TABLE III – UNIVARIATE ANALYSIS OF THE EFFECT OF VARIABLES ON THE T-CELL RESPONSE AGAINST AFP

	Patients with a positive T-cell response	Patients without a positive T-cell response	*p*-value
No. of patients	18	20	
Age (years)[1]	68.1 ± 6.8	65.5 ± 8.9	NS
Sex (M/F)	15/3	15/5	NS
AFP level (≦20/>20)	6/12	9/11	NS
Diff. degree of HCC (well/moderate or poor/ND)	5/9/4	6/5/9	NS
Tumor multiplicity (multiple/solitary)	14/4	9/11	NS
Vascular invasion (+/−)	7/11	4/16	NS
TNM factor			
(T1/T2–4)	2/16	11/9	0.006
(N0/N1)	18/0	19/1	NS
(M0/M1)	14/4	17/0	NS
TNM stage (I/II–IV)	2/16	11/9	0.006
Histology of nontumor liver (LC/chronic hepatitis)	16/2	17/3	NS
Liver function (Child A/B/C)	12/6/0	12/6/2	NS
Etiology (HCV/HBV/others)	14/3/1	18/1/1	NS

NS, no statistical significance; ND, not determined.
[1]Data expressed as mean ± SD.

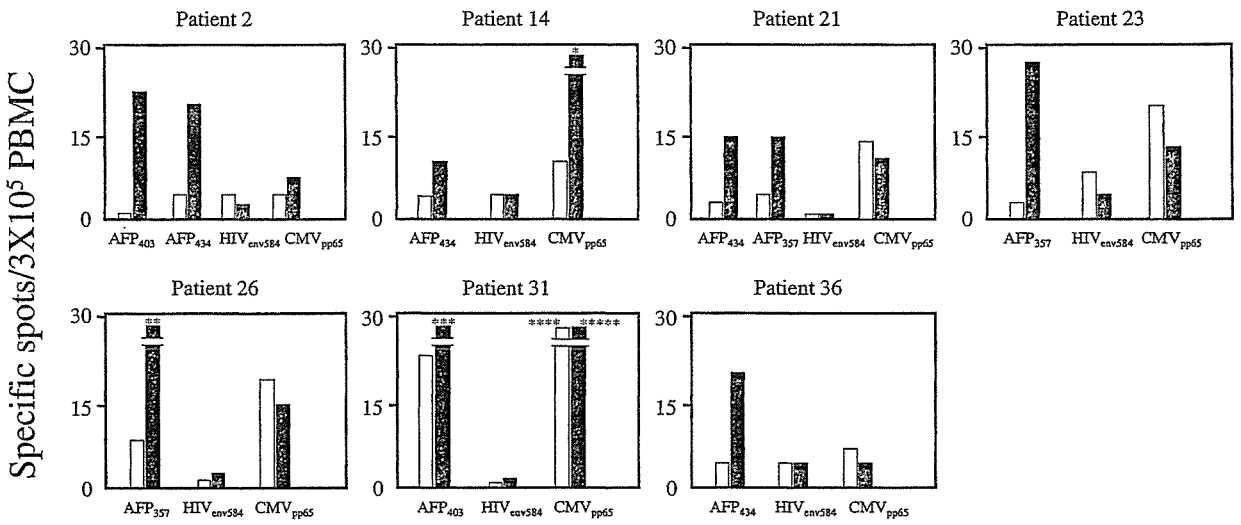

FIGURE 6 – The induction of AFP-specific T-cell responses in HCC patients after treatment of HCC. Direct *ex vivo* analysis (IFN-γ ELISPOT assay) of peripheral blood T-cell responses to AFP-, HIV- or CMV-derived peptides were performed before (open bar) and after (solid bar) HCC treatment. Only patients with a significant change in the T-cell response to peptides AFP_{403}, AFP_{424}, AFP_{434}, AFP_{357}, or AFP_{414} were included in the figure. A significant change in the IFN-γ response was defined as a more than twofold increase and the presence of more than 10 specific spots after HCC treatment. The data are expressed as the number of IFN-γ producing cells before and after treatment. The characteristics of the patients are shown in Table I, and the peptide sequences are described in Table II. * denotes 188 specific spots; **, 177 specific spots; ***, 59 specific spots; ****, 68 specific spots and *****, 81 specific spots.

results with those reports, we believe that AFP-specific T-cell responses in patients with advanced HCC are as strong as other tumor associated antigen-specific T-cell responses, and that the newly identified AFP epitopes are immunogenic.

For the analysis of clinical factors and frequency of AFP-specific IFN-γ producing cells, we obtained evidence that the frequency of the patients with advanced tumor stages for the group with AFP-specific immune responses was significantly higher ($p = 0.006$) than that for the group without the responses (Table III). In other words, tumor stages were associated with AFP-specific immune responses. These results might be explained by the invasion of tumor cells into micro vessels, extra capsules or lymph nodes that can induce T cells. In accordance with our results, a higher frequency of T cells against epithelial cell adhesion molecule, her-2/neu or CEA was also reported among patients with advanced colorectal cancer.[34,35]

Other factors, including serum AFP levels, histology of the nontumor liver, liver function and hepatitis viral infections were not significantly different between patients with and without positive T-cell responses. Specially, the frequency of peripheral AFP-specific T cells was not correlated with serum AFP levels. This result is consistent with the previously demonstrated results that frequencies and function of AFP-specific T cells were not reduced in HCC patient independent of serum AFP levels.[40] In the present study, AFP-positive T-cell responses were observed even in 4 of 8 (50%) patients with AFP-negative serum, and 2 of the 4 patients with AFP-negative serum but who were positive for AFP-specific T cells in the peripheral blood showed an increase in serum AFP

TABLE IV – CHARACTERISTICS OF PATIENTS STUDIED FOR T-CELL RESPONSIVENESS AFTER HCC TREATMENT

	Patients with increasing T-cell responsiveness	Patients without increasing T-cell responsiveness	*p*-value
No. of patients	7	10	
Age (years)[1]	69.1 ± 7.0	69.9 ± 7.1	NS
Sex (M/F)	6/1	8/2	NS
AFP level (≦20/>20)	1/6	3/7	NS
Diff. degree of HCC (well/moderate or poor/ND)	3/3/1	4/3/3	NS
Tumor multiplicity (multiple/solitary)	5/2	8/2	NS
Vascular invasion (+/−)	2/5	5/5	NS
TNM factor			
(T1–T2/T3–T4)	6/1	4/6	NS
(N0/N1)	7/0	10/0	NS
(M0/M1)	6/1	8/2	NS
TNM stage (I,II/III,IV)	6/1	3/7	0.049
Histology of nontumor liver (LC/chronic hepatitis)	7/0	10/0	NS
Liver function (Child A/B/C)	3/4/0	7/3/0	NS
Etiology (HCV/HBV/others)	7/0/0	9/1/0	NS
Positive T-cell responses before treatment (+/−)	2/5	7/3	NS
Treatments (PEIT/RF/TAE/chemotherapy)	1/2/4/0	1/0/8/1	NS

NS, no statistical significance; ND, not determined.
[1]Data expressed as mean ± SD.

during the follow-up period. In addition, it has been noted that tissue-AFP in HCC is positive in some patients with lower or negative-AFP.[31] Taken together, these results suggest that AFP-specific IFN-γ producing cells in the peripheral blood are more useful than serum AFP to detect HCC producing AFP at an early stage.

Also, 17 HCV infected and 3 HBV infected patients had AFP-specific T cells in the peripheral blood. AFP-specific CTLs could also be expanded in patients with HCV infection by *in vitro* peptide stimulation. Furthermore, the frequency of T cells reactive toward a single AFP epitope was equal or higher than that for a single HCV epitope.[41] These results suggest that immunotherapy of HCC could be possible independent of hepatitis viral infection, which causes host immune disorders because of the impairment of dendritic cells.[42–44]

Further, to understand host immune responses for HCC, the newly identified AFP epitopes were then used to analyze the immunological effects of HCC treatments, including tumor ablation, TAE and chemotherapy. The question regarding whether inhibition of HCC aided by antitumor treatments affects host cellular immune responses remains unknown. In the present study, we found that the frequency of AFP-specific T cells increased in 7 patients after HCC treatments and only increased for AFP but not for viral antigens. These results indicate that the effect of treatments on the host immune response is specific for HCC associated antigens.

For the analysis of factors associated with altered AFP-specific T-cell responses, we found that the ratio of patients with TNM Stage I or II is greater for patients with increasing T-cell responsiveness than for those without. Furthermore, 5 of the 7 patients did not show an AFP-specific T-cell response before treatment, but showed one afterward. These results suggest that HCC treatments have the possibility to restore tumor-specific T-cell responses, which are weak in patients with early stage HCC. Consistent with our findings, increased numbers of lymphocytes, natural killer cells and macrophages has been reported[45,46] to be present at the tumor site after percutaneous microwave coagulation therapy (PMCT). The mechanisms that enhance host immune responses because of HCC treatment are unknown, but the following are suggested. First, AFP antigen recognized by T cells may increase because of destruction of the tumor. Second, the inhibition of host immune responses by HCC is relaxed because of tumor ablation. Finally, the factors that enhance host immune responses, including cytokines, are induced by inflammation caused by HCC treatment.

Although further studies are necessary to understand the precise mechanisms, these results suggest that HCC treatments might be able to enhance host immune responses and that the newly identified AFP epitopes could be useful for analyzing host immune responses for HCC.

In conclusion, we identified and characterized novel HLA-A*2402-restricted T-cell epitopes derived from AFP. The newly identified epitope-specific T cells can be detected and induced by PBMC stimulation with these peptides in HCC patients. The frequency of AFP-specific T cells is the same as that of other immunogenic cancer associated antigen-derived epitopes in patients with advanced HCC, but is lower during the early stages of the tumor. On the other hand, anticancer treatments have the possibility to enhance the host immune responses and restore weak responses. These results may provide a rationale for T-cell-based immunotherapy against HCC, and suggest that the identified AFP epitopes could be a valuable component for HCC immunotherapy and for analyzing host immune responses.

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Effect of Hepatitis C Virus (HCV) NS5B-Nucleolin Interaction on HCV Replication with HCV Subgenomic Replicon

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We previously reported that nucleolin, a representative nucleolar marker, interacts with nonstructural protein 5B (NS5B) of hepatitis C virus (HCV) through two independent regions of NS5B, amino acids 208 to 214 and 500 to 506. We also showed that truncated nucleolin that harbors the NS5B-binding region inhibited the RNA-dependent RNA polymerase activity of NS5B in vitro, suggesting that nucleolin may be involved in HCV replication. To address this question, we focused on NS5B amino acids 208 to 214. We constructed one alanine-substituted clustered mutant (CM) replicon, in which all the amino acids in this region were changed to alanine, as well as seven different point mutant (PM) replicons, each of which harbored an alanine substitution at one of the amino acids in the region. After transfection into Huh7 cells, the CM replicon and the PM replicon containing NS5B W208A could not replicate, whereas the remaining PM replicons were able to replicate. In vivo immunoprecipitation also showed that the W208 residue of NS5B was essential for its interaction with nucleolin, strongly suggesting that this interaction is essential for HCV replication. To gain further insight into the role of nucleolin in HCV replication, we utilized the small interfering RNA (siRNA) technique to investigate the knockdown effect of nucleolin on HCV replication. Cotransfection of replicon RNA and nucleolin siRNA into Huh7 cells moderately inhibited HCV replication, although suppression of nucleolin did not affect cell proliferation. Taken together, our findings strongly suggest that nucleolin is a host component that interacts with HCV NS5B and is indispensable for HCV replication.

Hepatitis C virus (HCV) is a major cause of chronic hepatitis around the world (1, 7). Chronic infection with HCV results in liver cirrhosis and may lead to hepatocellular carcinoma (53, 54). HCV is an enveloped positive-strand RNA virus belonging to the genus *Hepacivirus* in the family *Flaviviridae*. The HCV RNA genome is ~9.6 kb in length and consists of a 5′ nontranslated region (NTR), a large open reading frame, and a 3′ NTR. The 5′ NTR contains an internal ribosome entry site, which mediates the translation of a single polyprotein of ~3,000 amino acid (aa) residues (61, 64). This polyprotein is cleaved by host and viral proteases into at least 10 different products (33). At the amino terminus of the polyprotein are the core protein, E1, and E2, followed by p7, a hydrophobic peptide with unknown function, and the nonstructural (NS) proteins NS2, NS3, NS4A, NS4B, NS5A, and NS5B. The 3′ NTR consists of a short variable sequence, a poly(U)-poly(UC) tract, and a highly conserved X region and is critical for HCV RNA replication and HCV infection (13, 29, 69, 71).

HCV is unique among positive-strand RNA viruses in that it causes persistent and chronic infections. In addition, the high mutation rate of the gene encoding the E2 protein allows it to escape host immune surveillance, which is strongly associated with chronic inflammation of the liver (19, 23, 66, 67). As a result, HCV replication has become a target for the treatment of chronically infected individuals. The RNA-dependent RNA polymerase (RdRp) NS5B is the central catalytic enzyme in HCV RNA replication. Several recombinant and catalytically active forms of NS5B have been expressed and purified from insect cells and *Escherichia coli*, and these proteins have provided insights into the biochemical and catalytic properties of NS5B (2, 12, 34, 68). Studies of HCV replication in vitro have to overcome several difficulties, since replication requires all or most NS proteins and/or host proteins and occurs at the membrane. An understanding of the biology of HCV replication has been facilitated by the development of subgenomic and full-length HCV replicons, which express HCV proteins and replicate their RNA when transfected into human hepatoma-cell-derived Huh7 cells and other cell lines (22, 24, 35).

Nucleolin is a major nucleolar phosphoprotein, and nucleolin-specific antibodies have been used to identify nucleoli (14, 59). Nucleolin has been shown to be an RNA chaperone and/or shuttling protein for various host and viral components in nucleoli, nucleoplasm, cytoplasm, and the plasma membrane (18, 37, 41). We previously reported that the transient expression of NS5B causes the redistribution of endogenous nucleolin from the nucleus to the cytoplasm and that nucleolin and NS5B interact, in vitro and in vivo, through two independent regions of NS5B, aa 208 to 214 and 500 to 506. We also showed that the C-terminal region of nucleolin inhibited NS5B RdRp activity through this interaction in vitro (20). Because full-length nucleolin was not available in that experimental condition (70), we could not determine the exact role of this interaction in vivo.

To further investigate the interaction between nucleolin and NS5B, we focused on NS5B aa 208 to 214. We prepared a

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series of mutant replicons in which each amino acid within this region was altered to alanine(s). Here, we report that the W208 residue is critical for transient HCV replication as well as for binding to nucleolin in vivo. HCV replication was considerably inhibited in cells in which endogenous nucleolin was transiently down-regulated by small interfering RNA (siRNA). Our results strongly suggest the involvement of nucleolin in HCV replication through its interaction with NS5B and that nucleolin acts as a positive modulator of HCV replication.

MATERIALS AND METHODS

Construction of plasmids. The plasmid pNNRZ2RU (28), which harbors a subgenomic replicon derived from MT-2C cells infected with HCV (a genotype 1b isolate, M1LE [GenBank accession no. AB080299]) and contains wild-type M1LE replicon (M1LE/wild) cDNA, was digested with MluI and BglII, and the obtained fragment was inserted into the MluI and BglII sites of the vector pGL3Basic (Promega) to create pGL3-*MluI-BglII*. The intermediate vector pGL3-*MluI-BglII*-S2321 was constructed by introducing the point mutation S2321 of NS5A into the MluI and SacI sites of pGL3-*MluI-BglII* by site-directed mutagenesis using primers carrying the necessary nucleotide changes. Subsequently, mutations were introduced into pGL3-*MluI-BglII*-S2321, which was digested with MluI and BglII. The resulting DNA fragments were subsequently ligated into the MluI and BglII sites of pNNRZ2RU. Plasmids containing the individual NS5B substitutions W208A, K209A, S210A, K211A, K212A, C213A, and P214A and the 7-amino-acid alanine substitution, cm211, were constructed by introducing each mutation into the Eco81I and NdeI sites of pGL3-*MluI-BglII*-S2321 by site-directed mutagenesis using primers carrying the necessary nucleotide changes.

The vector pNKFLAG (49) was used to express amino-terminally FLAG-tagged proteins. The plasmid pNNRZ2RU was subcloned by PCR using the primers 5′-TATCGAGCTCGATGTCAATGTCCTACTCATGGACAGGT-3′ (NS5B For), which contains an artificial initiation codon downstream of the SacI site, and 5′-ATGGATGGATCCGCGGGGTCGGGCGCGAGACAGGCT-3′ (NS5Bt Rev), which contains a BamHI site. NS5Bt, containing full-length NS5B truncated by 21 aa at the C terminus, was subcloned into the SacI and BamHI sites of pNKFLAG to create pNKFLAGNS5Bt.

The plasmid pNKGST/Nucleolin (20) was used for the expression of glutathione-*S*-transferase (GST)-fused nucleolin proteins. FLAG-labeled plasmids containing the individual NS5B substitutions W208A, K209A, S210A, K211A, K212A, C213A, and P214A and the 7-amino-acid alanine substitution cm211 were constructed by introducing fragments of pGL3-*MluI-BglII*-S2321 containing each mutation into the EcoRI and SmaI sites of pNKFLAGNS5Bt.

The sequences of all the constructs were confirmed using the dideoxy sequence method. The plasmids pLMH14 and pLMH14/GHD (40) were used as templates for replicon RNA LMH14 and LMH14/GHD, respectively.

Cell culture. We used two kinds of Huh7 cells, one derived from our own laboratory's original Huh7 cells, designated Huh7-DMB (56), and the other cured of MH14 gamma interferon, designated cured MH14 (40). Huh7-DMB cells were used for colony-forming assays, and cured MH14 cells were used for luciferase assays. Both types of Huh7 cells were grown in Dulbecco's modified Eagle's medium (Gibco-BRL, Invitrogen Life Technologies) supplemented with 10% fetal bovine serum, 2 mM L-glutamine, nonessential amino acids, 100 U of penicillin, and 100 μg of streptomycin.

In vitro transcription and purification of RNA. All plasmids harboring replicon RNA were linearized with XbaI and column purified (PCR purification kit; Promega). RNA was synthesized and purified as described previously (56).

RNA transfection and selection of G418-resistant cells. Subconfluent Huh7 cells were trypsinized, washed once with phosphate-buffered saline (PBS) that does not contain Ca and Mg [PBS(−)], and resuspended at 10^7 cells/ml in OPTI-MEM (Gibco-BRL, Invitrogen Life Technologies). One hundred nanograms of *neo* replicon RNA, with or without 1 μM of each siRNA, was added to 400 μl of each cell suspension in a cuvette with a gap width of 0.4 cm (Bio-Rad). The mixture was immediately transfected into Huh7 cells by electroporation with a GenePulser II system (Bio-Rad) set to 270 V and 975 μF. Following a 10-min incubation at room temperature, the cells were transferred into 10 ml of growth medium and seeded into a 10-cm-diameter cell culture dish. To select G418-resistant cells, the medium was replaced with fresh medium containing 1 mg/ml of G418 (GENETICIN; Gibco-BRL, Invitrogen Life Technologies) 24 h after transfection. After changing the medium twice per week for 4 weeks, the colonies were stained with Coomassie brilliant blue (0.6 g/liter in 50% methanol–10% acetic acid).

DNA transfection. Using the same electroporation protocol as described above, 500 ng of pCI-Neo (Promega), which encodes a neomycin resistance marker under the control of a cytomegalovirus (CMV) promoter/enhancer, with or without 1 μM of each siRNA, was transfected into Huh7 cells. G418-resistant cells were selected in medium containing 0.5 mg/ml G418. Four weeks after transfection, the colonies were stained with Coomassie brilliant blue.

Using DMRIE-C reagent (Invitrogen Life Technologies), 300 ng of pGL3 control (Promega), encoding luciferase under the control of a CMV promoter/enhancer, was cotransfected with or without 2 μM of each siRNA according to the manufacturer's instructions. Luciferase activity was assayed 48 and 72 h after transfection.

RNA transfection and luciferase assay. We used a luciferase assay to monitor luciferase replicon activity. Briefly, cured MH14 cells seeded onto 48-well plates were transfected with 250 ng of luciferase replicon RNA, with or without 2 μM of each siRNA, using DMRIE-C reagent according to the manufacturer's instructions. Cell proteins were extracted in a lysis buffer supplied in the Dual-Luciferase Reporter Assay system (Promega), and their luciferase activity was measured. Each assay was performed at least in triplicate, and means and standard deviations were determined.

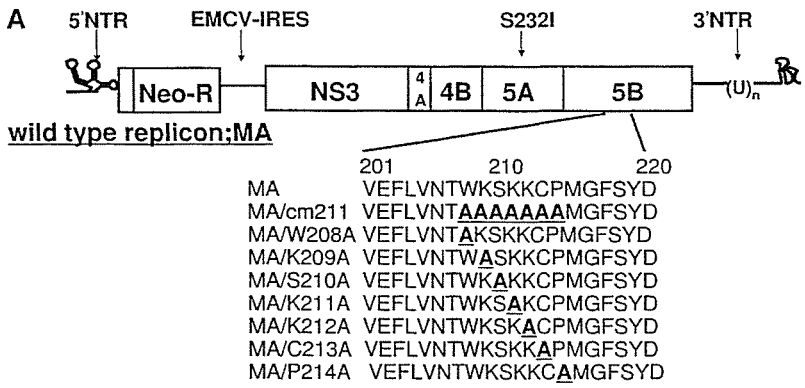
Preparation of cell extracts, coprecipitation with glutathione resin, and Western blot analysis. COS1 cells were transiently transfected using the calcium-phosphate method. The cells were harvested, washed with PBS(−), and sonicated in PBS lysis buffer [PBS(−) containing 150 mM NaCl, 1.0% Triton X-100, 1 mM EDTA, and 1 mM dithiothreitol] containing 10 μg each of aprotinin and leupeptin per ml. Total cell lysates were diluted 10-fold with PBS lysis buffer, mixed with 20 μl of glutathione-Sepharose 4B beads (glutathione resin) (Amersham Biosciences), and incubated for 3 h on a rotator in a cold room. After extensive washing with PBS(−) containing 1.0% Triton X-100, the bound proteins were eluted, fractionated by sodium dodecyl sulfate (SDS)–10% polyacrylamide gel electrophoresis (PAGE), transferred onto nitrocellulose membranes, and subjected to Western blot analysis with anti-FLAG M2 monoclonal antibody (Sigma). The proteins were visualized using enhanced chemiluminescence according to the manufacturer's instructions (Amersham Biosciences). As a loading control, the nitrocellulose membranes used for Western blot analysis with anti-FLAG M2 monoclonal antibody were reprobed with anti-GST monoclonal antibody (Santa Cruz Biotechnology, Inc.) according to the manufacturer's instructions (Amersham Biosciences).

siRNA. We purchased siRNA for luciferase GL3 duplex (si-Luc), siRNA for nonspecific control RNA duplex (si-Mix), siRNA for nucleolin (si-Nuc) (GGA AGACGGUGAAAUUGAU-deoxyribosylthymine [dT]dT), and siRNA for HCV (CCUCAAAGAAAAACCAAAC-dTdT) from B-Bridge International, Inc., and we purchased siRNA for GFP from QIAGEN.

Western blot analysis for endogenous nucleolin. Using the electroporation protocol described above, 1 μM of each siRNA was transfected into Huh7-DMB cells. After 48 h, the cells were harvested, washed with PBS(−), and sonicated in PBS lysis buffer. Total cell lysates were fractionated by SDS–10% PAGE, transferred onto nitrocellulose membranes, and subjected to Western blot analysis with rabbit polyclonal anti-nucleolin antibody (103C) (20), mouse monoclonal anti-nucleolin antibody (C23, sc-8031; Santa Cruz Biotechnology, Inc.), and mouse monoclonal anti-β-actin antibody (Sigma). The proteins were visualized by enhanced chemiluminescence according to the manufacturer's instructions (Amersham Biosciences).

RESULTS

We previously reported that NS5B from HCV subtype 1b isolate JK-1 and nucleolin interact in vitro and in vivo and that two regions of NS5B, amino acids 208 to 214 and 500 to 506, are both indispensable for binding to nucleolin. We also reported that the C-terminal region of nucleolin inhibited the RdRp activity of NS5B in a dose-dependent manner (20). Although the effect of full-length nucleolin could not be determined, because we could not obtain recombinant full-length nucleolin, these results strongly suggested that nucleolin may be a component of the HCV replication complex and, through its interaction with NS5B, may modulate HCV replication. To further investigate this question, we determined the biological effect of the interaction between NS5B from HCV subtype 1b



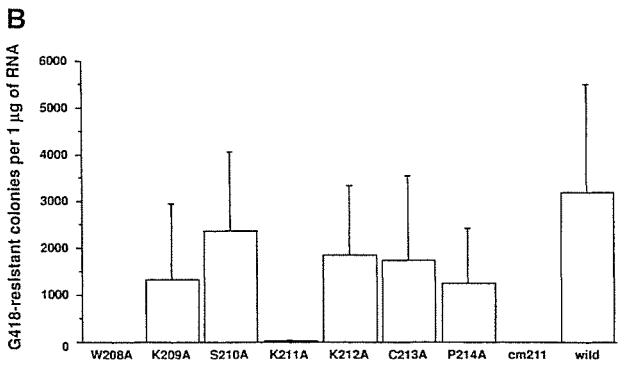


FIG. 1. (A) Schematic representation of the mutant replicons used in this study. MA is a replicon carrying the point mutation S232I in NS5A and was used as the wild-type replicon. The mutant replicons MA/cm211, MA/W208A, MA/209A, MA/S210A, MA/211A, MA/212A, MA/213A, and MA/214A carry NS5B mutations in MA, as shown. Numbering starts from the beginning of NS5B. EMCV, encephalomyocarditis virus; IRES, internal ribosome entry site. (B) Effect of each mutation on HCV RNA replication. Huh7-DMB cells were electroporated with 1 μg of each replicon RNA, and G418-resistant cells were selected with 1 mg/ml G418 and stained 4 weeks later. This figure shows the mean number of G418-resistant cell colonies per 10-cm-diameter cell culture dish per 1 μg replicon RNA.

isolate M1LE and nucleolin on HCV replication using an HCV subgenomic replicon system.

Scanning of aa 208 to 214 in an HCV subgenomic replicon. First, we tested the importance of NS5B aa 208 to 214, a region essential for nucleolin binding, in HCV RNA replication. For this purpose, we prepared eight mutant replicons (Fig. 1A). The wild-type replicon was represented by MA, in which S232 of NS5A was altered to I, because this mutant replicon can efficiently replicate in Huh7 cells (36, 56). In the replicon MA/cm211, each of the amino acids at positions 208 to 214 of NS5B was changed to alanine, whereas in the replicons MA/W208A, K209A, S210A, K211A, K212A, C213A, and P214A, each individual amino acid residue was changed to alanine. All of these mutant replicons were transfected into Huh7-DMB cells, which were selected with G418, and the number of G418-resistant colonies was used as an indication of HCV RNA replication. In cells transfected with MA/cm211 and MA/W208A, we observed no G418-resistant colonies, whereas in cells transfected with the six other point mutant replicons, as well as in cells transfected with MA/K211, we detected G418-resistant colonies, but they were fewer than those detected with wild-type replicon MA (Fig. 1B). Our negative control, the mutant replicon M1LE/5B-VDD, in which the GDD motif of NS5B was mutated to VDD, yielded no G418-resistant colonies (data not shown). The results of this experiment indicated that the region of NS5B at aa 208 to 214, especially W208, is essential for HCV RNA replication.

Interaction between nucleolin and NS5B. Although we have shown that NS5B from isolate JK-1 binds to nucleolin, it was necessary to show this in isolate M1LE. Due to the poor recovery of soluble full-length NS5B, we utilized NS5Bt (68), a soluble form of NS5B in which the C-terminal 21 aa were truncated, to dissect the interaction between NS5B and nucleolin. Previously, we confirmed that these 21 deleted amino acids were not essential for this interaction (20). FLAG-NS5Bt and GST-nucleolin were transiently coexpressed in COS1 cells, after which the lysates were subjected to a GST pull-down assay and the bound proteins were immunologically detected with anti-FLAG M2 and anti-GST antibodies. We found that GST-nucleolin could bind FLAG-NS5Bt from the M1LE isolate, whereas GST could not, indicating that nucleolin interacts with NS5B in both JK-1 and M1LE isolates (Fig. 2). To determine the essential region/residues of NS5B required for its binding to nucleolin, we again focused on aa 208 to 214 using the alanine scanning method (3). We prepared FLAG-NS5Bt/cm211, in which aa 208 to 214 were all replaced by alanine residues, and showed that it could not bind to GST-nucleolin in an in vivo immunoprecipitation assay (Fig. 2), indicating that aa 208 to 214 of NS5B in both M1LE and JK-1 isolates constitute a critical region for the binding of nucleolin. To identify the exact residue(s) within aa 208 to 214 critical for binding to nucleolin, we prepared seven alanine-substituted point mutants in which each amino acid was replaced by alanine, and we tested the ability of each point mutant to bind to GST-nucleolin. Using an in vivo immunoprecipitation assay, we found that of the seven point mutants, only FLAG-NS5Bt/W208A could not bind to GST-nucleolin (Fig. 2), indicating that W208 of NS5B is essential for this binding and may be essential for HCV replication.

Suppression of endogenous nucleolin by siRNA. To identify the siRNA sequence that knocks down the expression of endogenous nucleolin, we used the prediction services of

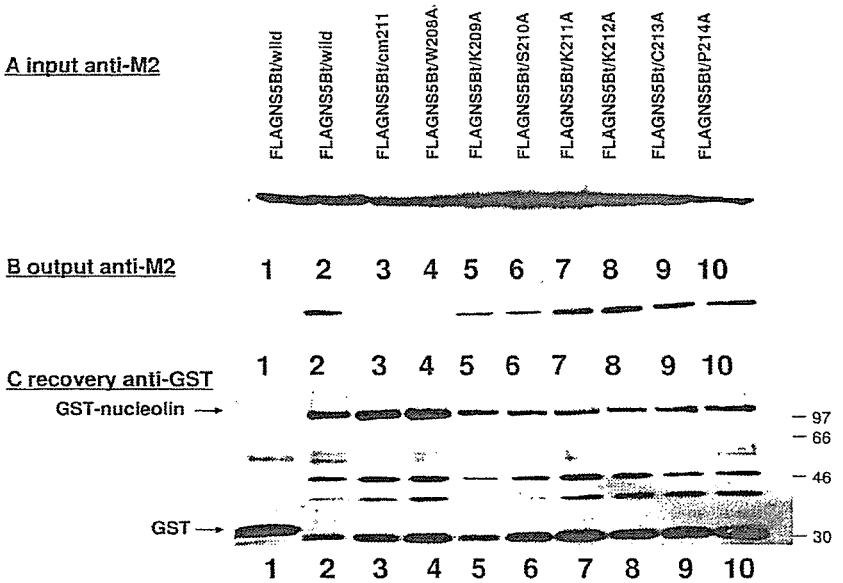


FIG. 2. Interaction between nucleolin and NS5B of HCV isolate M1LE and an essential residue for this interaction. COS1 cells were transiently cotransfected with mammalian expression vectors expressing FLAG-NS5Bt proteins (lanes: 1 and 2, wild type; 3, cm211; 4, W208A; 5, K209A; 6, S210A; 7, K211A; 8, K212A; 9, C213A; 10, P214A) and GST protein alone (lane 1) or GST-nucleolin protein (lanes 2 to 10). (A) Input of FLAG-NS5Bt proteins. Total lysates were fractionated by SDS–10% PAGE and subjected to Western blot analysis with anti-FLAG M2 monoclonal antibody. (B) Output of FLAG-NS5Bt proteins. Coprecipitants by glutathione resin were washed with PBS(−) containing 1.0% Triton X-100, fractionated by SDS–10% PAGE, and subjected to Western blot analysis with anti-FLAG M2 monoclonal antibody. (C) Recovery of GST or GST-nucleolin proteins. The nitrocellulose membrane used for Western blot analysis of coprecipitants with anti-FLAG M2 antibody was reprobed with anti-GST antibody. Molecular masses (kilodaltons) are indicated to the right of the panel.

iGENE (Tsukuba, Japan). We selected one sequence, si-Nuc, and, as a control for siRNA transfection, we utilized siRNA for luciferase (si-Luc) (GL3 luciferase duplex). Forty-eight hours after electroporation of each siRNA, at a concentration of 1 μM, into Huh7-DMB, the lysates were analyzed by Western blotting analysis with two kinds of antibody to nucleolin. We found that both anti-nucleolin antibodies detected the expression of endogenous nucleolin. Although si-Nuc efficiently knocked down the expression of endogenous nucleolin, si-Luc did not (Fig. 3), showing the specificity of the former. In addition, real-time PCR showed that si-Nuc decreased nucleolin mRNA by about one-third compared with si-Luc (data not shown).

Effect of nucleolin suppression on HCV replication. To test the effect of nucleolin knockdown on HCV RNA replication, we transfected 1 μM of si-Nuc or si-Luc along with 100 ng of replicon MA RNA into Huh7-DMB cells and selected the cells with G418. As shown in Fig. 4, we found that cotransfection of si-Nuc reduced the number of G418-resistant colonies, whereas cotransfection of si-Luc did not (Fig. 4). As a control for the efficient transfection of siRNA, we used si-HCV, which targets the HCV internal ribosome entry site and can efficiently suppress HCV replication, as described previously (51). Using this siRNA, we observed no G418-resistant colonies, indicating that siRNA was efficiently transfected under these experimental conditions. To rule out the possibility that suppression of nucleolin may have a detrimental effect on cells and may inhibit HCV RNA replication, we transfected pCI-Neo, which encodes a neomycin resistance gene under the control of a CMV promoter/enhancer, into Huh7-DMB cells, with or without si-Nuc and si-Luc, and selected the cells with 0.5 mg/dl G418. We found that the suppression of nucleolin expression did not significantly reduce the number of G418-resistant colonies (data not shown). In addition, massive cell death was not observed after the transfection of any siRNA (data not shown). These results indicate that the transient suppression of nucleolin may not affect cell proliferation but that nucleolin may affect the HCV replication complex itself.

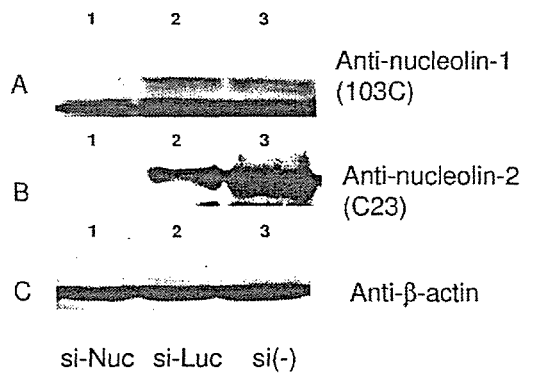


FIG. 3. Knockdown of endogenous nucleolin by siRNA. Huh7-DMB cells were electroporated with 1 μM si-Nuc and si-Luc. After 48 h, total cell lysates were fractionated by SDS–10% PAGE and subjected to Western blot analysis with the anti-nucleolin antibodies anti-nucleolin-1 (103C) in A and anti-nucleolin-2 (C23) in B and anti-β-actin antibody in C. Lanes: 1, cells transfected with si-Nuc; 2, cells transfected with si-Luc; 3, no siRNA [si(−)].

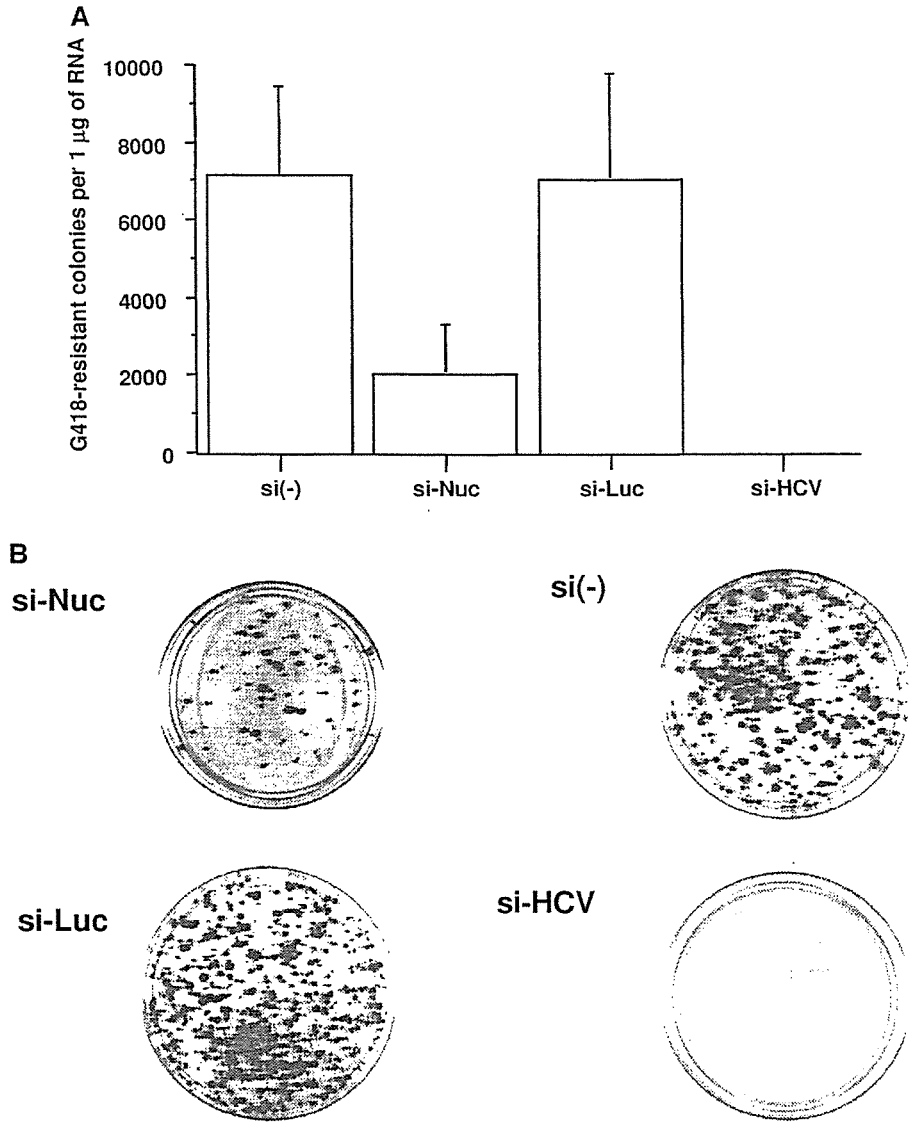


FIG. 4. Effect of suppression of endogenous nucleolin on HCV replication in the MA replicon. Huh7-DMB cells were electroporated with 1 μg of in vitro-transcribed MA RNA plus si-Nuc, si-Luc, si-HCV, or no siRNA [si(−)], and G418-resistant cells were selected with 1 mg/ml G418 and were stained 4 weeks later. (A) Mean number of G418-resistant colonies per 10-cm-diameter cell culture dish per 1 μg replicon RNA. Error bars indicate the standard deviations of the results from at least three independent experiments. (B) Visualization of G418-resistant colonies, as described in Materials and Methods.

Because the knockdown effect of siRNA does not continue for more than 3 weeks after transient transfection, the number of G418-resistant colonies may not be a good indicator of HCV RNA replication. We therefore performed a transient replication assay using a replicon in which the neomycin resistance gene was replaced by a luciferase gene, and luciferase activity was used as a marker of HCV RNA replication. Transfection of MH14 RNA, which was used as the wild-type replicon, into a subline of Huh7 cells resulted in highly efficient luciferase activity, whereas a polymerase-defective RNA replicon of MH14, MH4GHD, in which the catalytic GDD motif of NS5B polymerase was replaced by an inactive GHD motif, was used as a negative control (Fig. 5A). si-HCV and si-Luc suppressed the luciferase activity even at 24 h after transfection, but other siRNAs did not affect the luciferase activity, and luciferase activities in these siRNAs were similar to that of the control (no siRNA) at this point (Fig. 5B). We found that cotransfection of si-Nuc moderately suppressed both luciferase activity at 72 h after transfection and relative luciferase activity, whereas cotransfection of si-GFP and si-Mix did not (Fig. 5B and C). Cotransfection of si-HCV and si-Luc almost completely suppressed luciferase activity at 72 h after transfection. In a transient replication assay, the suppression of endogenous nucleolin also inhibited HCV replication.

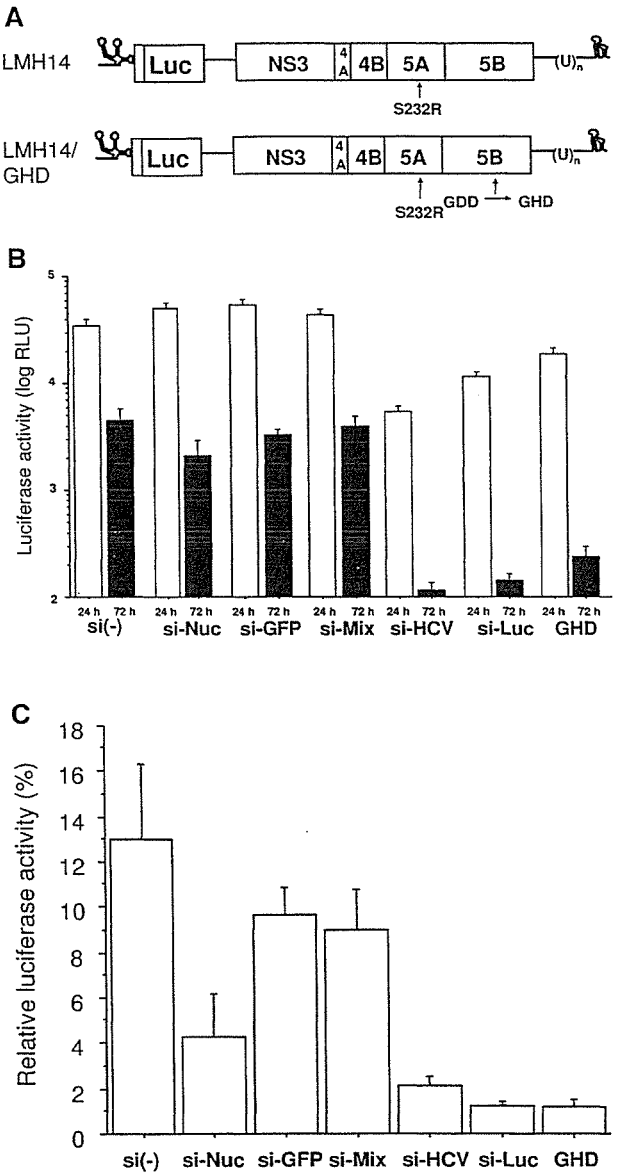


FIG. 5. Effect of suppression of endogenous nucleolin on HCV replication in the LMH14 replicon. (A) Schematic representation of the luciferase replicon. In the LMH14 replicon, the neomycin resistance gene was replaced by a luciferase gene, and S232 of NS5A was replaced by R. In the LMH14/GHD replicon, the NS5B GDD motif in LMH14 was changed to GHD and used as a negative control. (B) Cells were transfected with in vitro-transcribed LHH14 or LMH14/GHD RNA along with 2 μM of si-Mix, si-GFP, si-Nuc, si-Luc, si-HCV, or no siRNA [si(−)] using the DMRIE-C reagent, and luciferase activity (relative light units [RLU]) was measured 24 and 72 h after transfection. Shown are the activities at 24 and 72 h. Error bars indicate the standard deviations of the results from at least three independent experiments. (C) Activity at 24 h was used as an indication of each transfection. Shown are the ratios of activity (percent) at 72 h relative to that at 24 h. Error bars indicate the standard deviations of the results from at least three independent experiments.

To rule out the cytotoxic effects of the suppression of endogenous nucleolin, we transfected pGL3 control, with or without each siRNA, and measured luciferase activity 48 and 72 h after transfection. We found that cotransfection of each siRNA did not inhibit luciferase activity at both 48 and 72 h (Fig. 6), indicating that both suppression of nucleolin and transfection of siRNA did not have detrimental effects on transfected cells.

DISCUSSION

HCV replication has been found to take place in a distinctly altered membrane structure, or membranous web, of the endoplasmic reticulum (11). When HCV NS proteins are coexpressed in stable cell lines harboring replicons, they colocalize to these membrane structures, indicating that they might form a complex (16, 39, 47). These nonstructural proteins, together with host factors, form the viral replicase, the complex in which viral replication is thought to take place. The in vitro level of the RdRp activity of NS5B is low (12), indicating that cofactors, whether viral and/or host proteins and/or the appropriate cellular environment, are necessary for optimal activity of HCV RdRp. HCV NS5B has been reported to interact with NS3, NS4A, NS4B, NS5A, and NS5B itself (9, 48, 57, 65). Using an HCV subgenomic replicon, we previously reported the critical role of the interaction between NS5A and NS5B and the oligomerization of NS5B itself in HCV replication (36, 56). NS3 and NS4B have been shown to be positive and negative regulators, respectively, of NS5B in the replication complex (46).

In addition to interacting with HCV nonstructural proteins, NS5B has been reported to interact with many host proteins, including a SNARE-like protein (62); eIF4AII, an RNA-dependent ATPase/helicase; a component of the translation initiation complex (30), protein kinase C-related kinase 2, which specifically phosphorylates NS5B (27); and p68, a human RNA helicase I (15). The suppression of protein kinase C-related kinase 2 has been reported to reduce the phosphorylation of NS5B and to inhibit HCV RNA replication (27), and the suppression of p68 has been reported to inhibit the synthesis of negative-strand HCV RNA from the positive strand (15).

Several host proteins have been shown to interact with RdRp of other RNA viruses. For example, in poliovirus, an RdRp and an RdRp precursor interact with human Sam68 (38) and heterogeneous nuclear ribonucleoprotein C1/C2 (5), respectively, and modulate RdRp activity directly or indirectly. Bromo mosaic virus RdRp and tobacco mosaic virus RdRp interact with eukaryotic initiation factor 3 and eukaryotic initiation factor 3-related factor, altering RdRp activity (45, 50).

Here and in a previous report, we identified and characterized the interaction between nucleolin and HCV NS5B (20). Nucleolin was originally identified as a common phosphoprotein of growing eukaryotic cells, although its function is not completely understood. Nucleolin is a multifunctional protein that shuttles between the nucleus and cytoplasm. In addition, it is expressed on the surface of various cells, acting as a receptor for various ligands, including lipoproteins (55), cytokines, growth factors (6, 52, 60), the extracellular matrix (10, 18, 25), bacteria (58), and viruses (4, 8, 21, 41–44).

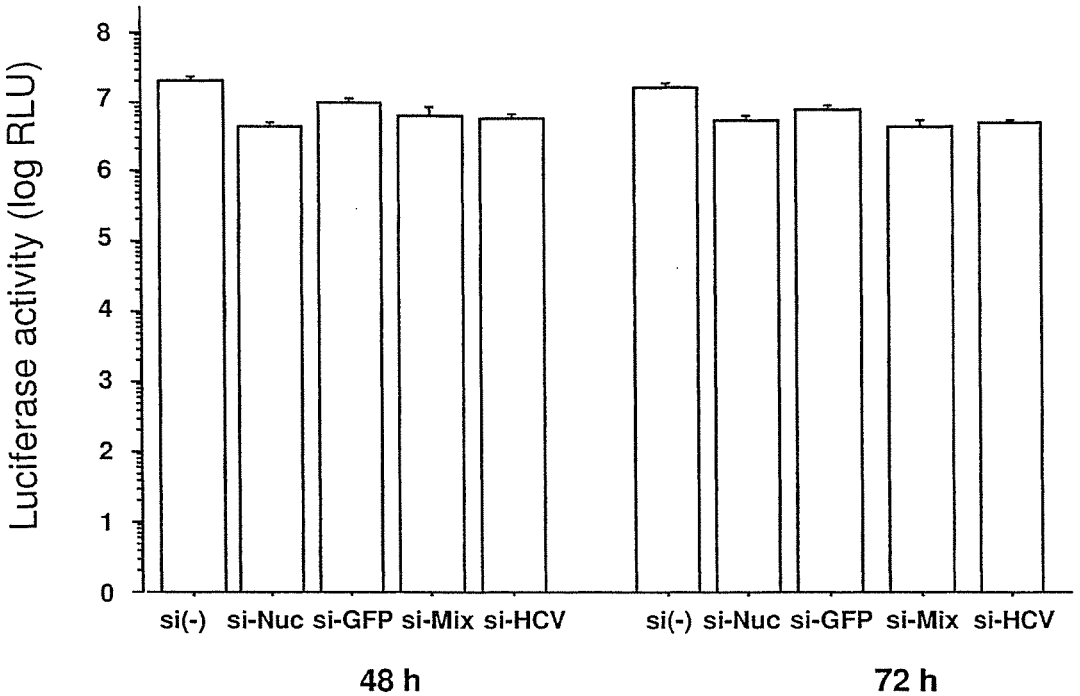


FIG. 6. Effect of suppression of endogenous nucleolin on cell proliferation. The plasmid pGL3 control, encoding the luciferase gene under the control of the CMV promoter/enhancer, was cotransfected with 2 μM of si-Mix, si-GFP, si-Nuc, si-HCV, or no siRNA [si(−)] using DMRIE-C reagent, and luciferase activity was measured 48 and 72 h after transfection. The error bars indicate the standard deviations of the results from at least three independent experiments.

We found that recombinant C-terminal nucleolin proteins can bind NS5B and inhibit its RdRp activity in a dose-dependent manner (20), suggesting that nucleolin may affect HCV replication by interacting with NS5B. The direct interaction of nucleolin with HCV NS5B in vivo and in vitro was shown to require two critical stretches of NS5B. Here, we showed that within one of these regions, aa 208 to 214, the W208 residue was critical for both binding of nucleolin and HCV replication. Transient down-regulation of endogenous nucleolin by siRNA considerably inhibited HCV replication in Huh7 cells. These results strongly indicate that nucleolin has an important role in HCV replication through its direct interaction with NS5B.

Our finding of an important positive role for nucleolin in HCV replication is apparently inconsistent with previous findings of an inhibitory role for nucleolin. It was previously reported that purified C-terminal nucleolin proteins inhibited the RdRp activity of NS5B in vitro. The latter result, however, may have been due to the use of recombinant truncated nucleolin proteins, because recombinant full-length nucleolin was not available (70). Taken together, however, these results indicate that N-terminal nucleolin may be important for the positive function of nucleolin in HCV replication, although the NS5B-binding region is within the RGG domain and RNA-binding domain 4 is at the C terminus.

Transfection of the mutant replicon containing NS5B W208A, which could not bind nucleolin, led to almost no HCV replication. By contrast, the suppression of nucleolin by siRNA moderately inhibited HCV replication, a result also observed with the transient assay using luciferase reporter replicon and G418-resistant colony formation. While HCV replication was completely inhibited by MA/W208A, replication was only partially inhibited by si-Nuc, indicating that si-Nuc can transiently suppress, but cannot eliminate, expression of endogenous nucleolin. Recently, nucleolin was reported to inhibit cell cycle progression after heat shock and genotoxic stress by increasing complex formation with human replication protein A (26). When pGL3 control or pCI-Neo was cotransfected with si-Nuc, the luciferase activity or the number of G418-resistant colonies was not reduced, strongly suggesting that the moderate inhibition of nucleolin expression did not have severe cytotoxic effects on siRNA-transfected cells. More efficient suppression of nucleolin may result in more severe inhibition of HCV RNA replication. It is therefore important to determine whether nucleolin is dispensable in mammalian cells as it is in *Saccharomyces pombe* (17) and *Saccharomyces cerevisiae* (31), since nucleolin may constitute a putative therapeutic target to inhibit HCV replication.

Using a clustered alanine substitution mutant library (CM) of NS5B, we previously showed that two stretches of NS5B amino acids, aa 208 to 214 and 500 to 506, were critical for nucleolin binding. According to the crystal models of NS5B, the former stretch is in the palm and the latter stretch is in the bottom of the thumb domain. We focused on identifying residues in aa 208 to 214 that are essential for nucleolin binding and HCV replication, as the CM mutant of aa 500 to 506 was defective in RdRp activity in vitro and HCV replication in vivo (36, 48, 49). We found that the W208 residue was critical for

both nucleolin binding and HCV replication. This residue is exposed to solvent at the edge of the palm and is not close to the catalytic pocket.

Nucleolin may stabilize monomeric NS5B, making it ready for oligomerization to NS5B, or it may facilitate the formation of a complex between NS5B and template RNA. In both cases, a substoichiometric amount of nucleolin may be required transiently at a step prior to the catalytic RdRp reaction of NS5B. Efforts to determine the contribution of amino acid residues 500 to 508 to nucleolin binding and HCV replication in vivo are ongoing and may reveal further correlations. We found that another mutant replicon, MA/K211A, reduced the number of G418-resistant colonies compared with the wild type and the other mutants. Because K211A of NS5Bt is close to the pocket of catalytic activity and did not affect binding to nucleolin, K211 may contribute to the structural integrity of the pocket or the heat-stable property of RdRp as reported previously (36).

Efficient HCV replication and infection in tissue-cultured cells by using full-length HCV RNA replicons have been reported previously (32, 63, 72). HCV replication occurs in differentiated subcellular fractions and involves dynamic complexes of structural proteins, nonstructural proteins, and HCV RNA demarcated by membrane structures. It is therefore of great interest to determine whether nucleolin is involved in such HCV-replicating intermediates in compartmented subcellular structures.

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Cytotoxic T Cell Responses to Human Telomerase Reverse Transcriptase in Patients With Hepatocellular Carcinoma

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Human telomerase reverse transcriptase, hTERT, has been identified as the catalytic enzyme required for telomere elongation. hTERT is expressed in most tumor cells but seldom expressed in most human adult cells. It has been reported that 80% to 90% of hepatocellular carcinomas (HCCs) express hTERT, making the enzyme a potential target in immunotherapy for HCC. In the current study, we identified hTERT-derived, HLA-A*2402–restricted cytotoxic T cell (CTL) epitopes and analyzed hTERT-specific CTL responses in patients with HCC. Peptides containing the epitopes showed high affinity to bind HLA-A*2402 in a major histocompatibility complex binding assay and were able to induce hTERT-specific CTLs in both hTERT cDNA-immunized HLA-A*2402/K^b transgenic mice and patients with HCC. The CTLs were able to kill hepatoma cell lines depending on hTERT expression levels in an HLA-A*2402–restricted manner and induced irrespective of hepatitis viral infection. The number of single hTERT epitope-specific T cells detected by ELISPOT assay was 10 to 100 specific cells per 3×10^5 PBMCs, and positive T cell responses were observed in 6.9% to 12.5% of HCC patients. hTERT-specific T cell responses were observed even in the patients with early stages of HCC. The frequency of hTERT/tetramer$^+$CD8$^+$ T cells in the tumor tissue of patients with HCC was quite high, and they were functional. In conclusion, these results suggest that hTERT is an attractive target for T-cell–based immunotherapy for HCC, and the identified hTERT epitopes may be valuable both for immunotherapy and for analyzing host immune responses to HCC. (HEPATOLOGY 2006;43:1284-1294.)

Hepatocellular carcinoma (HCC) is the most frequent primary malignancy of the liver and has gained much clinical interest because of its increasing incidence.[1-3] Although current advances in therapeutic modalities have improved the prognosis of HCC patients,[4-6] the survival rate is still not satisfactory. One of the reasons for the poor prognosis is the high rate of recurrence after treatment. To protect against recurrence, tumor antigen-specific immunotherapy is an attractive strategy. Although many tumor-specific antigens have been identified in various cancers, the number of HCC-specific antigens known is still limited.

Human telomerase reverse transcriptase, hTERT, has been identified as the catalytic enzyme required for telomere elongation.[7-10] Recently, several results regarding hTERT-specific cytotoxic T cell (CTL) responses were reported for humans and mice.[11-20] These reports revealed that hTERT-specific CTLs induced by stimulation with peptides or DNA-based immunization kill cancer cell lines that have high levels of hTERT, suggesting that hTERT-reactive T cell clones are not deleted from the human T cell repertoire and that hTERT may be a useful tumor-specific antigen as a target for T-cell–based immunotherapy for cancers. However, the existence of hTERT-specific CTLs and the relationship between immunological

Abbreviations: HCC, hepatocellular carcinoma; hTERT, human telomerase reverse transcriptase; CTL, cytotoxic T cell; HIV, human immunodeficiency virus; PBMC, peripheral blood mononuclear cell; AFP, alpha-fetoprotein; CMV, cytomegalovirus; FCS, fetal calf serum; TIL, tumor infiltrating lymphocyte; PCR, polymerase chain reaction; TRAP, telomerase repeat amplification protocol; IFN-γ, interferon gamma.

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responses and clinical factors have not been well characterized in patients with HCC.

In the current study, we first attempted to identify HLA-A*2402–restricted T cell epitopes derived from hTERT and then analyzed hTERT-specific immunological responses in HCC patients.

Patients and Methods

Patient Population. The study examined 72 HLA-A24–positive patients with HCC who were admitted to Kanazawa University Hospital between January 2002 and December 2004, consisting of 48 men and 24 women ranging from 46 to 81 years of age with a mean age of 67 ± 9 years. HCCs were detected by imaging modalities such as dynamic computed tomography (CT) scan, magnetic resonance imaging, and abdominal arteriography. The diagnosis of HCC was histologically confirmed by taking ultrasound-guided needle biopsy specimens in 29 cases, surgical resection in four cases, and autopsy in four cases. For the remaining 35 patients, the diagnosis was based on typical hypervascular tumor staining on angiography in addition to typical findings, which showed hyperattenuated areas in the early phase and hypoattenuation in the late phase on dynamic CT.[21] All subjects were negative for antibodies to human immunodeficiency virus (HIV) and gave written informed consent to participate in this study in accordance with the Helsinki declaration. Eleven healthy blood donors with HLA-A24, who did not have a history of cancer and were negative for hepatitis B surface antigen and anti-HCV antibody, served as controls.

Laboratory and Virologic Testing. Blood samples were tested for hepatitis B surface antigen and HCV antibody by commercial immunoassays (Fuji Rebio, Tokyo, Japan). HLA-based typing of peripheral blood mononuclear cells (PBMCs) from patients and normal donors was performed using complement-dependent microcytotoxicity with HLA typing trays purchased from One Lambda.

The serum alpha-fetoprotein (AFP) level was measured by enzyme immunoassay (AxSYM AFP, Abbott Japan, Tokyo, Japan), and the pathological grading of tumor cell differentiation was assessed according to the general rules for the clinical and pathological study of primary liver cancer.[22] The severity of liver disease (stage of fibrosis) was evaluated according to the criteria of Desmet et al.,[23] using biopsy specimens of liver tissue, where F4 was defined as cirrhosis.

Synthetic Peptides. To identify potential HLA-A24–binding peptides within hTERT, the sequence was reviewed using a computer-based program, which was employed by accessing the World Wide Web site BioInformatics and Molecular Analysis Section for HLA peptide binding predictions (available from http://bimas.cit.nih.gov). The HLA-A24–restricted epitopes derived from HIV envelope protein,[24] cytomegalovirus (CMV) pp65,[25] and HCV NS3 were used as control peptides to test for T cell responses, and the HLA-A2–restricted epitope derived from AFP[26] was used as a control peptide for HLA-A24 stabilization assay as previously described. Peptides were synthesized at Mimotope (Melbourne, Australia) and Sumitomo Pharmaceuticals (Osaka, Japan). They were identified using mass spectrometry, and their purities were determined to be greater than 80% by analytical high-pressure liquid chromatography.

Cell Lines. Three human hepatoma cell lines, HepG2, HuH6, and HuH7, were cultured in Dulbecco's minimum essential medium (Gibco, Grand Island, NY) with 10% fetal calf serum (FCS) (Gibco).

T2-A24 cells, which were T2 cells transfected with HLA-A*2402,[25] were cultured in RPMI 1640 medium containing 10% FCS and 800 μg/mL G418 (GibcoBRL, Grand Island, NY). The HLA-A*2402 gene-transfected C1R cell line (C1R-A24)[27] was cultured in RPMI 1640 medium containing 10% FCS and 500 μg/ml of hygromycin B(Sigma, St Louis, MO), and K562 was cultured in RPMI 1640 medium containing 10% FCS. All media contained 100 U/mL penicillin and 100 μg/mL streptomycin (GibcoBRL, Grand Island, NY).

Plasmid Construction. The plasmid which contains hTERT cDNA was subcloned as previously described.[28] In brief, the EcoRI-SalI fragment containing the hTERT cDNA was subcloned from pCI-Neo-hTERT, which was provided by Dr. Seishi Murakami (Cancer Research Institute, Kanazawa University). The fragment was subcloned into the EcoRI-SalI sites of the plasmid pNKZ-FLAG (pNKZ-FLAG-hTERT).

Injection of hTERT cDNA Into HLA-A*2402/K^b Transgenic Mice. Transgenic mice expressing the α1 and α2 domains from the HLA-A*2402 molecule and the α3 domain from the murine H-2K^b molecule,[29] kindly provided by Sumitomo Pharmaceuticals (Osaka, Japan), were bred in a specific-pathogen–free environment at the animal facility in Kanazawa University. For immunization with the hTERT cDNA, mice were injected with 50 μL cardiotoxin (Latoxan, Rosans, France) (10 μmol/L) per leg into the tibialis anterior muscles on both sides. Five days after injection of the cardiotoxin, the vector pNKZ-FLAG-hTERT containing the hTERT cDNA was injected into the same part of the muscle. Mice immunized with the plasmid pNKZ-FLAG were also used as negative controls. Splenocytes harvested on day 7 after the

Table 1. Characteristics of the Patients Studied

Clinical Diagnosis	No. of Patients	Sex M/F	Age (yr) Mean ± SD	ALT (IU/L) Mean ± SD	AFP (ng/mL) Mean ± SD	Etiology (B/C/Others)	Child-Pugh (A/B/C)	Diff. degree* (Wel/Mod/ Por/ND)	Tumor size** (Large/ Small)	Tumor multiplicity (Multiple/ Solitary)	Vascular Invasion (+/−)	TNM Stage (I/II/IIIA/ IIIB/IIIC/IV)
HCC patients	72	48/24	67 ± 9	66 ± 36	1722 ± 7029	9/59/4	43/25/4	15/21/1/35	44/28	39/33	15/57	30/26/9/1/2/4
Normal donors	11	8/3	35 ± 2	ND	ND	ND	ND	ND	ND	ND	ND	ND

*Histological degree of HCC; wel: well differentiated, mod: moderately differentiated, por: poorly differentiated, ND: not determined.
**Tumor size was divided into either "small" (≤2 cm) or "large" (> 2 cm).

injection of cDNA were tested directly *ex vivo* for IFN-γ production using an ELISPOT assay.

Preparation of PBMCs and Tumor-Infiltrating Lymphocytes. PBMCs were isolated as previously described.[30,31] Fresh PBMCs were used for the CTL assay, and the remaining PBMCs were resuspended in RPMI 1640 medium containing 80% FCS and 10% dimethyl sulfoxide (Sigma, St. Louis, MO) and cryopreserved until used. Tumor-infiltrating lymphocytes (TILs) were isolated by mechanical homogenization of tumors, which were resected by surgical treatment and cryopreserved as described until used.

Major Histocompatibility Complex Binding Assay. Peptide binding assays were performed as previously described.[31,32] The data were expressed as % mean fluorescence intensity (MFI) increase, which was calculated as follows: Percent MFI increase = (MFI with the given peptide − MFI without peptide)/(MFI without peptide) × 100.

ELISPOT Assay. ELISPOT assays were performed as previously described[31] with the following modifications. Three hundred thousand unfractionated PBMCs or 100,000 TILs with 10,000 T2-A24 cells were added in duplicate cultures of RPMI 1640 medium containing 5% FCS together with the peptides at 10 μg/mL. For the mouse assay, 2×10^5 spleen cells were used for each well. The number of specific spots was determined by subtracting the number of spots in the absence of antigen from that in the presence of antigen. Responses were considered positive for the human ELISPOT assay if more than 10 specific spots were detected and if the number of spots in the presence of antigen was at least twofold greater than that in the absence of antigen.

Cytotoxicity Assay. hTERT-derived peptide-specific T cells were expanded from PBMCs in 96-well round-bottomed plates (NUNC, Naperville, IL) as previously described.[30] Briefly, 400,000 cells per well were stimulated with 10 μg/mL synthetic peptide, 10 ng/mL rIL-7, and 100 pg/mL rIL-12 (Sigma) in RPMI 1640 medium supplemented with 10% heat-inactivated human AB serum, 100 U/mL penicillin, and 100 μg/mL streptomycin. The cultures were re-stimulated with 10 μg/mL peptide, 20 U/mL of rIL-2 (Sigma) and 1×10^5 mytomicin C-treated autologous PBMCs on days 7 and 14. On days 3, 10, and 17, 100 μL RPMI with 10% human AB serum and 10 U/mL rIL-2 (final concentration) were added to each well. Cytotoxicity assays were performed as previously described.[31]

Tetramer Staining and Flow Cytometry. Peptide $hTERT_{461}$-specific tetramer was purchased from Medical Biological Laboratories Co., Ltd (Nagoya, Japan). Tetramer staining was performed as previously described.[33] In brief, PBMCs and TILs were stained with CD4-FITC, CD14-FITC, CD19-FITC, CD8-PerCP (BD PharMingen, San Diego, CA), and tetramer-PE (10 μL) for 30 minutes at room temperature. Cells were washed, fixed with 0.5% paraformaldehyde/phosphate-buffered saline, and analyzed on a FACSCalibur flow cytometer. Data analysis was undertaken with CELLQuest software (Becton Dickinson, San Jose, CA).

Telomerase Assay. Telomerase activity was measured by two methods according to the manufacturer's directions. First, a polymerase chain reaction (PCR)-based telomerase repeat amplification protocol (TRAP) assay was carried out with a TRAPEZE ELISA telomerase detection kit (Intergen Co. Ltd., Auckland, New Zealand). The products of the PCR were fractionated by electrophoresis on a 10% polyacrylamide gel and then visualized by staining with SYBR-Green I (Molecular Probes, Eugene OR). Second, a TRAP enzyme-linked immunosorbent assay (ELISA) was used to quantitatively measure telomerase activity with a TRAPEZE ELISA telomerase detection kit (Intergen Co. Ltd.). Cell extracts were prepared from HepG2, HuH6, and HuH7 cells and used at 0.01 μg per assay. Telomerase activity was also measured in the tumor of 10 patients with HCC who received surgical treatment. Cell extracts were prepared from resected tumors and used at 0.1 μg per assay.

Statistical Analysis. Fisher's exact test (2-sided *P*-value) and the unpaired Student's *t* test were used to analyze the effect of variables on immune responses in HCC patients.

Results

Patient Profiles. The clinical profiles of the patients are shown in Table 1. The tumors of 37 patients were

Table 2. Peptides

Peptide	Source	Start Position	Amino Acid Sequence	HLA Restriction	Score*
$hTERT_{1088}$	hTERT	1088	TYVPLLGSL	HLA-A24	432
$hTERT_{845}$	hTERT	845	CYGDMENKL	HLA-A24	317
$hTERT_{167}$	hTERT	167	AYQVCGPPL	HLA-A24	300
$hTERT_{461}$	hTERT	461	VYGFVRACL	HLA-A24	280
$hTERT_{324}$	hTERT	324	VYAETKHFL	HLA-A24	240
$hTERT_{1009}$	hTERT	1009	AYRFHACVL	HLA-A24	200
$hTERT_{385}$	hTERT	385	RYWQMRPLF	HLA-A24	200
$hTERT_{637}$	hTERT	637	DYVVGARTF	HLA-A24	150
$hTERT_{622}$	hTERT	622	RFIPKPDGL	HLA-A24	72
$hTERT_{869}$	hTERT	869	DFLLVTPHL	HLA-A24	42
HIV env_{584}	HIV envelope	584	RYLRDQQLL	HLA-A24	720
CMV $pp65_{328}$	CMV pp65	328	QYDPVAALF	HLA-A24	168
HCV $NS3_{1031}$	HCV NS3	1031	AYSQQTRGL	HLA-A24	200
AFP_{137}	AFP	137	PLFQVPEPV	HLA-A2	3

*Estimated half-time of dissociation from the HLA-A24 or -A2 allele (min).

histologically classified as 15 well, 21 moderately, and 1 poorly differentiated HCC. Other patients were diagnosed with HCC based on typical CT findings and an elevation of AFP. The tumors were categorized as "large" (>2 cm) in 44 cases and "small" (≤2 cm) in 28 cases, and as "multiple" (≥2 nodules) in 39 cases and "solitary" (single nodule) in 33 cases. Vascular invasion of the HCC was observed in 15 cases. According to the TNM staging of the Union Internationale Contre Le Cancer (UICC) classification system (6th version),[34] 30, 26, 9, 1, 2, and 4 patients were classified as having stages I, II, IIIA, IIIB, IIIC, and IV disease, respectively.

Selection of Potential HLA-A24–Binding Peptides Within hTERT. To identify potential HLA-A24–binding peptides, the amino acid sequences of hTERT were analyzed using a computer program designed to predict HLA-binding peptides based on the estimation of the half-time dissociation of the HLA–peptide complex. Ten peptides were selected according to the half-time dissociation scores (Table 2). Two of the 10 peptides have been reported to contain HLA-A*2402–restricted epitopes (peptides $hTERT_{461}$ and $hTRERT_{324}$).[35] Next, MHC stabilization assays were performed to test the HLA-A*2402–binding capacity of these peptides using T2-A24 cells. Most peptides increased HLA-A24 expression, indicating that they bound and stabilized the HLA complex on the cell surface (Fig. 1). Peptide $CMVpp65_{328}$, which is identified as a strong binder of the HLA-A*2402 molecule,[25] also increased HLA-A24 expression. Percent MFI increase of the tested peptides except for peptides $hTERT_{1009}$, $hTERT_{385}$, and $hTERT_{622}$ was greater than that of peptide AFP_{137}, which is HLA-A2 restricted.[26]

Immunogenicity of hTERT Peptides in HLA-A*2402/K^b Transgenic Mice. To determine whether these HLA-A24–binding peptides include HLA-A*2402–restricted T cell epitopes, HLA-A*2402/K^b transgenic mice were immunized with hTERT cDNA, and the spleen cell responses were evaluated by interferon gamma (IFN-γ) ELISPOT. Six of 10 hTERT-derived peptides were recognized by the spleen cells of at least one of the primed mice (Fig. 2). Peptides $hTERT_{1009}$, $hTERT_{385}$, $hTERT_{622}$, and $hTERT_{869}$ were not recognized by any mice. These results show that peptides $hTERT_{1088}$, $hTERT_{845}$, $hTERT_{167}$, $hTERT_{461}$, $hTERT_{324}$, and $hTERT_{637}$ may be immunogenic and contain the epitopes restricted by HLA-A*2402.

T Cell Responses to hTERT-Derived Peptides Assessed by IFN-γ ELISPOT Analysis in HCC Patients. To determine whether these HLA-A24–binding peptides could be recognized by the T cells of patients with HCC, PBMC responses were evaluated by IFN-γ ELISPOT. Six of 10 hTERT-derived peptides were recognized by PBMCs of at least one patient, and 29 of 72 patients (40.3%) responded to at least one of the analyzed hTERT-derived peptides. An overview of all responses is shown in Fig. 3A. Single hTERT epitope-specific IFN-γ–producing cells were detected in 6 (8.3%), 6 (8.3%), 9 (12.5%), 5 (6.9%), 9 (12.5%), and 9 (12.5%) of 72 patients in response to the stimulation with peptides $hTERT_{1088}$, $hTERT_{845}$, $hTERT_{167}$, $hTERT_{461}$, $hTERT_{324}$, and $hTERT_{637}$, respectively. Peptides $hTERT_{1009}$, $hTERT_{385}$, $hTERT_{622}$, and $hTERT_{869}$ were not recognized by any patient.

The peptides recognized by PBMCs of the patients with HCC were comparable to those recognized by spleen cells of hTERT cDNA-immunized HLA-A*2402/K^b transgenic mice. These peptides also displayed a relatively high affinity for the HLA-A*2402 molecule compared with the negative control peptide (Fig. 1). The strength of the hTERT-specific T cell responses assessed by the frequencies of IFN-γ–producing cells in the PBMC population is between 10 and 100 specific cells per 3×10^5 PBMCs. Peptide $CMVpp65_{328}$, which includes an

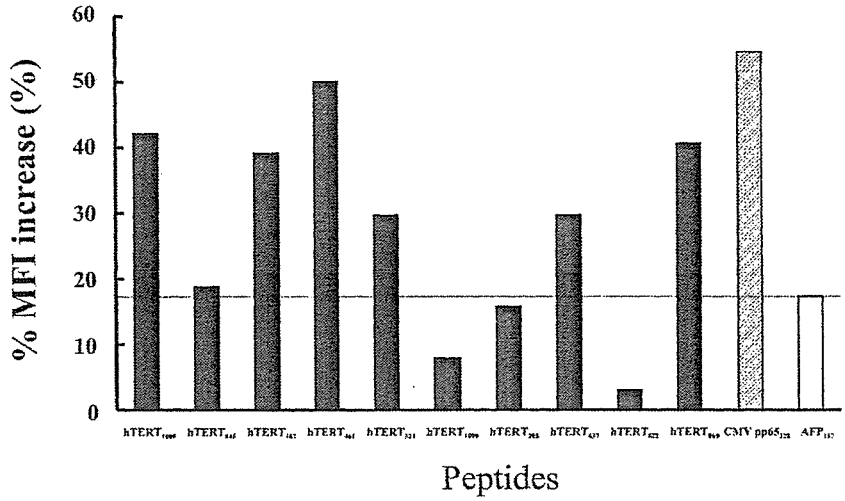

Fig. 1. MHC binding affinity. TAP-deficient T2-A24 cells were cultured for 16 hours at 26°C to enhance the expression of peptide-receptive cell surface molecules. They were incubated with individual peptides at 10 μg/mL at 37°C for 2 hours, washed, and stained with anti-HLA-A24 monoclonal antibody, anti-mouse immunoglobulin-conjugated FITC, and 1 μg propidium iodide per milliliter. The data are expressed as the percent mean fluorescence intensity (MFI) increase for live, propidium iodide-negative cells. Peptide CMVpp65$_{328}$, a previously identified CMV pp65-derived peptide known to be a strong binder to HLA-A24; was used as a positive control. Peptide AFP$_{137}$, a previously identified AFP-derived peptide known to be HLA-A2 restricted, was used as a negative control. The experiment was performed three times, and a representative result is shown. MHC, major histocompatibility complex; HLA, human leukocyte antigen; FITC, fluorescein isothiocyanate; CMV, cytomegalovirus.

epitope derived from the CMV pp65 protein, and HCVNS3$_{1031}$, which includes an epitope derived from the HCV NS3 protein, were also recognized by PBMCs of 31 of 72 (40%) and 12 of 51 (24%) patients with HCC, respectively. Conversely, no patients showed positive T cell responses against peptide HIVenv$_{584}$ derived from the HIV envelope protein, suggesting that these T cell responses were antigen-specific.

In contrast to the results for HCC patients, the ELISPOT assays for the healthy donors did not show more than 10 specific spots for all hTERT-derived peptides (Fig. 3B). The numbers of specific spots (mean ± SD) in the healthy donors were 1.4 ± 1.7, 0.6 ± 0.8, 0.8 ± 1.1, 0.7 ± 1.2, 0.5 ± 0.7, 0.6 ± 1.2, 2.0 ± 2.6, 1.7 ± 2.6, 1.6 ± 3.4, and 1.9 ± 2.9 for hTERT$_{1088}$, hTERT$_{845}$, hTERT$_{167}$, hTERT$_{461}$, hTERT$_{324}$,

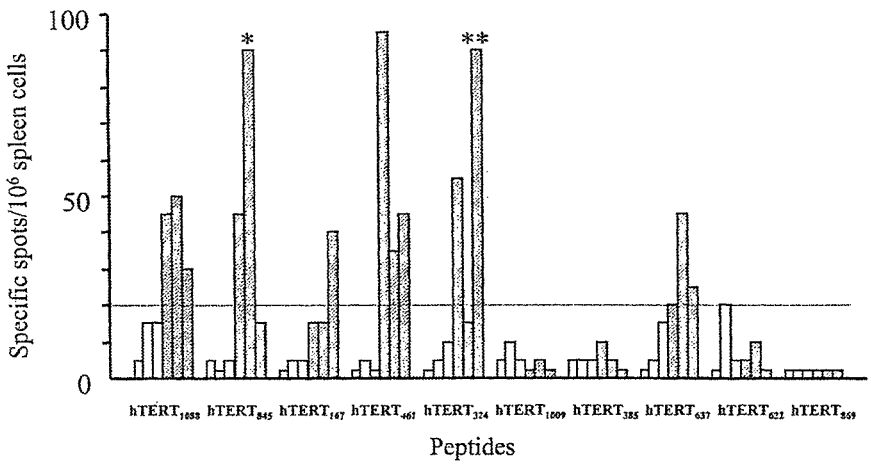

Fig. 2. Direct *ex vivo* analysis (IFN-γ ELISPOT assay) of spleen cell responses to hTERT-derived peptides in hTERT cDNA (hatched bars) or β-gal cDNA (open bars)-immunized HLA-A*2402/K^b transgenic mice. The immunization was performed in three mice for each cDNA. A positive T cell response was defined as more than 20 specific spots/1 × 10^6 spleen cells, which was the maximum response in β-gal cDNA-immunized mice. The peptide sequences are described in Table 2. * denotes 450 specific spots, ** denotes 130 specific spots. IFN-γ, interferon gamma; hTERT, human telomerase reverse transcriptase.

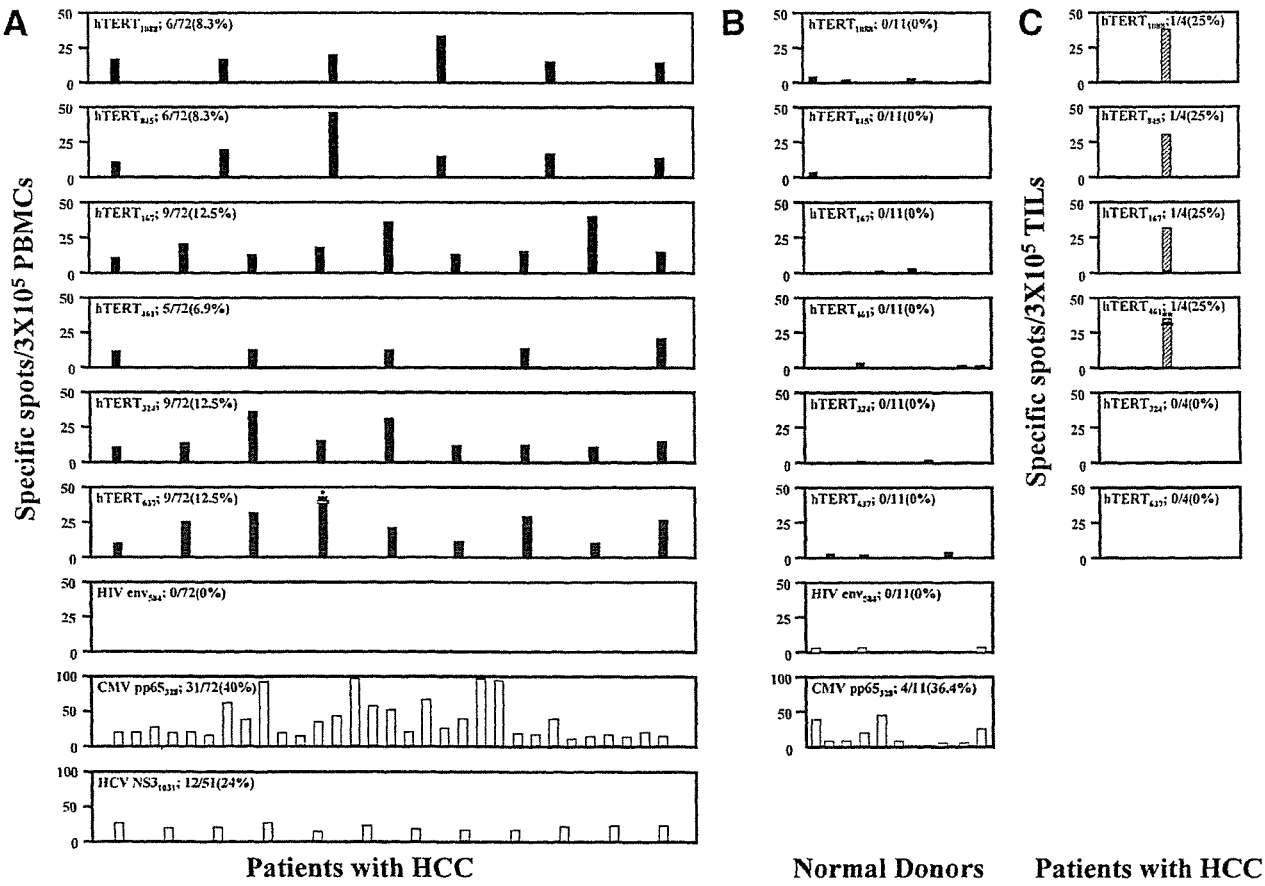

Fig. 3. Direct *ex vivo* analysis (IFN-γ ELISPOT assay) of peripheral blood T cell responses to hTERT-derived peptides (peptides $hTERT_{1088}$, $hTERT_{845}$, $hTERT_{167}$, $hTERT_{461}$, $hTERT_{324}$, and $hTERT_{637}$; solid bars) or control peptides (Peptides $HIVenv_{584}$, $CMVpp65_{328}$ and $HCVNS3_{1031}$; open bars) in HCC patients (A) and normal donors (B). Direct *ex vivo* analysis of tumor-infiltrating lymphocyte responses to hTERT-derived peptides (hatched bars) in HCC patients (C). Only significant IFN-γ responses are included in A and C. Responses were considered positive if more than 10 specific spots were detected and if the number of spots in the presence of antigen was at least twofold greater than that in the absence of antigen. The peptide sequences are described in Table 2. The data for peptides $hTERT_{1009}$, $hTERT_{385}$, $hTERT_{622}$, and $hTERT_{869}$ are excluded because there was no positive T cell response. * denotes 100 specific spots. ** denotes 243 specific spots. IFN-γ, interferon gamma; hTERT, human telomerase reverse transcriptase; HCC, hepatocellular carcinoma.

$hTERT_{1009}$, $hTERT_{385}$, $hTERT_{637}$, $hTERT_{622}$, and $hTERT_{869}$ peptides, respectively. The proportion of normal donors who showed positive T cell responses to CMV protein-derived peptides and the frequencies of the specific T cells were virtually the same as those of the HCC patients (Fig. 3B).

In ELISPOT assay using TILs, IFN-γ–producing T cells responding to peptides $hTERT_{1088}$, $hTERT_{845}$, $hTERT_{167}$, and $hTERT_{461}$ were detected as shown in Fig 3C, suggesting that hTERT-specific TILs were functional.

Cytotoxic Activity Against hTERT-Derived Peptides in HCC Patients. All hTERT-derived peptides were tested for their potential to induce HLA-A24–restricted CTLs from PBMCs of HCC patients with HLA-A24. Each peptide was tested on at least 10 patients. After three rounds of stimulation with the synthetic peptides, responder cells that had been stimulated with peptides $hTERT_{1088}$, $hTERT_{845}$, $hTERT_{167}$, $hTERT_{461}$, $hTERT_{324}$, and $hTERT_{637}$ lysed the peptide-pulsed C1R-A*2402 cells as shown in Fig. 4. Conversely, other peptides failed to induce CTLs specific for the corresponding peptides.

Cytotoxic Activity of hTERT Peptide-Specific CTLs Against Hepatoma Cell Lines. To examine whether hTERT peptide-specific CTLs induced from PBMCs of HCC patients lyse hepatoma cell lines that express hTERT, we first checked the telomerase activity in three hepatoma cells. TRAP assays showed that the three hepatoma cells expressed hTERT; however, the expression in HuH6 cells was lower than that in HepG2 or HuH7 cells (Fig. 5A). The results were confirmed in the TRAP ELISA, which is a quantitative measurement of telomerase activity. The expression levels of hTERT in HepG2 and HuH7 cells were more than twofold higher than the level in HuH6 cells (Fig. 5B).

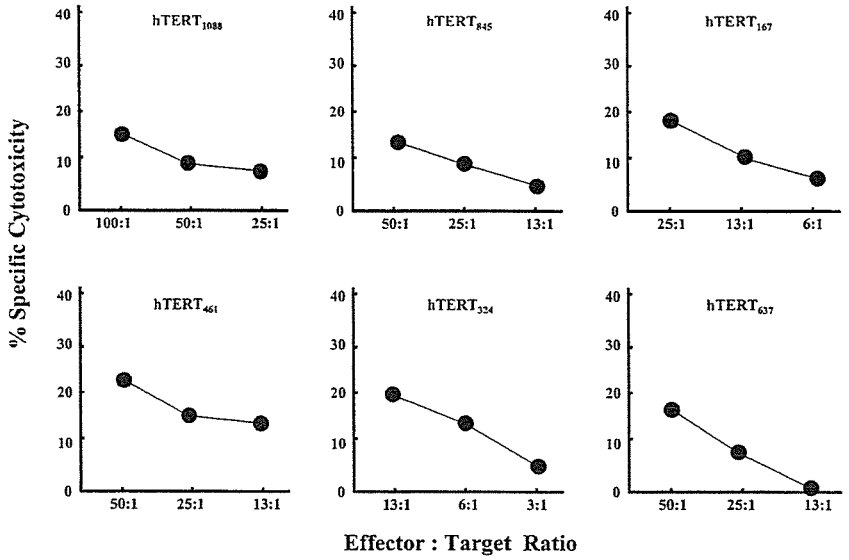

Fig. 4. Cytotoxicity of hTERT-specific T-cell lines derived with peptide in patients with HCC. The cytotoxicity of the T-cell lines was determined by a standard 6-hour cytotoxicity assay at various effector to target (E/T) ratios against C1R-A*2402 cells pulsed with one of the hTERT-derived peptides listed in Table 2. The data are indicated as the percent specific cytotoxicity, which is calculated as follows: (cytotoxicity in the presence of specific peptide) − (cytotoxicity in the absence of peptide). hTERT, human telomerase reverse transcriptase; HCC, hepatocellular carcinoma.

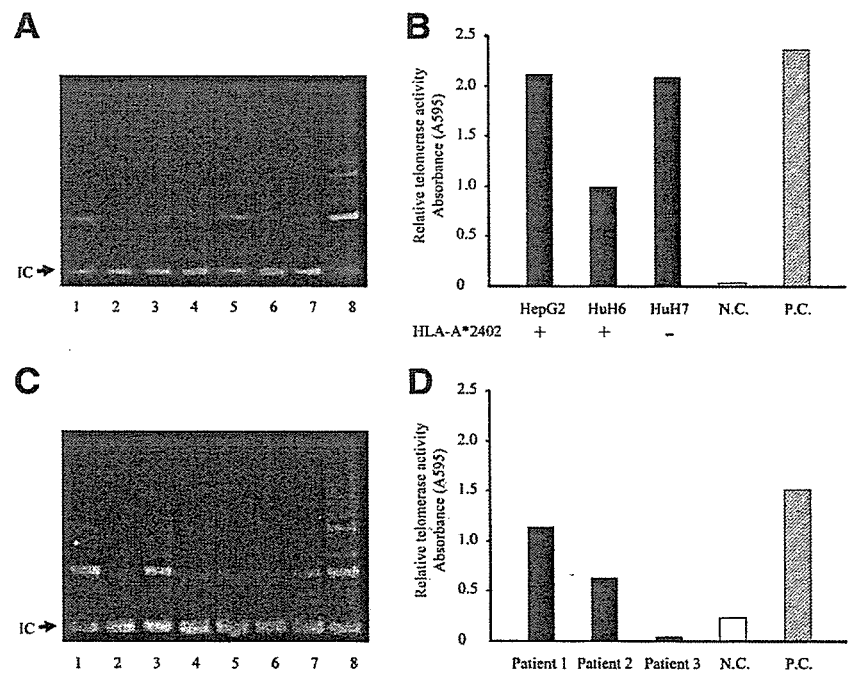

Fig. 5. Telomerase activity in hepatoma cell lines (A, B) and tumors resected by surgical treatment (C, D). A TRAP assay was carried out with 0.01 μg and 0.1 μg cell extract from hepatoma cell lines and tumors, respectively. The products of the PCR were fractionated by electrophoresis on a 10% polyacrylamide gel and then visualized by staining with SYBR-Green I. The TRAP internal control (IC) is shown for each extract. A: Lane 1; HepG2, Lane 2; HepG2 with heat, Lane 3; HuH 6, Lane 4; HuH 6 with heat, Lane 5; HuH 7, Lane 6; HuH 7 with heat, Lane 7; negative control, Lane 8; positive control. B: Lanes 1, 3, and 5, HCCs from three different patients; Lanes 2, 4, and 6, HCCs from three different patients with heat; Lane 7, negative control; Lane 8, positive control. Relative telomerase activity was measured with a TRAPEZE ELISA telomerase detection kit (TRAP ELISA) in hepatoma cell lines (C) and tumors resected by surgical treatment (D). Molecular typing of the HLA-A allele for hepatoma cell lines was performed with genomic DNA using standard site-specific oligonucleotide PCR. NC, negative control; PC, positive control; TRAP, telomerase repeat amplification protocol; PCR, polymerase chain reaction; HCC, hepatocellular carcinoma.

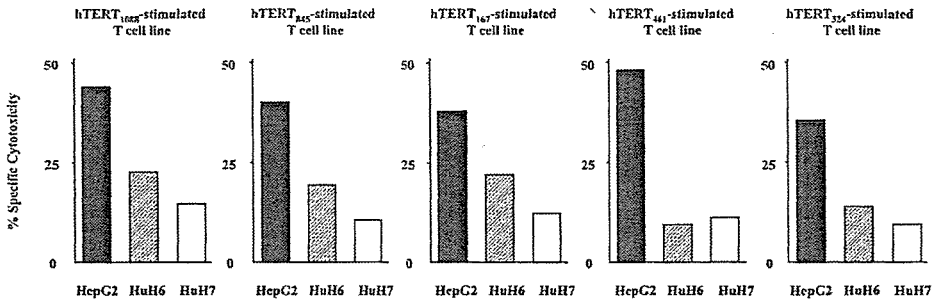

Fig. 6. Cytotoxicity of hTERT-specific T-cell lines derived with peptide against hepatoma cell lines. HepG2 (solid bar) highly expresses hTERT and has HLA-A*2402. HuH 6 (hatched bar) shows low expression of hTERT and has HLA-A*2402. HuH 7 (open bar) shows hTERT expression of the same level as HepG2 but does not have HLA-A*2402. The cytotoxicity was determined by a standard 6-hour cytotoxic assay (E/T ratio of 50:1). hTERT, human telomerase reverse transcriptase.

We next examined the cytotoxicity of hTERT peptide–specific CTLs against these hepatoma cell lines. As shown in Fig. 6, peptides $hTERT_{1088}$, $hTERT_{845}$, $hTERT_{167}$, $hTERT_{461}$, and $hTERT_{324}$-specific CTLs showed cytotoxicity against HepG2 cells, which highly express hTERT and has the HLA-A*2402 molecule. In contrast, the CTLs did not show cytotoxicity against HuH7 cells, which express hTERT at the same level as HepG2 cells but do not have HLA-A*2402. In addition, the cytotoxicity of hTERT-specific CTLs induced with peptides $hTERT_{1088}$, $hTERT_{845}$, $hTERT_{167}$, and $hTERT_{324}$ against HuH6 cells, which express HLA-A*2402 and a low level of hTERT, was weak compared with the cytotoxicity against HepG2 cells. The difference was even more marked in the cytotoxicity of CTLs induced with peptide $hTERT_{461}$, and the CTLs were not cytotoxic to HuH6 cells.

Telomerase activity was also detected in the tumor of 3 of 10 patients with HCC (Fig. 5C and D). All of the three patients showed hTERT-specific T cell responses in ELISPOT assay.

Detection of $hTERT_{461}$ Tetramer$^+$ and CD8$^+$ T Lymphocytes in PBMCs and TILs. To analyze the character of hTERT specific T cells in patients with HCC more precisely, we examined the frequencies of $hTERT_{461}$ tetramer$^+$ cells in PBMCs and TILs, and compared them with the results of ELISPOT assay. PBMCs and TILs were stained with CD4-FITC, CD14-FITC, CD19-FITC, CD8-PerCP, and tetramer-PE as described in Patients and Methods. At least 1×10^5 cells in the $CD8^+CD4^-CD14^-CD19^-$ gate were then analyzed for tetramer staining as shown in Fig. 7A.

As indicated in Fig. 7B, the frequencies of $CD8^+$ $CD4^-CD14^-CD19^-hTERT_{461}$ tetramer$^+$ cells in peripheral blood were 0.03% to 0.71% of $CD8^+$ T cells (patients 1-15). The frequencies in the patients with positive responses for ELISPOT assay were 0.06% to 0.71%. Interestingly, 7 of 10 patients without positive responses for ELISPOT assay showed 0.07% to 0.26% $CD8^+$ $CD4^-CD14^-CD19^-hTERT_{461}$tetramer$^+$ cells. These results suggest that dysfunctional hTERT-specific T cells exist in patients with HCC. Conversely, the frequency of $CD8^+CD4^-CD14^-CD19^-hTERT_{461}$ tetramer$^+$ cells in TILs was quite high (2.73%), and they were functional (patient 16).

hTERT-Specific T Cell Responses and Clinical Features of HCC Patients. To evaluate the status of hTERT-specific T cell responses in patients with HCC,

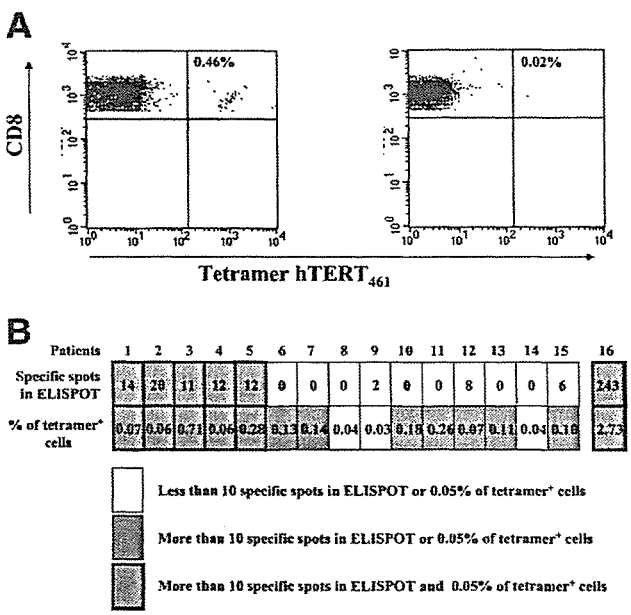

Fig. 7. Detection of hTERT-specific, HLA-A*2402-tetramer$^+$, $CD8^+CD4^-CD14^-$ $CD19^-$ T lymphocytes in the peripheral blood and tumor. PBMCs isolated from representative patients with HCC (A) were stained with tetrameric complexes and antibodies and analyzed on a FACSCalibur™ flow cytometer. Analysis of the association between the frequency of tetramer$^+$ cells and IFN-γ-producing cells detected in ELISPOT assay (B). Tetramer staining and ELISPOT assay were performed in 16 patients using PBMCs (patients 1-15) and TILs (patient 16). hTERT, human telomerase reverse transcriptase; PBMC, peripheral blood mononuclear cell; HCC, hepatocellular carcinoma; IFN-γ, interferon gamma; TIL, tumor-infiltrating lymphocyte.