存的な感染性を示し、HepG2 細胞には全く感受性を示さなかった。そこで、HCV のエンベロープ蛋白質を発現させた CHO 細胞と 293T 細胞を用いて、それぞれ HCVpv/CHO と HCVpv/293T を作製した。両シュードタイプウイルスとも高マンノース型のエンベロープ蛋白質を保持していた。HCVpv/CHO は hFGFR5 依存的に HepG2 細胞に高い感染性を示したのに対し、HCVpv/293T は hCD81 依存的に Huh7 細胞に高い感染性を示した。また、慢性C型肝炎患者血清中には HCVpv/293T に対する高い中和抗体が高率に検出されたのに対し、HCVpv/CHO に対する中和抗体価は低かった。患者血清中に存在する天然の HCV 粒子も、HCVpv と同様に HepG2 細胞と Huh7 細胞に結合した。HCV 粒子の HepG2 細胞への結合は可溶型 hFGFR5 や抗 hFGFR5 抗体で、また、Huh7 細胞への結合は抗 hCD81 抗体によって阻害された。

D. 考察

hFGFR5 は HCV の新しい受容体候補分子であることが示された。293T 細胞で作製した HCVpv はレトロウイルスで作製したシュードタイプと同様に hCD81 依存的な感染指向性を示し、CHO 細胞で作製すると hFGFR5 依存的に感染することが示された。また、C型肝炎患者血清中にも同様な親和性を示す HCV 粒子が存在することが示された。

E. 結論

C型肝炎患者の体内には hCD81-tropic と hFGFR5-tropic な HCV が産生されている可能性が示唆された。この性状の違いは、感染細胞の種類（肝細胞、リンパ球など）に起因するのか、あるいは同一細胞における感染経過の違いによるものなのかは今のところ不明である。hCD81-tropic な HCV は大量に産生されて、hCD81 との相互作用を介して、主に免疫機構の攪乱やクリオグロブリン血症などの肝外病変の発症に関与し、hFGFR5-tropic な HCV は少量産生され、抗体に中和されずに持続感染に関与しているのかも知れない。今後、異なる細胞親和性を示す HCV 粒子の産生様式と感染論学的な意義を明らかにしたい。

F. 健康危険情報

特になし。

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H. 知的所有権の出願・登録状況

特になし。

厚生労働科学研究費補助金（肝炎等克服緊急対策研究事業）

C型肝炎新規治療開発に資するプロテオーム解析を用いた治療標的分子の網羅的検索系とヒト肝細胞キメラマウスHCV感染モデルを用いた実証系の開発に関する研究班

分担研究報告書

ヒト肝細胞キメラマウスを用いた肝炎ウイルスの感染実験

分担研究者　高橋祥一　広島大学自然科学研究支援開発センター　助手

研究要旨：肝炎ウイルスに感染する小動物モデルの作製は、生体内における肝炎ウイルス増殖メカニズムの解明・新薬の開発に必須である。本研究において、マウス肝臓が高度にヒト肝細胞に置換された、ヒト肝細胞キメラマウスに野生型や変異株型のB型およびC型肝炎ウイルスを投与し、持続感染させることに成功した。このモデルマウスは抗ウイルス剤の効果判定に有用であり、さらにはウイルス蛋白の機能解析にも有用であった。本モデルマウスは、新規抗ウイルス剤の効果判定のスクリーニングや肝炎ウイルスの分子生物学的検討などの *in vivo* 研究に広く応用が可能であると思われる。

A.研究目的

肝炎ウイルスに感染する小動物モデルは確立されておらず、生体内における感染・複製のメカニズムの解明は困難である。本研究は、マウス肝臓が高度にヒト肝細胞に置換されたヒト肝細胞キメラマウスを用いて肝炎ウイルス感染マウスを作製し、肝炎ウイルスの基礎的研究に有効利用することを目的とする。

B.研究方法

Albプロモーター下にuPAを高発現し、生後、肝細胞がアポトーシスを起こすAlb-uPA Tgマウスと重症免疫不全であるSCIDマウスを交配させたuPA-SCIDマウスにヒト肝細胞を経脾的に投与し、マウス肝臓が高度にヒト肝細胞に置換された、ヒト肝細胞キメラマウス（キメラマウス）を用いる。キメラマウスへB型（HBV）およびC型肝炎ウイルス（HCV）陽性患者血清やHBVを産出する細胞培養上清を経静脈的に投与する。投与後、定時的にマウス血液を採取し、血中HBV-DNAおよびHCV-RNAを測定し、感染確認後、抗ウイルス剤と投与する。

なお、血清を用いる患者には、あらかじめ本研究目的を説明し、同意を得た。

また、マウスの処置の際は、ジエチルエーテルを用いた麻酔下に行い、マウスの苦痛はごく軽度にとどまるものと思われる。

C.結果

・ HBV陽性血清の投与の投与により、10^7～10^9 コピー/mLのウイルス血症が長期にわたり持続した。肝免疫組織学的検討において、ヒトアルブミン陽性のヒト肝細胞は、HBc-Ag陽性であり、置換されたヒト肝細胞に特異的にHBVが感染していることが確認された。このHBV感染マウスに30 mg/kg/日のラミブジンを経口投与すると、血中HBV-DNAは著明に低下した。

・ 1.4倍長のHBV ゲノムを組み込んだplasmidを作製し、HepG2細胞にstable transfectionし、上清中に約10^6 コピー/mLのHBVを恒常的に産出する細胞を作製した。野生型のYMDD株と同時に、ラミブジン耐性であるYVDD株を産出する細胞も作製した。これらの細胞の培養上清をキメラマウスへ投与することにより、HBV感染が確認された。これらの感染マウスに30 mg/kg/dayのラミブジンを経口投与したところ、YMDD株感染マウスでは血中HBV-DNAは低下したが、YVDD株感染マウスでは低下しなかった。

・ 培養上清の投与によるHBV感染マウスの血清をnaïveなキメラマウスに投与したところ、血中にHBV-DNAが検出され、Passageも可能であることが確認された。また、高容量の培

養上清や濃縮による高濃度のHBVを投与することにより、マウスへの感染性が向上した。
・ 変異株を用いてHBV蛋白の機能の解析を行った。e抗原の機能解析のため、e抗原を欠失させたコンストラクトを作製し、その培養上清をキメラマウスに投与したところ、HBV感染が成立した。このことより、e抗原はHBVの感染・複製には必須ではないことが示された。
・ HCV陽性患者血清の投与により、10^6～10^7 copy/mLのウイルス血症が長期にわたり持続した。肝免疫組織学的検討ではヒトアルブミン陽性細胞はコア蛋白陽性であり、置換されたヒト肝細胞に特異的にHCVが感染していることが確認された。このHCV感染マウスへ7000単位/kg/dayのIFN-αを連日筋注したところ、血中HCV-RNA量は感度以下に低下した。またIFN-αの投与中止により、HCV-RNAは再陽性化した。

D.**考察**
キメラマウスを用いて有効な肝炎ウイルス感染モデルマウスが作製された。作製したモデルは、既知の抗ウイルス剤の効果判定に有用あり、今後、新規候補となる抗ウイルス剤の生体内における効果判定としても有用であると思われる。

E.**結論**
リバースジェネティクス法により種々の変異ウイルスを血中に有するマウスの作製が可能であり、生体内における肝炎ウイルスの分子生物学的な検討に、広く応用が可能であると思われる。

F.**健康危機情報**
特になし

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H.**知的財産権の出願・登録状況**
今回の研究内容については特になし

Ⅲ. 研究成果の刊行に関する一覧表

研究成果の刊行に関する一覧表

雑誌

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Ⅳ. 研究成果の刊行物・別刷

G to A Hypermutation of Hepatitis B Virus

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G to A hypermutation of the human immunodeficiency virus type 1 (HIV-1) is induced by a deaminase APOBEC3G and is related to host antiviral defense. APOBEC3G has also been found to reduce the replication of HIV-1 by an unknown mechanism. This enzyme also reduces the production of hepatitis B virus, although the mechanism for this action has not been clearly elucidated. The hypermutated hepatitis B virus (HBV) is rarely found in usual sequencing analyses. Using peptide nucleic acid mediated by polymerase chain reaction clamping, we detected the hypermutated HBV DNA in 1 of 8 patients with acute HBV infection and 4 of 10 with chronic HBV infection. In the latter group, hypermutated genomes were found only in eAb-positive patients. As much as 72.5% of G residues were mutated in the hypermutated clones. G to A substitutions were predominant in almost all clones sequenced compared with other substitutions. G to A mutated viral genomes also were found in HepG2–derived cell lines that continuously produced HBV into the supernatant. Both alpha and gamma interferon reduced virus production in these cell lines, but they did not alter the frequency of the hypermutation. Transcripts of APOBEC3G, as well as some other deaminases, were found in these cell lines. In conclusion, our results show that part of the minus strand DNA of HBV is hypermutated both *in vitro* (HepG2 cell lines) and *in vivo*. The role and mechanism of hypermutation in reducing HBV replication should be further investigated to understand the anti-HBV defense system. (HEPATOLOGY 2005;41:626-633.)

Hepatitis B virus (HBV) is a small enveloped DNA virus that replicates in hepatocytes in a noncytolytic manner. Chronic infection with the virus often leads to chronic hepatitis and liver cirrhosis. Hepatocellular carcinoma arises in chronic carriers at a higher frequency than noninfected individuals.[1-4]

The replication cycle of the HBV includes pregenome RNA synthesis and reverse transcription, resulting in the production of the minus strand DNA, which serves as a template of the plus strand DNA.[5] The life cycle of this virus resembles that of the human immunodeficiency virus 1 (HIV-1), which also replicates through reverse transcription.[6]

Recent reports showed that a cytosine deaminase APOBEC3G (apolipoprotein B mRNA-editing enzyme, catalytic polypeptide-like 3G), which is packaged in HIV-1 virions, induces G to A hypermutation to a nascent reverse transcript of HIV-1, which contributes in part to the innate antiviral activity.[7-10] The antiviral activity of APOBEC3G is species specific[11,12] and may represent the different actions of the protein.[13,14] The virion infectivity factor encoded by lentivirus genomes associates with APOBEC3G to prevent the enzyme from being packaged into virions and triggers its proteasomal degradation.[15-18] The negative strand DNA of the HBV might be a target of such antiviral deaminase activity. In fact, naturally occurring HBV genomes bearing the hallmarks of retroviral G to A hypermutation have been reported in clones obtained from 2 HBV carriers.[19] Both of these clones represented subgenomes arising from reverse transcrip-

Abbreviations: HBV, hepatitis B virus; HIV-1, human immunodeficiency virus type 1; APOBEC3G, apolipoprotein B mRNA-editing enzyme, catalytic polypeptide-like 3G; HBsAg, hepatitis B surface antigen; HBeAg, hepatitis B early antigen; PCR, polymerase chain reaction; PNA, peptide nucleic acid.

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tion of packaged spliced mRNA. However, such hypermutated genomes have otherwise never been reported, nor deposited in DNA databases. Moreover, whether such hypermutated sequences are generated in liver cells or in leukocytes is unknown.

Inhibition of HBV replication by APOBEC3G was observed recently in a transient transfection system.[20] However, no induction of hypermutations to the HBV genome was observed. Instead, prevention of pre-genome RNA packaging was observed.

The aims of the current study were to determine the frequency of viral genomes with G to A substitutions in HBV carriers and patients with acute HBV infection, and to determine whether the hypermutated sequences are generated in hepatic cell lines. We identified such hypermutated viral genomes in 5 of 18 HBV carriers and patients with acute HBV infection and the expression of known deaminases that are potentially responsible for the hypermutation in cultured hepatoma cell lines.

Materials and Methods

Serum Samples. Serum samples from 18 adult Japanese patients with HBV infection were studied. At the time of the study, 8 of these patients had acute HBV infection and tested positive for immunoglobulin M anti–hepatitis B core antibody. The remaining 10 patients were chronic carriers. All serum samples were stored at −80°C until examined. All patients were negative for serum markers of both hepatitis C virus and HIV-1 infection, and none was on antiviral treatment.

Serological Markers of HBV Infection. Hepatitis B surface antigen (HBsAg) was detected by enzyme immunoassay (Roche Diagnostics, Basel, Switzerland), and hepatitis B early antigen (HBeAg) as well as anti-HBe were detected by radioimmunoassay (Abbott Diagnostics, Abbott Park, IL). HBV DNA was determined by transcription-mediated amplification and hybridization-protection assay (Chugai Diagnostics, Tokyo, Japan), and the results were expressed as log genome equivalents per milliliter. The lower detection limit of this assay is 3.7 log genome equivalents/mL (equivalent to 5,000 copies/mL). The antibody against hepatitis C virus was tested for by the third-generation enzyme immunoassay (Roche Diagnostics).

Analysis of HBV DNA in Cell Lines That Stably Produce HBV. Two cell lines known to produce wild-type HBV and one cell line known to produce lamivudine-resistant HBV (with mutations of L528M and M552V) were created by transfecting 1.4 genome length sequences of HBV to HepG2 cell lines. These cell lines produced HBV that showed a similar sedimentation in sucrose density gradient centrifugation to HBV extracted from the serum of carriers (M. Tsuge et al., manuscript in preparation) and could infect human hepatocyte chimeric mice (manuscript in preparation). These cell lines were grown in Dulbecco's modified Eagle's medium supplemented with 10% (vol/vol) fetal bovine serum at 37°C and 5% CO_2. Cells were seeded to semiconfluence in 6-well tissue culture plates and then treated with media containing interferon alpha or gamma. After 3 days of interferon treatment, the cells were harvested and lysed with 250 μL lysis buffer (10 mmol/L Tris-HCl [pH 7.4], 140 mmol/L NaCl, 0.5% [vol/vol] NP-40) followed by centrifugation for 2 minutes at 15,000*g*. Replicative intermediate of the HBV was immunoprecipitated and subjected to Southern blot analysis and quantitative analysis by light cycler. The effect of lamivudine was analyzed similarly, except that cells were harvested after 5 days of treatment.

Detection of Hypermutated Clones by Polymerase Chain Reaction With PNA Clamping, Cloning, and Sequencing. HBV DNA was extracted from 100 μL serum or culture supernatant by SMITEST (Genome Science Laboratories, Tokyo, Japan) and was dissolved in 20 μL H_2O. The first round of polymerase chain reaction (PCR) was performed with an outer primer set (PLF1 and BR112 [Table 1]) and a second-round PCR with an inner primer set (PLF2 and PLR2 [Table 1]). The peptic nucleic acid (PNA) oligonucleotide, initially designed to detect lamivudine-resistant variant genome,[21] was an 18-mer (PNA 552 [Table 1]) that exactly matched the 18-nucleotide sequence of the original YMDD sequence of DNA polymerase/reverse transcriptase, which contained GG and TG sequences (AGT TAT ATG GAT GAT GTG). The PCR with PNA clamping was performed in a total volume of 25 μL, consisting of a reaction buffer (100 mmol/L Tris-HCl [pH 8.3], 50 mmol/L KCl and 15 mmol/L $MgCl_2$), 0.2 mmol/L each of dNTPs, 1 μL of the DNA solution, 12.5 pmol each primer, 150 pmol PNA 552, and 1 unit of Taq DNA polymerase (Gene Taq, Wako Pure Chemicals, Tokyo, Japan) together with 0.2 μg anti-Taq high (Toyobo Co., Osaka, Japan). The amplification conditions included an initial denaturation at 95°C for 4 minutes and 25 cycles of amplification (denaturation at 95°C for 45 seconds, PNA annealing at 73°C for 2 minutes, annealing and extension of primer at 63°C for 50 seconds), followed by a final extension at 63°C for 7 minutes. Part of the X gene was amplified with an outer primer pair (HBV1 and HBV2) and an inner primer (PLF2 and HBV2) (Table 1) for the first- and second-round amplifications, respectively. The amplification for the first-round PCR included initial denaturation at 95°C for 4 minutes and 25 cycles of amplification (denatur-

Table 1. Oligonucleotides and PNAs Used in the Current Study

Primer	Sequence
	HBV amplification
PLF1	5′-GGT ATG TTG CCC GTT TGT CC-3′
BR112	5′-TTC CGT CGA CAT ATC CCA T-3′
PLF2	5′-CCT ATG GGA GTG GGC CTC AG-3′
PLR2	5′-CCA ATT ACA TAT CCC ATG AAG TTA AGG GA-3′
HBV1	5′-CCG GAA AGC TTG AGC TCT TCT TTT TCA CCT CTG CCT AAT CA-3′
HBV2	5′-CCG GAA AGC TTG AGC TCT TCA AAA AGT TGC ATG GTG CTG G-3′
BR109	5′-AAG GGA GTA GCC CCA ACG TT-3′
	PNA
PNA552	H2N-CAC ATC ATC CAT ATA ACT-CON2H
PNA552V	H2N-CAC ATC ATC CAC ATA ACT-CON2H
	Amplification of mRNAs of deaminases
APO1a	5′-CAG AGC ACC ATG ACT TCT-3′
APO1d	5′-ATT GTG GCC AGT GAG CTT CA-3′
APO2a	5′-AGA AGG AAG AGG CTG CTG TG-3′
APO2b	5′-AGA ACG GCT GCC TGC CAA CT-3′
APO2c	5′-GAA GGC TGG CAG GAT GGT GT-3′
APO2d	5′-CAG GTG ACA TTG TAC CGC AG-3′
APO3Aa	5′-TCT TAA CAC CAC GCC TTG AG-3′
APO3Ad	5′-GAA GAT GCG CAG TCT CAC GT-3′
APO3Ba	5′-AGA GCG GGA CAG GGA CAA GC-3′
APO3Bb	5′-GCG TAT CTA AGA GGC TGA AC-3′
APO3Bd	5′-CGA AGG ACC AAA GGG TCA TT-3′
APO3Be	5′-ACA AGT AGG TCT GGC GCC GT-3′
APO3Ca	5′-AGG ACG CTG TAA GCA GGA AG-3′
APO3Cb	5′-CCG ATG AAG GCA ATG TAT GG-3′
APO3Cc	5′-GTC GTC GCA GAA CCA AGA GA-3′
APO3Cd	5′-GAT GTG TAC CAG GTG ACC TG-3′
APO3Da	5′-CTG GGA CAA GCG TAT CTA AG-3′
APO3Dd	5′-AGT CTG AGA TGA AGA GGT GG-3′
APO3Fa	5′-CTT GGG TCC TGC CGC ACA GA-3′
APO3Fd	5′-TCA TCC TTG GCC GGC TAG TC-3′
APO3Ga	5′-GAC TAG CCG GCC AAG GAT GA-3′
APO3Gb	5′-CAC AGT GGA GCG AAT GTA TC-3′
APO3Gc	5′-GTT CGG AAT ACA CCT GGC CT-3′
APO3Gd	5′-ACT CCT GGT CAC GAT GCA GC-3′

ation at 95°C for 45 seconds, PNA annealing at 73°C for 2 minutes, primer annealing at 60°C for 1 minute, and extension of primer at 63°C for 4 minutes), followed by the final extension at 63°C for 7 minutes. The second-round amplification was performed under the same conditions without a primer extension for 3 minutes. The estimated error rate of the Taq DNA polymerase was 1.76×10^{-5} per site in amplifying approximately 10^2 copies of plasmid under the same conditions as described previously and cloning and sequencing.[21] Products (1 μL each) of the second-round of PNA PCR were subjected to PCR with primers PLF2 and BR109 for 35 cycles (94°C, 1 minute; 58°C, 1 minute; 72°C, 1.5 minutes) after initial denaturation at 94°C for 4 minutes and followed by the final extension at 72°C for 7 minutes. Amplicons were purified by electrophoresis on 2% (wt/vol) agarose gel and cloned into pGEM-T Easy Vector (Promega, Madison, WI) with the standard method, and then transformed into *Escherichia coli* JM 109 (Takara Shuzo Co., Otsu, Japan). Sequencing was performed in the ABI PLISMTM 310NT Genetic analyzer (Applied Biosystems, Tokyo, Japan) with Big Dye terminator version 3.0 Cycle Sequencing Ready Reaction kit (Applied Biosystems). Ten independent clones from each serum sample of patients or supernatant of cell cultures were sequenced for analysis and compared for nucleotide sequences obtained by direct sequencing of PCR products. Hypermutation was defined as clones with a statistically significant number of G to A substitutions.

Sequence Analysis. Nucleotide sequences were aligned and parameters of hypermutation were evaluated with Hypermut Program Package[22] (http://www.hiv.lanl.gov/HYPERMUT/hypermut.html). We used nucleotide sequences obtained by direct sequencing as reference sequences and tentatively labeled clones with a statistically significant ($P < .05$ by Fisher's exact test) number of G to A substitutions as "hypermutated."

Detection of mRNA of Known Deaminases by Reverse Transcription and PCR. Total RNA was extracted from HepG2 cell lines by using cell-to-cDNAII kit (Ambion, Austin, TX). The extracted RNA was reverse transcribed with random primer and M-MLV reverse transcriptase (ReverTra Ace, TOYOBO, Osaka, Japan) at 42°C for 60 minutes according to the instructions provided by the manufacturer. Synthesized cDNAs were used to detect mRNAs of known deaminases using primers listed in Table 1. Each of these primers was carefully designed to amplify only the target member of the APOBEC families. Amplification of specific deaminases was confirmed by amplifying each deaminase cDNA by using cDNAs obtained from organs reported to be positive for the expression of each deaminase. The amplicons were analyzed in 2% agarose gel, and the nucleotide sequences were confirmed by direct sequencing.

Results

Frequent Detection of G to A Substituted HBV Genomes by PCR With PNA Clamping in Patients With Acute or Chronic Hepatitis B Virus Infection. Using PCR with PNA clamping, clones with multiple G to A substitutions were found (Table 2). In contrast, only small numbers of other substitutions were identified in these clones. A hypermutated genome of HBV was found in 1 of 8 patients with acute HBV infection and 4 of 10 patients with chronic HBV infection (Table 2). We cloned and sequenced more than 20 clones without PNA and found no hypermutated clones. Among patients with chronic HBV infection, hypermutated clones were identified only in eAb-positive patients (Table 2). Figure 1

Table 2. Nucleotide Substitutions of Clones Amplified by PCR With PNA Clamping and Clinical Features of Patients With Acute and Chronic Hepatitis B Virus Infections

Patient	No. of Substitutions*		No. of Clones†	Pre-core‡	CP§	eAg	eAb	HBV DNA	ALT
	G to A	Other							
A-1	27	3	8 (1)	G	A/G	42	0	5.1	2,517
A-2	13	4	8	G	A/G	7.8	88	6.1	3,778
A-3	12	2	5	A/G	A/G	190	0	<3.7	1,417
A-4	11	0	4	G	A/G	58.3	0	4.5	2,550
A-5	11	3	9	G	A/G	170	0	8.3	175
A-6	7	7	9	A/G	Mixed	260	0	7.8	28
A-7	1	2	4	G	Mixed	0.1	99.4	4.1	2,295
A-8	1	1	3	A	T/A	0.7	91	7.1	6,183
C-1	152	2	10 (10)	A	T/A	0.3	100	5.5	394
C-2	44	12	9 (4)	A/G	T/A	18.2	73.4	6.2	340
C-3	30	4	10 (1)	A/G	T/A	0.3	97	7.3	53
C-4	23	1	3	G	A/G	140	0	5.9	2,770
C-5	22	1	8 (1)	A	T/A	0.4	95	6.5	105
C-6	19	9	9	A/G	Mixed	200	0	8.2	113
C-7	18	5	7	G	T/A	170	0	6.6	31
C-8	17	1	7	G	A/G	200	0	7.7	92
C-9	12	4	7	G	T/A	180	0	>8.8	56
C-10	6	4	7	A	A/G	2.5	95	8.3	267

*Total number of nucleotide substitutions in 10 clones compared with sequences obtained by direct sequencing.

†Number of different clones of 10 clones sequenced. Figures in parentheses represent the number of clones with hypermutation (those with a statistically significant number of G to A substitutions).

‡Nucleotide sequence of codon 28 of pre-core protein (nucleotide 1896).

§Nucleotide sequence of basic core promoter (nucleotides 1762 and 1764). Mixed represents mixture of A/G and T/A.

illustrates hypermutations found in an eAb-positive patient with chronic HBV infection (C-1 in Table 2). As much as 72.5% (29 of 40) of G residues were mutated in such hypermutated clones. Hypermutation was found in both the envelope/polymerase region (Fig. 1A) and x region (Fig. 1B) of HBV genome obtained from this patient. Preference of G to A mutation was similar with those reported in HIV-1; that is, G residues in GA sequences were the most frequently hypermutated (Fig. 2). In contrast, the G residues in CxG context were less frequently substituted (Fig. 2). Numerous G to A nucleotide substitutions were identified in clones lacking a statistically significant number of G to A hypermutations (Table 2). The number of such substitutions was apparently greater than "other substitutions" (Table 2). There was no relationship between the degree of hypermutation and serum alanine aminotransferase concentration or HBV DNA level (Table 2).

Fig. 1. G to A hypermutations detected in sequences of HBV DNA in sera extracted from an HBe antibody-positive HBV carrier (Patient C-1, Table 2) by PCR with PNA clamping. (A) DNA sequence alignment in the HBs antigen/polymerase region of the HBV. The nucleotide sequences that were obtained by direct sequencing were used as a reference sequence (**top line**). The target sequence of PNA annealing is **underlined**. (B) DNA sequence alignment in the x region of the HBV.

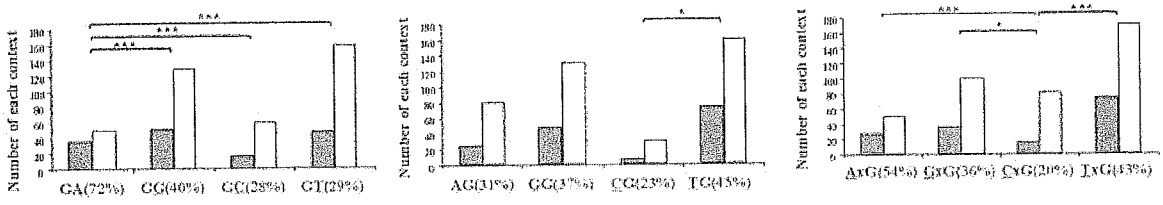

Fig. 2. Preferred nucleotide contexts of G to A hypermutation in 10 clones from patient C-1 (Table 2). The preferred nucleotide letter one letter after (left), one letter before (middle), and two places before (right, x = any) the target G residue. **Open bars:** number of occurrences of each context in the sequence analyzed. **Gray bars:** number of G residues mutated to A. The percentage in parentheses represents the rate of mutated G residues.*$P < .05$, *** $< .001$ (Fisher's exact test or chi-square test).

G to A Hypermutation in HBV-Producing Cell Lines. We established HepG2 cell lines that continuously produced HBV into the medium and examined the frequency of hypermutation. Hypermutated clones were identified in one of these cell lines (Table 3 and Fig. 3). The preference of G to A mutation was similar to that found in serum samples obtained from patients (data not shown). Various levels of HBsAg, HBeAg, and HBV DNA were released into the medium from these cells (Table 3). No relationship was found between the frequency of the hypermutated genome and intracellular intermediates of HBV DNA and HBsAg and HBeAg levels (Table 3). Figure 4 shows replicative intermediates of the HBV produced in these cell lines detected by Southern blot analysis (Fig. 4). No noticeable difference was observed between a cell line with hypermutated genomes and those without hypermutated genomes (lanes 1 and 2 in Fig. 4).

G to A Hypermutation During Antiviral Treatment. We treated the cell lines with alpha and gamma interferon and lamivudine. Both interferons reduced HBV DNA production from these cells in a dose-dependent manner (Fig. 5). The frequency of G to A hypermutation did not increase in those treated cell lines (Fig. 6), suggesting that G to A hypermutation is not responsible for antiviral defense through these interferons. Treatment of a cell line with lamivudine resulted in marked reductions in the production of HBV in the supernatant as well as intracellular viral intermediates (Fig. 7) and completely abolished identification of G to A substitution (Fig. 6). A similar reduction of detection of hypermutated clones was observed in serum samples obtained from patients who were treated with lamivudine (data not shown).

Table 3. Nucleotide Substitutions of Clones Amplified by PCR With PNA Clamping in Three Cell Lines That Produce the Hepatitis B Virus

Cell Line	No. of Substitutions* G to A	No. of Substitutions* Other	No. of Clones†	eAg	HBs Ag	HBV DNA
Cell line 1	102	0	10 (7)	17	4.7	5.2
Cell line 2	19	0	7	10	4.9	4.6
Cell line 3	21	1	6	14	2.8	4.6

*Total number of nucleotide substitutions in ten clones compared with sequences of the transfected clone.

†Number of different clones of 10 clones sequenced. The figure in parentheses represents the number of clones with hypermutation (those with a statistically significant number of G to A substitutions). Codon 28 of the pre-core gene of the transfected clone was wild (Trp), and nucleotides 1762/1764 were T/A.

Expression of Deaminases in HepG2 Cell Lines. We examined the expression of known deaminases to see whether any such enzymes are active in HepG2 cells. As shown in Fig. 8, mRNA expression of 5 of 8 of these deaminases was detected, although the expression level of some deaminases was very low. mRNA of Apobec3G, a key enzyme for the hypermutation of HIV-1, was expressed in HepG2 cells, but the cDNA of this enzyme was only found by nested PCR. The expression level of the mRNA was similar in HBV-producing cells with various levels of hypermutations of HBV as well as parent HepG2 cells (detected by only nested PCR).

Discussion

In this study, we detected the mutated HBV genome in some patients by using PCR with PNA clamping. PNA is a DNA analog in which the ribose-phosphodiester backbone of DNA has been replaced by *N*-(2-aminoethyl) glycine linkages.[23] The PNA anneals strongly to DNA like a complementary DNA, but with higher affinity.[23] The annealing of the PNA to the target sequence thus prevents amplification of the target DNA in the PCR. In our previous study,[21] we attempted to block the amplification of lamivudine-sensitive wild-type YMDD motif strain and detected a very small amount (1/10,000) of YMDD motif mutant. Because the target sequence of this system contained many Gs with GA and GG (AGT TAT ATG GAT GAT GTG), we assumed that we could detect very rare hypermutated genomes.

Because we did not detect any hypermutated sequence without PNA, we assumed that the rate of the hypermutated genome is very low. This low frequency of hyper-

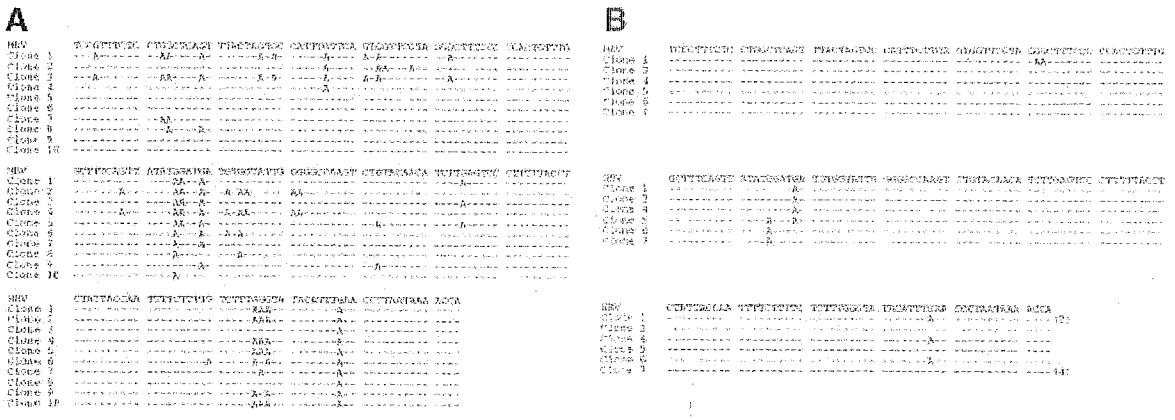

Fig. 3. G to A hypermutations detected in sequences of the HBV DNA (produced by HBV DNA-transfected cell lines to the supernatants). The nucleotide sequences of the transfected clone were used as a reference sequence (**top line**). DNA sequence alignments in the HBs antigen/polymerase region of cell line 1 (A) and cell line 2 (B) of the HBV. Numbers in parentheses are numbers of clones.

mutated genomes accounts for the lack of reports of such sequences with only one exception until recently,[19] in which the presence of two clones of hypermutated sequences in spliced genomes was reported. One may assume that the rare hypermutated genome might be produced in peripheral blood mononuclear cells because the HBV genome was previously found in such cells.[24-28] However, we showed that these genomes are found in HBV-transfected cell lines. Our results clearly demonstrate that hypermutation actually occurs in hepatocytes. The reason(s) for such a low frequency of hypermutation is not clear. The low expression level of deaminases in hepatocytes might account for the low frequency. In fact, we observed a very low expression level of APOVEC3G (transcripts was only detected by nested PCR [Fig. 8]) in HepG2 cell lines.

Recently, Turelli et al.[20,29] suggested that overexpression of APOBEC3G inhibits the replication of HBV by preventing encapsidation of the virus. However, they did not observe an increase in G to A hypermutation. In contrast, Rosler et al.[30] reported that G to A substitutions significantly increased in HepG2 cells when co-transfected with APOBEC3G cDNA. They found only 50 G to A substitutions by cloning 223 clones,[30] suggesting that the frequency of G to A substitutions is rare despite overexpression of APOBEC3G. Our preliminary data suggest that overexpression of APOBEC3G does not produce a

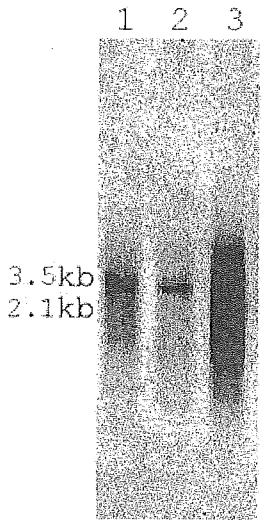

Fig. 4. Southern blot analysis of the HBV DNA extracted from cell lines that stably produce HBV into the supernatant. Two YMDD wild-type virus sequences (lanes 1 and 2) and one YVDD mutant virus sequence (lane 3) were transfected into the HepG2 cell line.

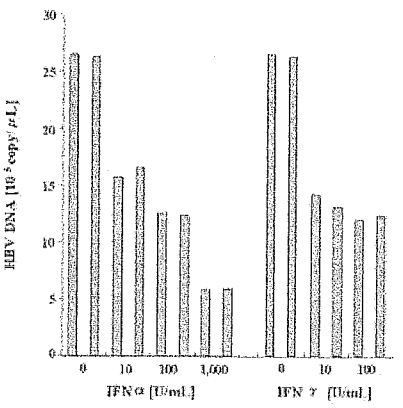

Fig. 5. Effects of interferon alpha and gamma on production of HBV DNA by cell line 1. Experiments were performed in duplicate with increasing amounts of each interferon.

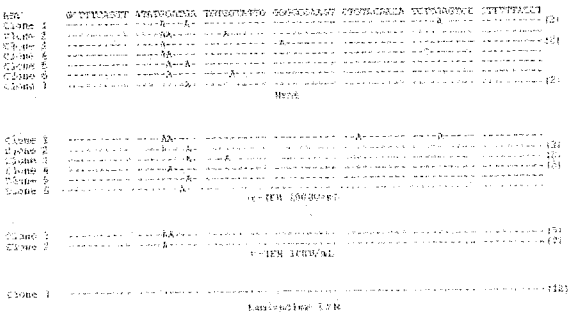

Fig. 6. Nucleotide sequence substitutions around YMDD motif of reverse transcriptase detected by PCR with PNA clamping after treating a HepG2 cell line (cell line 2 in Table 3). The nucleotide sequence of the transfected clone was used as a reference sequence (**top line**). Cells were treated with interferons and lamivudine as shown in Figs. 5 and 7, respectively.

noticeable increase in HepG2 cells by our detection method (C. Noguchi and K. Chayama, unpublished data). However, the method employed to detect hypermutation is not quantitative. Moreover, no antibody to detect APOBEC3G is available. Measurement of activity of this enzyme might be necessary to address this issue.

Because the patterns of hypermutations found in patients as well as cell lines are in agreement with strong dinucleotide preferences of a retroviral genome[31-35] edited by APOBEC3G,[7-9] we assume that hypermutations might also be induced by a similar enzyme. As pointed out by Turelli et al.,[20-29] another deaminase including APOBEC3F might be responsible for the generation of hypermutation. We actually detected the expression of deaminases in HepG2 cell lines. The expression levels of these deaminases are very low because they were detected by only two-stage PCR with one exception (only APOBEC3F was detected by a single-stage PCR).

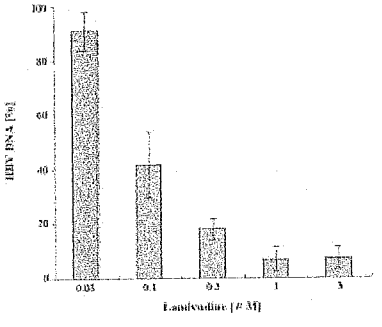

Fig. 7. Effects of lamivudine on production of HBV DNA by cell line 1. After 5 days of lamivudine treatment, the HBV DNA in core particles was immunoprecipitated and quantitated by real-time PCR. Data are mean ± SD of 4 independent experiments.

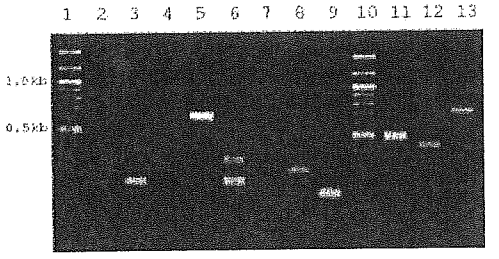

Fig. 8. Agarose gel electrophoresis of mRNAs of known deaminases amplified by reverse transcription-polymerase chain reaction. Lane 1: molecular weight size marker; lane 2: APOBEC1; lane 3: APOBEC2; lane 4: APOBEC3A; lane 5: APOBEC3B; lane 6: APOBEC3C; lane 7: APOBEC3D; lane 8: APOBEC3F; lane 9: APOBEC3G; lane 10: molecular weight size marker. Only mRNA of APOBEC3F was detected by one-stage PCR. To confirm the predictability of the assay, 3 negative mRNAs in Hep3G (APOBEC1, 3A and 3D) were amplified by using mRNAs from tissues known to express it. Lanes 11 and 12: APOBEC1 and APOBEC3A from the ileum; lane 13: APOBEC3D from the duodenum. All detected cDNAs were cloned, and nucleotide sequences were confirmed.

However, other possibilities should not be ignored. For example, some viral proteins might prevent such editing activity of deaminase by associating with this enzyme, as virion infectivity factor does in HIV-1–infected cells. Possibly the edited HBV genomes are degraded in liver cells rapidly by removal of the U residues by uracil DNA glycocylase followed by cellular nucleases.[36]

We found hypermutated genomes only in patients positive for eAb. The G to A nucleotide substitution of codon 28 of pre-core protein, which induces premature stop of this protein and basal core promoter mutations (A1762T/G1764A), might be related to the clearance of eAg.[28] Further studies should be conducted to investigate the relationship between G to A substitutions in these regions by deaminase(s), production of eAg, and replication efficacy of the virus.

A recent study showed that the amount of HBV DNA reduction occurs noncytopathologically through the action of cytokines, especially interferon alpha/beta and gamma.[37,38] We thus examined whether interferon can alter the occurrence of hypermutation. However, the results showed no increase in the number of hypermutation in HepG2–derived cell lines treated by interferon alpha and gamma (Fig. 6). Thus, the antiviral action of the mechanism responsible for G to A substitution in liver cells is likely to be independent of the action of interferon.

In conclusion, numerous innate intracellular defense systems exist, and the precise pathways of such systems are not fully understood. The role of editing of the HBV genome in such defense systems should be further investigated to understand the natural antiviral mechanisms and to develop an antiviral strategy against HBV.

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