer containing orthophenylenediamine. The plate was incubated at room temperature for 30 min, and then 50 μL of 4 N H_2SO_4 are added to each well. The absorbance at 492 nm was recorded and positive and negative results were scored as described by Li *et al.* [9].

Statistical analyses

Statistical analyses were performed using the chi-square test, and $P < 0.05$ was considered significant.

RESULTS

Age-specific prevalence of anti-HEV and anti-HAV

Basic patterns of age-specific prevalence of anti-HEV were similar in the three examination years (Fig. 1). The prevalence of anti-HEV increased gradually until 40–49 years old, and then decreased slowly with age. In contrast, the prevalence of anti-HAV was almost nil in people younger than 20 years, increased steeply at a certain age range, and then reached 80–90% in older people in all of the three years of examination. The age range at which the prevalence of anti-HAV started to increase sharply was 20–29 years in 1974, 30–39 years in 1984 and 40–49 years in 1994. Thus, it shifted by 10 years at each examination year. Anti-HAV was significantly more prevalent than anti-HEV in all age ranges over 30 years in 1974 ($P < 0.001$ in all). Similarly, anti-HAV was significantly more prevalent in all age ranges over 40 years in 1984 ($P < 0.001$ in all), and in all age ranges over 50 years in 1994 ($P < 0.001$ in all).

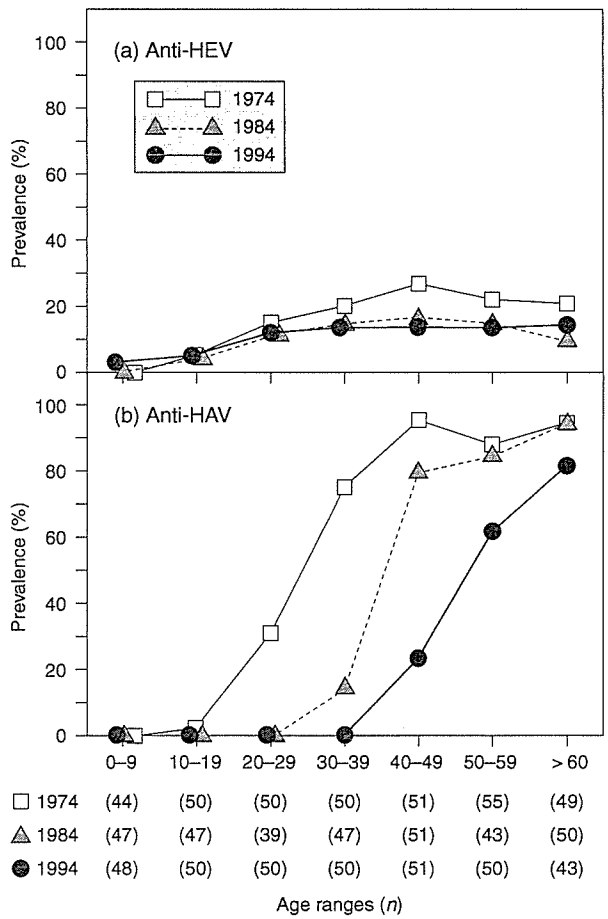

Fig. 1 Age-specific prevalence of anti-HEV and anti-HAV in Japan at three different times. Number of individuals tested in each age group and year is indicated below in parentheses.

Sex- and age-specific prevalence of anti-HEV

Figure 2 illustrates the prevalence of anti-HEV in serum samples from different age groups of healthy Japanese volunteers collected in 1974, 1984 and 1994 stratified by

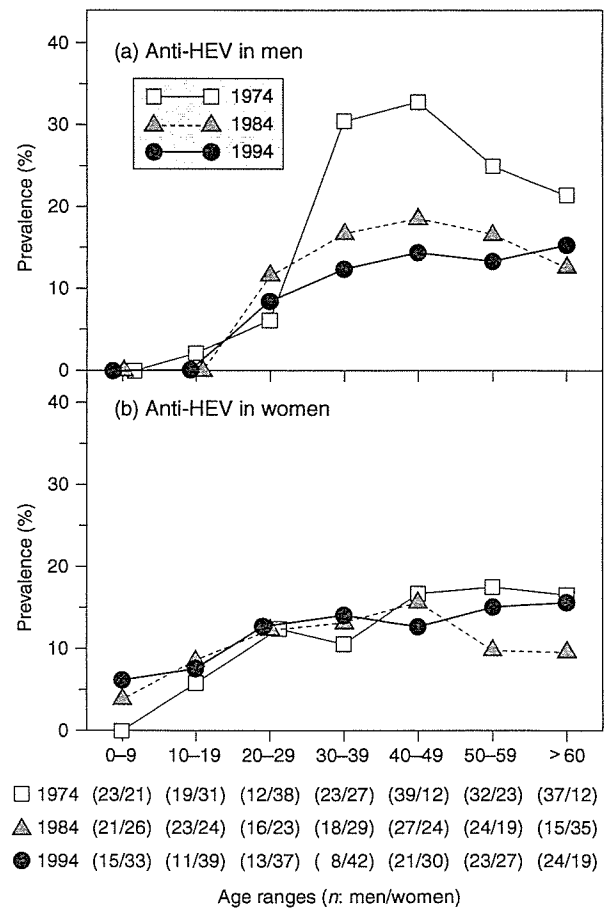

Fig. 2 Sex and age-specific prevalence of anti-HEV in Japan at three different times. Numbers of men/women tested in each age group and year are indicated below in parentheses.

sex and age. Although basic profiles of the prevalence of anti-HEV did not differ between men and women, anti-HEV in men was significantly more frequent in 1974 (21.6%) than in 1984 (11.1%, $P = 0.012$) and 1994 (10.4%, $P = 0.013$); the difference is attributed to a high frequency of anti-HEV in age groups older than 30 years in 1974. In sharp contrast, the age-specific prevalence of anti-HEV in women stayed unchanged in the three different years examined (11.0% in 1974, 10.6% in 1984 and 11.9% in 1994). Differences in the prevalence of anti-HEV between men and women were significant only in the year 1974 ($P = 0.008$). Age-specific prevalence of anti-HAV was quite similar between males and females in each year of examination (data not shown).

DISCUSSION

Many immunological methods have been developed for the determination of anti-HEV utilizing natural and recombinant viral proteins as antigens. They are, however, disappointingly nonspecific and have been shown to yield discrepant results using the same panel of sera with or without anti-HEV [10]. Lack of reliable serological assays for the detection of anti-HEV has hampered the accurate examination of exposure to HEV in various epidemiological and clinical settings.

Recently, Li *et al.* [11] succeeded in developing VLP using a recombinant baculovirus containing the coding sequences for capsid protein of the virus. The VLP appear to have similar antigenicity to the authentic HEV particles [12]. Using these VLP, a novel ELISA for anti-HEV has been developed that is sensitive and specific in seroepidemiological surveys for HEV infection. A considerable proportion of Japanese adults (around 10–20%) appear to have had previous exposure to HEV, although Japan is not endemic for hepatitis E [9,13].

The clinical features of HAV infection are similar to those of HEV infection, in that they both are transmitted by the faecal–oral route and cause acute hepatitis without chronic sequelae. In the present study, serological markers of HAV and HEV infection were determined and compared among healthy Japanese volunteers at three different time points (1974, 1984 and 1994). Age-specific prevalence of anti-HAV increased steeply and reached 90% at a certain age range dependent on the year of examination. The age range at which anti-HAV increased shifted by 10 years in subsequent time points, indicating that HAV infection was endemic several decades ago in Japan and has been contained thereafter. This would be mainly because of an improvement of sanitary conditions in Japan since the 1950s.

Age-specific profiles distinct between anti-HAV and anti-HEV during the last 20 years in Japan would be not only of epidemiological but also of clinical relevance. The prevalence of anti-HAV increasing with age involving by far the most aged individuals signifies a life-long immunoprotection against HAV. By sharp contrast, the prevalence of anti-HEV did not increase linearly with age, and peaked in individuals aged 40–49 years. Furthermore, unlike anti-HAV that has become increasingly absent in younger age groups, anti-HEV was detected in younger individuals aged 20–29 years in both men and women, and among women aged <20 years, in the three examination years. Similar age-specific profiles of anti-HEV have been reported in India [14]. Prevalence of anti-HEV in Japanese individuals younger than 30 years old was somewhat higher in the present study, than the almost zero prevalence reported by Li *et al.* [9]. Although there was some difference in percentages, the basic pattern of age-specific prevalence of anti-HEV was almost similar between the two studies.

Based on the age-specific distribution of anti-HEV, the exposure to HEV has not decreased during the past 20 years in Japan, unlike that to HAV. This implies that the principal transmission route of HEV may be different from that of HAV and would not be prevented only by improved sanitary conditions, despite the lower infectivity and transmissibility of HEV than HAV [14]. Zoonotic transmission of HEV through domestic and wild animals may account for the perpetuation of HEV infection, and deserves to be examined in future studies. Recently, transmission of HEV from pigs and deer to human beings has been reported in Japan [15,16].

In conclusion, exposure to HEV has stayed unchanged during the last 20 years in Japan, contrasting with HAV, which has diminished over the same period. These results warrant closer attention to infection with HEV, especially because it can induce fulminant hepatitis not only in pregnant women in developing countries [14], but also in sporadic cases in developed countries [15].

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The efficacy and safety of thymosin alpha-1 in Japanese patients with chronic hepatitis B; results from a randomized clinical trial

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SUMMARY. Thymalfasin (thymosin alpha-1; Tα1) is a 28-amino acid polypeptide that has shown efficacy in the treatment of chronic hepatitis B virus (HBV) infection. The objective of this study was to evaluate the long-term, dose-related efficacy and safety of Tα1 treatment in chronic hepatitis B patients with positive HBV-DNA and abnormally high alanine aminotransferase (ALT) levels. A total of 316 patients were randomized to receive either 0.8 or 1.6 mg of Tα1 monotherapy for 24 weeks. At the end of the 72-week observation period (12 months after cessation of therapy), 36.4% of patients in the 1.6-mg treatment group achieved normalization of ALT, 30% achieved clearance of HBV-DNA by branched DNA *vs* 15% by transcription-mediated amplification, and 22.8% achieved clearance of HBe-antigen. Patients in the 0.8-mg treatment group achieved similar efficacy rates, although patients with advanced fibrosis demonstrated a significantly better response rate when treated with 1.6 mg of Tα1 monotherapy *vs* 0.8 mg (as determined by intragroup analysis; patients were not stratified by liver biopsy). All adverse drug reactions were mild and most involved the fluctuation of liver enzymes, which was most likely related to the positive immune effects caused by the response to Tα1 treatment. Adverse event incidence was similar in the 1.6- and 0.8-mg treatment groups. In conclusion, Tα1 at doses of 0.8 and 1.6 mg exhibits long-term efficacy against hepatitis B with a good safety profile.

Keywords: chronic hepatitis B, thymalfasin, thymosin alpha-1.

INTRODUCTION

Chronic hepatitis B affects nearly 350 million people worldwide and is a leading cause of liver cirrhosis and hepatocellular carcinoma [1–3]. Early and effective intervention may help terminate hepatitis B virus (HBV) replication and promote long-term disease remission.

Over the last three decades, research has focused on the development of antiviral and immunomodulatory therapies to treat patients with HBV. Currently, interferon alpha and lamivudine are two widely used therapies. Interferon alpha has reasonably good efficacy with initial response rates of 30–40% compared with 10–20% among untreated controls. However, of those who responded to interferon alpha therapy, 56% relapsed within the first year after discontinuation of therapy (median 3.1 months) [4]. In addition, interferon alpha has a poor side-effect profile, leading to inadequate compliance and frequent need for dose reduction [3–5]. Once-daily lamivudine rapidly produces a suppression of HBV-DNA replication [6,7]. However, approximately 90% of

Abbreviations: ALT, alanine aminotransferase; anti-HBe, hepatitis B e-antibody; bDNA, branched DNA; HBeAg, hepatitis B e-antigen; HBV, hepatitis B virus; MHC, major histocompatibility complex; NK, natural killer; Tα1, thymosin alpha-1; TMA, transcription-mediated amplification.

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patients relapse once therapy is stopped [8]. Adefovir dipivoxil, a nucleotide analogue, is also capable of rapidly inducing suppression of HBV-DNA, but long-term efficacy is in question because of low rates of hepatitis B e-antigen (HBeAg) seroconversion [9]. Moreover, adefovir-resistant mutations have also been reported [10]. Therefore, the development of new therapeutic agents with long-term efficacy is needed to reduce morbidity and mortality rates among patients with chronic hepatitis B.

Thymalfasin (thymosin alpha-1, Tα1) is an immune-modulating peptide that has been shown to enhance Th1 cytokine production as well as T-cell differentiation and maturation [11]. Several clinical studies have shown that treatment with Tα1 monotherapy results in significantly higher sustained response rates when compared with controls [12–18] and exhibits no significant side effects [14–19]. Moreover, complete virological response tends to increase or accumulate gradually after the cessation of Tα1 therapy [14,17].

Tα1 therapy is used in many countries worldwide for the treatment of chronic hepatitis B. This study evaluates the dose-related efficacy and safety of Tα1 in Japanese chronic HBV patients.

METHODS

This 72-week multicentre, randomized study investigated the safety and efficacy of Tα1 at two different doses. A total of 316 Japanese patients with chronic hepatitis B from 49 medical institutions in Japan were randomized to receive either 0.8 or 1.6 mg of Tα1 monotherapy six times a week for the first 2 weeks, and then twice a week for the subsequent 22 weeks. Efficacy was determined by clinical test values of alanine aminotransferase (ALT), HBV-DNA, HBeAg and hepatitis B e-antibody (anti-HBe) during 24 weeks of Tα1 administration and during the 48-week follow-up period. For the determination of HBV-DNA level by branched DNA (bDNA), a Quantiplex HBV-DNA kit was used (standard value: <0.70 Meq/mL; Daiichi Pure Chemicals Co, Ltd, Tokyo, Japan). During the course of the study, the more sensitive transcription-mediated amplification (TMA) assay became available for the determination of HBV-DNA level. Thus, HBV-DNA level was also tested by TMA using an HBV Amplify Standard & Luminescent reagent kit DNA probe (standard value: <3.7 LGE/mL; Chugai Diagnostic Science Co, Ltd, Tokyo, Japan). SAS™ (SAS Institute Inc., Cary, NC, USA) software was used for statistical analyses. Changes in HBeAg and anti-HBe levels were assessed by the chi-squared test; changes in the ALT and HBV-DNA levels were assessed by Mann–Whitney *U*-test. The two-tailed significance level was set at 5%, and multiplicity was not considered. This study was conducted in compliance with current GCP and with the Declaration of Helsinki, and was approved by the institutions' Ethics Committees.

Eligible patients included men and women ≥18 years of age who were HBV-DNA positive, HBeAg positive with elevated ALT, and with histologically diagnosed chronic hepatitis confirmed by liver biopsy taken within 48 weeks before the start of treatment. The concomitant use of glycyrrhizin, propagermanium, systemic glucocorticoids, interferon or lamivudine was prohibited.

RESULTS

Analysis of safety was performed on 310 patients, and analysis of efficacy was performed with results from 284 patients, excluding those with protocol violations.

As shown in Table 1, patient groups were similar in all respects except with regard to the degree of liver disease on entry. Due to a lack of stratification based on liver histology, the 1.6-mg treatment group had a higher ratio of advanced fibrosis (bridging fibrosis with lobular distortion, stage F3; $P = 0.018$) and inflammation (severe necro-inflammatory

Table 1 Baseline characteristics of the patients

	Group 1 (0.8 mg) (%)	Group 2 (1.6 mg) (%)	*P*-value
Age (years)			
Mean ± SD	36.6 ± 9.9	37.3 ± 10.6	0.545
n	139	144	
Gender			
Male	95 (68.3)	109 (75.7)	
Female	44 (31.7)	35 (24.3)	0.168
New Inuyama classification (fibrosis staging)			
F0	5 (3.6)	3 (2.1)	
F1	61 (43.9)	54 (37.5)	
F2	44 (31.7)	36 (25.0)	
F3	22 (15.8)	46 (31.9)	0.018
Unknown	7 (5.0)	5 (3.5)	
New Inuyama classification (activity grading)			
A0	3 (2.2)	4 (2.8)	
A1	63 (45.3)	39 (27.1)	
A2	50 (36.0)	73 (50.7)	
A3	14 (10.1)	21 (14.6)	0.010
Unknown	9 (6.5)	7 (4.9)	
History of IFN therapy			
No	92 (66.2)	80 (55.6)	
Yes	47 (33.8)	64 (44.4)	0.067
ALT level (IU/L)			
Mean ± SD	124.6 ± 129.50	144.5 ± 143.20	0.148
HBV-DNA level by TMA (LGE/mL)			
Mean ± SD	6.96 ± 1.28	6.90 ± 1.20	0.499
HBV-DNA level by bDNA (mEq/mL)			
Mean ± SD	578.7 ± 1038.00	662.6 ± 1132.00	0.792

Table 2 Response to thymosin alpha-1 therapy

	Group/Dose	24 Weeks (end of therapy) *n* (%)	72 Weeks (end of follow-up) *n* (%)
ALT	Group 1/0.8 mg	33/134 (24.6)	38/118 (32.2)
	Group 2/1.6 mg	37/137 (27)	43/118 (36.4)
HBV-DNA (−) (bDNA)	Group 1/0.8 mg	20/115 (17.4)	24/93 (25.8)
	Group 2/1.6 mg	20/117 (17.1)	27/90 (30)
HBV-DNA (−) (TMA)	Group 1/0.8 mg	8/129 (6.2)	14/104 (13.5)
	Group 2/1.6 mg	7/129 (5.4)	15/100 (15)
HBeAg (−) (seronegative)	Group 1/0.8 mg	4/103 (3.9)	18/80 (22.5)
	Group 2/1.6 mg	5/104 (4.8)	18/79 (22.8)
HBe (−) and Anti-HBe (+) (seroconversion)	Group 1/0.8 mg	4/103 (3.9)	15/80 (18.8)
	Group 2/1.6 mg	5/104 (4.8)	17/79 (21.5)

reaction, grade A3; $P = 0.01$) patients according to the New Inuyama classification for histopathological scoring of the liver [20].

Tα1 monotherapy exhibited equal efficacy when administered at either 0.8 or 1.6 mg, as shown in Table 2. The results in the 0.8-mg group and the 1.6-mg group, respectively, at 72 weeks showed that the rate of normalization of ALT was 32 and 36% ($P > 0.05$); clearance of HBV-DNA by the bDNA test was 26 and 30% ($P > 0.05$), and by TMA 14 and 15% ($P > 0.05$); clearance of HBeAg was 23 and 23% ($P > 0.05$); and the appearance of anti-HBe at 72 weeks was 19 and 22% ($P > 0.05$). At 72 weeks from baseline, both the 0.8- and 1.6-mg treatment groups showed significant improvement in ALT, HBV-DNA and anti-HBe levels, as shown in Fig. 1.

Evaluation of within-group progress demonstrated that patients with advanced fibrosis (stage F3) did show significant improvements in all HBV markers at 24 weeks when treated with 1.6 mg of Tα1 monotherapy *vs* 0.8 mg (Fig. 2). For these patients, changes in baseline ALT

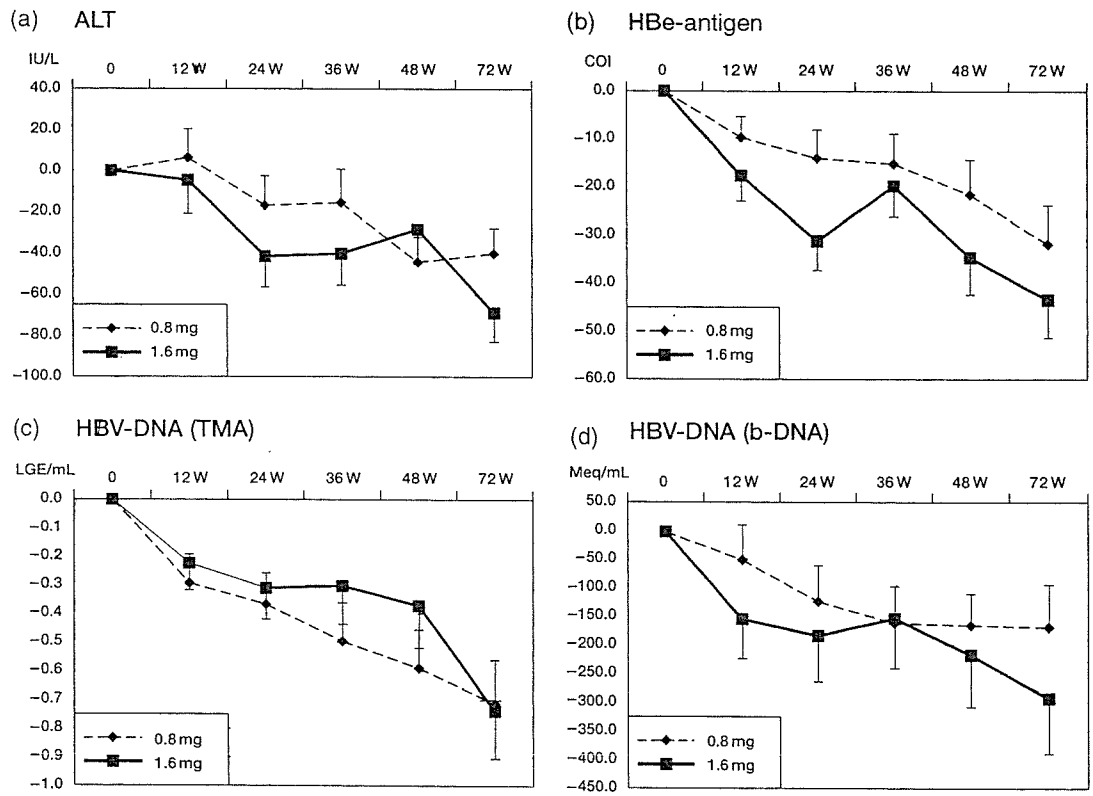

Fig. 1 Reduction from baseline in serum levels of ALT (a), HBeAg (b), HBV-DNA by TMA (c) and HBV-DNA by bDNA (d) for all patients in both treatment arms. All values are expressed as mean ± standard error (SE).

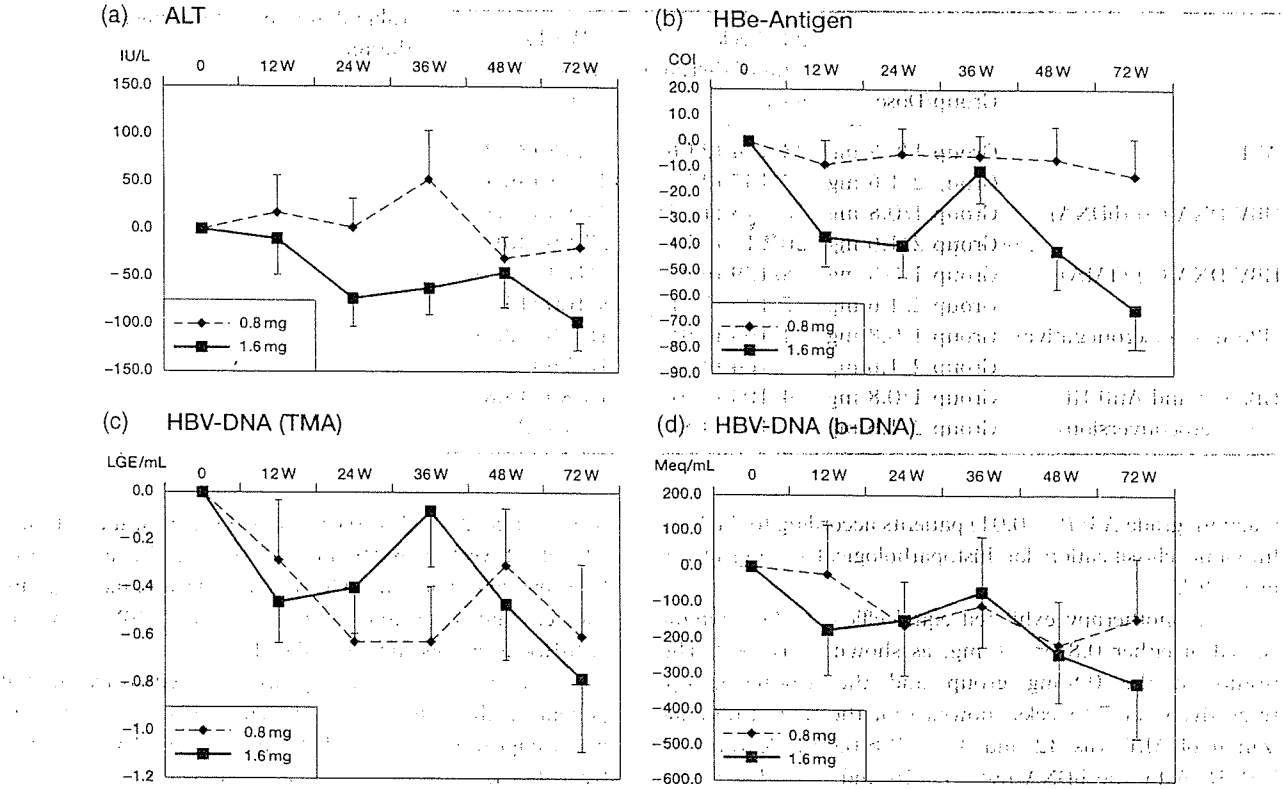

Fig. 2 Reduction from baseline in serum levels of ALT (a), HBeAg (b), HBV-DNA by TMA (c) and HBV-DNA by bDNA (d); stratified for patients with F3 fibrosis. All values are expressed as mean ± standard error (SE).

($P = 0.03$) and HBeAg ($P < 0.01$) levels were sustained at 72 weeks and were statistically superior in the 1.6-mg treatment group.

In the 310 patients followed up for 72 weeks, 1077 adverse events were reported, most of which were unrelated to the study drug. Of the total adverse events, 377 (38.7%) were considered possibly related to Tα1 and occurred in 120 patients (Table 3). There were 22 cases of transient exacerbation of liver function (11 cases in each dose group), which were classified as ALT flares and assumed to be associated with the immunomodulating action of Tα1. One patient had two flares during the 72-week period. Onset of the flares occurred from 2 to 64 weeks (median 19 weeks) from the start of treatment. All patients who experienced flares recovered uneventfully and there were no cases of death because of liver failure. Over the 72 weeks, only three (0.28%) adverse events were considered to be serious; one patient developed bile duct cholangiocarcinoma and two patients (0.43%) developed hepatocellular carcinoma. None of these three serious adverse events were considered to be due to Tα1. Between the two treatment groups, there were no statistical differences in the incidences, symptoms, or severity of adverse events.

Table 3 Incidence of adverse events

Variable*	Group 1 (0.8 mg)	Group 2 (1.6 mg)
Malaise	36 (23.8)	40 (26.5)
Nausea	14 (9.3)	16 (10.6)
Headache	12 (7.9)	18 (11.9)
Abdominal discomfort or pain	10 (6.6)	15 (9.9)
Anorexia	5 (3.3)	15 (9.9)
ALT elevation	11 (7.3)	11 (7.2)
AST elevation	5 (3.3)	10 (6.6)

*The adverse events shown are those that occurred in at least 5% of the patients in a treatment group. Although these adverse events were probably related to the hepatitis B, they were considered to be possibly related to thymosin alpha-1.

DISCUSSION

Tα1 is a 28-amino acid polypeptide which was originally isolated from bovine thymus extract (thymosin fraction 5) and is now chemically synthesized [21]. Tα1 treatment leads to the inhibition of chronic viral infection through a mechanism of cellular immune response modulation via an increase in the secretion of interferon-alpha, interferon-gamma, and cytokines such as IL-2, IL-3, and the differentiation and maturation of T cells [11,19]. Tα1 also increases

T-cell populations by blocking apoptosis [22] and increases natural killer (NK) cell activity in multiple animal models and normal human subjects [11]. In addition, Tα1 has direct antiviral properties as well as increasing the expression of major histocompatibility complex (MHC) class 1 molecules on infected cells [23].

Tα1 has been clinically used as a 6-month therapy for chronic hepatitis B in many studies. Zavaglia *et al.* [12] reported that the rate of HBV-DNA clearance after treatment with Tα1 (as determined by liquid phase hybridization) was 23% at 20 months. Mutchnick *et al.* [13] reported that the rate of HBeAg clearance was 23% and HBV-DNA (liquid phase hybridization) clearance was 20% in 49 cases at the end of a 6-month follow-up period. Similarly, Chien *et al.* [14] reported that the rate of HBeAg and HBV-DNA (liquid phase hybridization) clearance was 40% in 32 cases evaluated at 12 months of post-treatment follow-up. Interestingly, another study confirmed that the effectiveness of Tα1 appeared to increase after the completion of drug administration, especially at 12 months post-treatment [24].

In this randomized, multicentre study of chronic hepatitis B patients in Japan, Tα1 was administered at a dose of 0.8 or 1.6 mg twice weekly for 24 weeks, and a long-term observation was conducted at 72 weeks (12 months after cessation of therapy). Even though many of the patients in this study were considered difficult-to-treat (32% had advanced liver fibrosis and 44% were previously unresponsive to interferon therapy), treatment with Tα1 at a dose of 1.6 mg for 6 months resulted in significant improvements in ALT, HBV-DNA and HBeAg. Therefore, this study demonstrates the efficacy of Tα1 treatment.

There were no statistically significant differences in treatment efficacy with 0.8 or 1.6 mg of Tα1 monotherapy. However, patients were not stratified by liver biopsy, which may have influenced these results. A stratified, intragroup analysis demonstrated that patients with more serious disease exhibited superior results when treated with 1.6 mg *vs* 0.8 mg of Tα1. At 72 weeks, changes from baseline ALT and HBeAg levels were also statistically superior in the 1.6 mg treatment group. Therefore, it is suggested that the higher dose of 1.6 mg for 24 weeks be administered, especially in the case of advanced fibrosis.

Historical comparison suggests that Tα1 and conventional interferon therapies have similar efficacy, and that both are superior to placebo. Japanese patients who received interferon alpha-2a for 6 months had response rates for normalization/clearance at 24–48 weeks after completion of therapy of: 41.6% (10 of 24) for ALT; 27.8% (five of 18) for HBV-DNA (bDNA); and 15% (three of 20) for HBeAg [25]. Regarding HBV-DNA and HBeAg, although response rates are decreasing with the availability of increasing assay sensitivity from advances in assay methods, response rates are still considered to be similar to those reported by Iino *et al.* [25] when evaluated at 12–18 months after the start of interferon administration. With Tα1 therapy, the rates of clearance of HBV-DNA and HBeAg have the tendency to increase with time, even after completion of therapy [24], whereas there is a recurrence of chronic hepatitis B after completion of interferon therapy [4,5,26]. Similar positive results to Tα1 therapy were demonstrated in additional studies evaluating the efficacy of longer-term treatment with interferon therapy in Japan [25,27–31]. In contrast, the results for HBeAg clearance and seroconversion were only 15 and 5%, respectively, in trials where Japanese patients received placebo for 24 weeks [32,33].

Once-daily lamivudine is another therapy for the treatment of hepatitis B that rapidly produces a beneficial reduction in viral DNA [6,7]; however, approximately 90% of patients relapse once therapy is stopped [8]. In addition, lamivudine-resistant YMDD mutations are common and increase over time – from 14% at 1 year to 38% at 2 years and to 69% at 5 years [34]. Sustained biochemical and virological response rates tend to decrease over time because of the development of this drug resistance. In addition, deterioration of liver function and histology has been demonstrated in patients who develop YMDD mutations [34]. HBV, therefore, does not respond well to lamivudine therapy [35,36]. By contrast, treatment with Tα1 exhibited cumulative improvements, even after the completion of therapy, and no Tα1-resistant mutations have been reported [24].

In this study, the rate of progression to hepatocellular carcinoma was calculated to be 0.43% per year; however, the period of observation was too short to compare with the previously observed rates of 4.9% in 5 years and 6.6–7.7% in 10 years in non-treated patients [37,38]. In addition, the high prevalence of patients with advanced disease may have facilitated the appearance of the two cases of hepatocellular carcinoma seen in our study. ALT flares were seen in 22 patients and therapy with Tα1 was interrupted in 16, but all the patients recovered or had their flares managed by hospitalization. In fact, in the natural progression of chronic hepatitis B, transient exacerbations of liver function are commonly seen [39–41]. It has been suggested that the ALT flares are an essential component of natural remission. Therefore, a temporary elevation of ALT may occur in the course of therapy using a drug with a mechanism of intensifying the immune system and accelerating natural remission, such as Tα1 or interferon. Overcoming this exacerbation of liver function is an important part of the eventual therapeutic effect. When the exacerbation in liver function is observed during therapy, the patient should be checked for liver failure by evaluating bilirubin and prothrombin. As long as these values are acceptable, therapy should be continued.

Studies of concomitant Tα1 and interferon therapy are ongoing. A study by Saruc *et al.* [42] compared the outcomes of Tα1 and interferon alpha-2b combination therapy ($n = 27$) with lamivudine and interferon alpha-2b combination therapy ($n = 15$) in patients with HBeAg-negative chronic hepatitis B. At 26 weeks post-therapy, 74% of

patients treated with Tα1 plus interferon alpha-2b achieved a sustained response, defined as a loss of HBV-DNA and normalization of ALT, *vs* 53.3% of patients treated with lamivudine and interferon alpha-2b combination therapy. At 18 months post-therapy, the sustained response rates were 70% in the Tα1 plus interferon alpha-2b treated patients *vs* only 20% in the lamivudine alpha-2b treated patients [42]. More controlled trials with a longer duration of follow-up are needed to adequately evaluate the efficacy and safety of these novel combination therapies.

In conclusion, the results from the present study suggest that Tα1 therapy exhibits long-term efficacy against chronic hepatitis B, with no significant adverse effects. Tα1 leads to the normalization of ALT level and clearance of HBV-DNA and HBeAg at response rates similar to those seen in previous studies after treatment with interferon. The efficacy was dose-dependent for patients with advanced fibrosis, with a statistically significant superiority of the 1.6 mg over the 0.8 mg dose. Therefore, the administration of Tα1 at a dose of 1.6 mg may become a new safe and effective therapeutic option for difficult-to-treat hepatitis B patients.

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