厚生労働省科学研究費補助金（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

HCV のエンベロープ遺伝子を組み込んだ組換え VSV の作製

分担研究者　松浦善治　大阪大学微生物病研究所　教授

研究要旨： これまでにC型肝炎ウイルス(HCV)のエンベロープ蛋白質を被ったシュードタイプウイルス(HCVpv)を用いて感染機構の解析が進められてきたが、HCVpvはゲノムにエンベロープ遺伝子を持たないため、一度しか感染できず、二次感染は起こらない。本研究では水疱性口内炎ウイルス(VSV)のエンベロープ遺伝子を欠損させ、代わりにHCVのエンベロープ遺伝子を組み込んだ組換えVSV(HCVrv)を作製し、その感染様式を解析した。293TやHuh7細胞で作製したHCVrvは、Huh7細胞に最も高い感染性を示し、抗hCD81抗体やC型肝炎患者血清で感染が中和された。また、一部のHuh7細胞株では、HCVrvの感染拡大が確認された。自立増殖可能なHCVrvは、各種遺伝子型のHCVの感染機構の解析に有用であと考えられた。

A．研究目的

C型肝炎ウイルス(HCV)に感染すると肝硬変を経て高率に肝細胞癌を発症する。我が国には2百万人ものHCV感染者が存在すると推定され、既感染者に対する有効な肝癌進展阻止法の開発が急務である。近年、特定のクローン(JFH-1株)を用いたHCVの増殖系が確立されたものの、未だHCVの感染機構の詳細は明らかにされていない。これまでに我々は、HCVエンベロープ蛋白質を被ったシュードタイプ水疱性口内炎ウイルス(HCVpv)を作製し、その感染機構の解析を進めてきた。シュードタイプウイルスはゲノムにエンベロープ遺伝子を持たないため、一度しか感染できず、二次感染は起こらない。本研究では水疱性口内炎ウイルス(VSV)のエンベロープ遺伝子を欠損させ、代わりにHCVのエンベロープ遺伝子を組み込んだ組換えVSV(HCVrv)を作製し、その感染様式を解析し、新しいC型肝炎治療法の開発の可能性を探ることを目的とする。

B．研究方法

1a型H77株および1b型Con1株のHCVエンベロープ遺伝子を、VSVのエンベロープ遺伝子を欠損させたVSVのcDNAに挿入し、HCVrvを各種動物細胞で作製した。HCVrvの性状ならびに、感染様式をHCVpvおよびJFH-1株と比較した。

（倫理面への配慮）

本研究にあたっては、試料提供者、その家族、および同様の肝疾患患者の人権、尊厳、利益が保護されるよう十分に配慮する。具体的には、厚生労働省等で検討されている「ヒトゲノム解析研究に関する共通指針」に則り各研究実施機関の医学研究倫理審査委員会に申請し、インフォームドコンセントに係る手続きを実施し、また提供試料、個人情報を厳格に管理、保存する。

C．研究結果

HCVrvはHCVpvと同様に、293TやHuh7細胞で作製すると感染性を示すウイルスが得られ、これらのウイルスはHuh7細胞に最も高い感染性を示した。HCVrvの作製は37℃よりも、30℃で培養した方が高い感染価のウイルスが得られた。また、293TやHuh7細胞で作製したHCVrvは、HCVpvと同様に、抗hCD81抗体やC型肝炎患者血清で中和された。さらに、新たに樹立したJFH-1株の増殖効率の良いHuh7細胞では、HCVrvの感染の拡大が観察された。

D．考察

今回作製した組換えVSVは、これまでのシュードタイプウイルスやJFH-1ウイルスと同様に、hCD81依存的な感染性を示した。また、一部のHuh7細胞株では、HCVrvによって発現されたHCVエンベロープ蛋白質を利用して、感染を拡大していることが確認された。自立増殖可能な組換えVSVを用いることにより、各種遺伝子型のHCVの感染機構を詳細に解析することが可能になるものと思われる。

E．結論

1. HCVのエンベロープ遺伝子を組み込んだ組

換えウイルス(HCVrv)を作製した。
2. HCVrv は、Huh7 細胞に高い感染性を示し、抗 hCD81 抗体やC型肝炎患者血清で中和された。
3. 一部の Huh7 細胞株で、HCVrv の感染拡大が確認された。
4. 自立増殖可能な HCVrv は、各種遺伝子型の HCV の感染機構の解析に有用である。

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。

厚生労働省科学研究費補助金（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

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研究要旨：C肝炎ウイルス（HCV）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感染性HCVクローンを用いて、genotype 1a、1b および 2a の HCV 感染マウスが作製され、さらにはこれら HCV 感染マウスを用いて、HCV genotype 別のインターフェロン感受性の検討も可能であった。本モデルマウスは、HCV の IFN 抵抗性のメカニズムの解明や、新規抗ウイルス剤の効果判定のスクリーニングなどの *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

C 型慢性肝炎患者に対するインターフェロン（IFN）療法の治療成績は向上しつつあるも、未だ満足すべきものではない。この問題の克服のためには、どのようなウイルス側因子が IFN 抵抗性と関与しているのか、その解明が必要と思われる。しかし C 型肝炎ウイルス（HCV）は有効な動物モデルが確立されておらず、その基礎研究が困難である。我々は肝不全マウスおよび免疫不全マウスを交配させ作製した uPA/SCID マウスにヒト肝細胞を移植し、マウス肝細胞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、B 型（HBV）および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しHBVおよびHCV感染モデルマウスの作製に成功した。本研究は、HCVクローンを用いてリバースジェネティクス法により HCV 感染マウスを作製し、ウイルス変異と IFN 抵抗性を検討することを目的とする。

B．研究方法

キメラマウスに、以下の方法により、クローンを用いて HCV genotype 1a、1b および 2a を感染させた。

- Genotype 1a 感染性 HCV クローン pCV-H77C（金沢大学・本多先生、金子先生より供与）より *in vitro* transcription 法にて HCV RNA を合成し、マウス肝臓内に直接注入した。
- Genotype 1b の急性重症 C 型肝炎患者より HCV RNA を抽出し、クローニングにより、pHCV-KT9 を得た。PHCV-KT9 *in vitro* transcription 法にて HCV RNA を合成し、マウス肝臓内に直接注入した。また合成した HCV RNA を electroporation 法にて Huh7.5.1 細胞に transfection したところ、培養上清中に HCV RNA が検出されることが確認された。この 3 日後の培養上清 10 mL を 50 倍濃縮し、濃縮培養上清 200 uL をマウスに静脈内投与した。
- Genotype 2a 感染性 HCV クローン pJFH-1（国立感染症研究所・脇田先生より供与）より *in vitro* transcription 法にて HCV RNA を合成し、electroporation 法にて Huh7 細胞に transfection し、3 日後の培養上清 500 μL をマウスに静脈内投与した。

投与後、real time PCRにより血中HCV RNAを測定した。HCV感染マウス血清10 μLをnaïveなキメラマウスに投与し、感染を確認後、1000 IU/g/dayのIFN-alphaを2週間連日筋注した。

C．結果

HCV RNA を肝臓内注入（genotype 1a）および培養上清を投与（genotype 2a）したすべてのマウスにおいて、投与 2 週後、血中 HCV RNA は陽性となった。投与 6 週後、血中 HCV RNA は、2.4×10^7 copies/mL（genotype 1a）、2.5×10^5 copies/mL（genotype 2a）に上昇し、リバースジェネティクス法により genotype 1a および 2a の HCV 感染マウスが作製された。また genotype 1b の RNA を transfection した培養上清を投与したマウス 3 匹中 2 匹、または肝臓内に直接注入した 5 匹のマウスすべてにおいて HCV の感染が確認された。

これらのマウス血清を naïve なキメラマウスに投与したところ、感染が確認され、血中 HCV RNA は、8.5×10^6 copies/mL（genotype 1a）、1.7×10^5

copies/mL (genotype 2a) に上昇し、これらのクローンが感染性であることが、再確認された。

これらのマウスにIFN-alphaを2週間投与したところ、血中 HCV RNA は genotype 1a 感染マウスで 0.7 log、genotype 1b 感染マウスでは 1.2 log の低下であったが、genotype 2a 感染マウスではすべて感度以下 (10^3 copies/mL) に低下した。これらの結果より、genotype 1a および 1b は 2a に比べ、IFN 抵抗性であることが確認された。

D. 考察

リバースジェネティクス法により HCV genotype 1a および 2a 感染マウスが作製され、これらのマウスを用いて HCV genotype 間での *in vivo* における IFN 感受性の検討が可能であった。

E. 結論

これらの手法を用いることにより、今後，種々の変異HCVを血中に有するマウスの作製が可能であり，ウイルス変異と感染性およびIFN抵抗性のメカニズムの解明に有用であると思われる。また現在、臨床上最も問題となるgenotype 1bの感染マウスも作製中である。

F. 健康危機情報

特になし

G. 研究発表

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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

発表者氏名	論文タイトル名	発表誌名	巻号	ページ	出版年
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Dual effect of APOBEC3G on *Hepatitis B virus*

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G to A hypermutation of *Hepatitis B virus* (HBV) and retroviruses appears as a result of deamination activities of host APOBEC proteins and is thought to play a role in innate antiviral immunity. Alpha and gamma interferons (IFN-α and -γ) have been reported to upregulate the transcription of APOBEC3G, which is known to reduce the replication of HBV. We investigated the number of hypermutated genomes under various conditions by developing a quantitative measurement. The level of hypermutated HBV in a HepG2 cell line, which is semi-permissive for retrovirus, was 2.3 in 10^4 HBV genomes, but only 0.5 in 10^4 in permissive Huh7 cells. The level of APOBEC3G mRNA was about ten times greater in HepG2 cells than in Huh7 cells. Treatment of HepG2 cells with either IFN-α or -γ increased the transcription of APOBEC3G and hypermutation of HBV. These mRNAs and hypermutation of HBV genomes were induced more prominently by IFN-γ than by IFN-α. Both IFNs decreased the number of replicative intermediate of HBV. Overexpression of APOBEC3G reduced the number of replicative intermediate of HBV and increased hypermutated genomes 334 times, reaching 968 in 10^4 genomes. Deamination-inactive APOBEC3G did not induce hypermutation, but reduced the virus equally. Our results suggest that APOBEC3G, upregulated by IFNs, has a dual effect on HBV: induction of hypermutation and reduction of virus synthesis. The effect of hypermutation on infectivity should be investigated further.

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INTRODUCTION

Hepatitis B virus (HBV) is a small, enveloped DNA virus with partially double-stranded DNA as a genome (Ganem & Schneider, 2001; Seeger & Mason, 2000). The virus replicates through transcription of pregenome RNA and reverse transcription, like retroviruses (Skalka & Goff, 1993; Summers & Mason, 1982). Infection with HBV causes chronic hepatitis and often leads to liver cirrhosis and hepatocellular carcinoma (Wright & Lau, 1993; Bruix & Llovet, 2003; Ganem & Prince, 2004).

Recent reports have shown that a cytidine deaminase, APOBEC3G, which is packaged in human immunodeficiency virus (HIV) virions in non-permissive cells, induces G to A hypermutation to a nascent reverse transcript of HIV and serves as part of the innate antiviral activity (Mangeat *et al.*, 2003; Zhang *et al.*, 2003; Lecossier *et al.*, 2003; Harris *et al.*, 2003). Recent studies have demonstrated that a small number of HBV DNA in serum samples of patients with chronic HBV infection contains hypermutated genomes (Gunther *et al.*, 1997; Suspene *et al.*, 2005a; Noguchi *et al.*, 2005). We reported previously that there are small numbers of hypermutated genomes in serum samples of the majority of patients with chronic HBV infection and that G to A hypermutation could be induced in cultured liver cells derived from HepG2 cell lines (Noguchi *et al.*, 2005) using a peptide nucleic acid-mediated PCR clamping method. Suspene *et al.* (2005a) developed the more sensitive differential DNA denaturation (3D)-PCR method to detect hypermutated genomes and found that some APOBEC proteins induce G to A, and in some cases C to T, hypermutations in HBV DNA (Suspene *et al.*, 2005a). Why only a very small proportion of the HBV genome is hypermutated is unknown at present. Furthermore, the

mechanism that controls the level of APOBEC protein expression and degree of hypermutation has not been fully investigated. Recently, Tanaka *et al.* (2006) identified an interferon (IFN)-stimulated response element (ISRE) in the promoter region of APOBEC3G and showed that IFN-α upregulates transcription of APOBEC3G. Peng *et al.* (2006) also reported that IFN-α and -γ upregulate mRNA transcription of APOBEC proteins. However, these reports did not analyse whether increased numbers of APOBEC proteins actually increase hypermutation. More recently, Bonvin *et al.* (2006) demonstrated that IFN induces transcription of APOBEC proteins and increases hypermutation of HBV.

IFNs are cytokines that play a major role against many pathogens (Samuel, 2001; Colonna *et al.*, 2002; Grandvaux *et al.*, 2002). We also reported in a previous study that both IFN-α and -γ reduce virus replication in stably HBV-transfected cell lines without inducing a remarkable increase in G to A hypermutation (Noguchi *et al.*, 2005). However, the method used in previous experiments for detection of hypermutation was not as sensitive as the method of Suspene *et al.* (2005a, b) and not quantitative. To assess the level of hypermutation, a reliable measurement of hypermutated genome is needed. In the present study, we developed a new and sensitive method for the measurement of hypermutated genome levels. Using this method, we show here that both IFN-α and -γ increased the levels of hypermutated genomes in cultured cell lines. Furthermore, both IFNs increased the mRNA level of APOBEC3G. We also performed overexpression experiments to examine whether APOBEC3G and its inactive mutants increase the levels of hypermutation and reduce HBV replication.

METHODS

Plasmid constructs. The expression vector for haemagglutinin (HA)-tagged human APOBEC3G, pcDNA3/HA-A3G, was constructed as described previously (Kobayashi *et al.*, 2004). APOBEC3F cDNA was obtained by modifying APOBEC3F like (IMAGE clones from Open Biosystems) to have the same sequence as human APOBEC3F transcript variant 1 (GenBank NM_145298) and cloned into pcDNA3/HA (Invitrogen). APOBEC3G mutants were constructed using a QuikChange mutagenesis kit (Stratagene). The construction of wild-type HBV 1.4 genome length, pTRE-HB-wt, has been described previously (GenBank accession no. AB206816) (Tsuge *et al.*, 2005).

Cell culture and transfection. Huh7 and HepG2 cell lines were grown in Dulbecco's modified Eagle's medium supplemented with 10 % (v/v) fetal calf serum at 37 °C in 5 % CO_2. Cells were seeded to semi-confluence in six-well tissue culture plates. Transient transfection of the plasmids into HepG2 and Huh7 cell lines was performed using *Trans*IT-LT1 (Mirus) according to the instructions provided by the supplier. A plasmid encoding a secreted form of human placental alkaline phosphatase (SEAP) was co-transfected to adjust the transfection efficiency. The SEAP assay in the culture medium was performed using the Great EscAPe SEAP Reporter System 3 (BD Bioscience).

T23 cells are HepG2 cells stably transfected with the plasmid pTRE-HB-wt. They were cultured using a method described previously (Tsuge *et al.*, 2005). Cells were seeded to semi-confluence in six-well tissue culture plates and then treated with medium containing either IFN-α (Hayashibara Biochemical Laboratories) or IFN-γ (Shionogi & Co.). The cells were harvested 12–72 h after IFN treatment. Core-associated HBV DNA was extracted from the cells for HBV DNA quantification and quantitative analysis of G to A hypermutated genomes (Noguchi *et al.*, 2005).

Analysis of core-associated HBV DNA. The cells were harvested 4 days after transfection and lysed with 250 µl lysis buffer [10 mM Tris/HCl pH 7.4, 140 mM NaCl and 0.5 % (v/v) NP-40] followed by centrifugation for 2 min at 15 000 *g*. The core-associated HBV genome was immunoprecipitated from the supernatant by mouse anti-core monoclonal antibody anti-HBc determinant α (Institute of Immunology, Tokyo, Japan) and subjected to quantitative analysis after SDS/proteinase K digestion followed by phenol extraction and ethanol precipitation. Quantitative analysis was performed by real-time PCR using the 7300 Real-Time PCR system (Applied Biosystems). The primers used for amplification were #1, 5′-ACTTCAACCCCAACAMRRATCA-3′ (nt 2978–2999) [numbers are those of HBV subtype C reported by Norder *et al.* (1994)] and #2, 5′-AGAGYTTGKTGGAATGTKGTGGA-3′ (nt 24–1), where M is A/C, R is G/A, Y is T/C and K is G/T. The probe was a 6-carboxy-fluorescein (FAM)-labelled minor-groove binder (MGB) probe, 5′-(FAM)-TTAGAGGTGGAGAGATGG-(MGB)-3′ (nt 3184–3167). Real-time PCRs were set up in 25 µl TaqMan Universal Master Mix with 1 µl DNA solution, 0.9 µM each primer and 0.25 µM probe. The amplification conditions were 2 min at 50 °C, 10 min at 95 °C, followed by 40 cycles of amplification (denaturation at 95 °C for 15 s, annealing at 55 °C for 30 s and extension at 62 °C for 90 s).

Amplification and analysis of hypermutated HBV genomes by 3D-PCR. HBV DNA was extracted from 100 µl serum obtained from a chronic HBV carrier (genotype C) by SMITEST (MBL International) and was dissolved in 20 µl H_2O. Hypermutated genomes were detected by modified 3D-PCR using primers #1 and #2 and DNA solution from serum containing 8.0×10^7 or 2.3×10^5 copies of core-associated HBV DNA in 25 µl of 100 mM Tris/HCl pH 8.3, 50 mM KCl, 15 mM $MgCl_2$, 0.2 mM each dNTP, 10 pmol each primer and 1.25 U *Taq* DNA polymerase (Gene Taq, Nippon Gene Co.), together with 0.25 µg anti-*Taq* high (TOYOBO Co.). The amplification conditions included an initial denaturation step at 83–95 °C for 5 min, followed by 45 cycles of denaturation at 83–95 °C for 1 min, annealing at 50 °C for 30 s, extension at 72 °C for 30 s followed by 10 min of final extension. Amplicons were separated by electrophoresis on 2 % (w/v) agarose gel, cloned and sequenced in an ABI PRISM 3130 Genetic Analyzer with a BigDye Terminator version 3.1 cycle sequencing ready reaction kit (Applied Biosystems). The PCR products were also analysed on Hanse Analytik (HA)-yellow gel as described previously (Suspene *et al.*, 2005b; Tsuge *et al.*, 2005; Abu-Daya *et al.*, 1995).

Quantitative analysis of hypermutated genomes by real-time PCR. Hypermutated genomes were quantified by real-time PCR using the 7300 Real-Time PCR system (Applied Biosystems) and the above primers and probes. The amplification conditions included activation at 95 °C for 10 min followed by initial denaturation at 88 °C for 20 min and 45 cycles of amplification (denaturation at 88 °C for 15 s, annealing at 50 °C for 30 s and extension at 62 °C for 90 s). We chose 88 °C as this temperature is appropriate for detection of about 20 % hypermutated genomes. There are 200–300 such hypermutated genomes in 10^4 genomes present in HepG2 cells transiently transfected with APOBEC3G. The buffer comprised 10 mM Tris/HCl pH 8.3, 50 mM KCl, 3 mM $MgCl_2$, 10 mM EDTA, 60 nM Passive Reference 1 (Applied Biosystems), 0.2 mM each dNTP, 0.9 µM each primer, 0.25 µM probe, 5×10^6 copies of HBV DNA

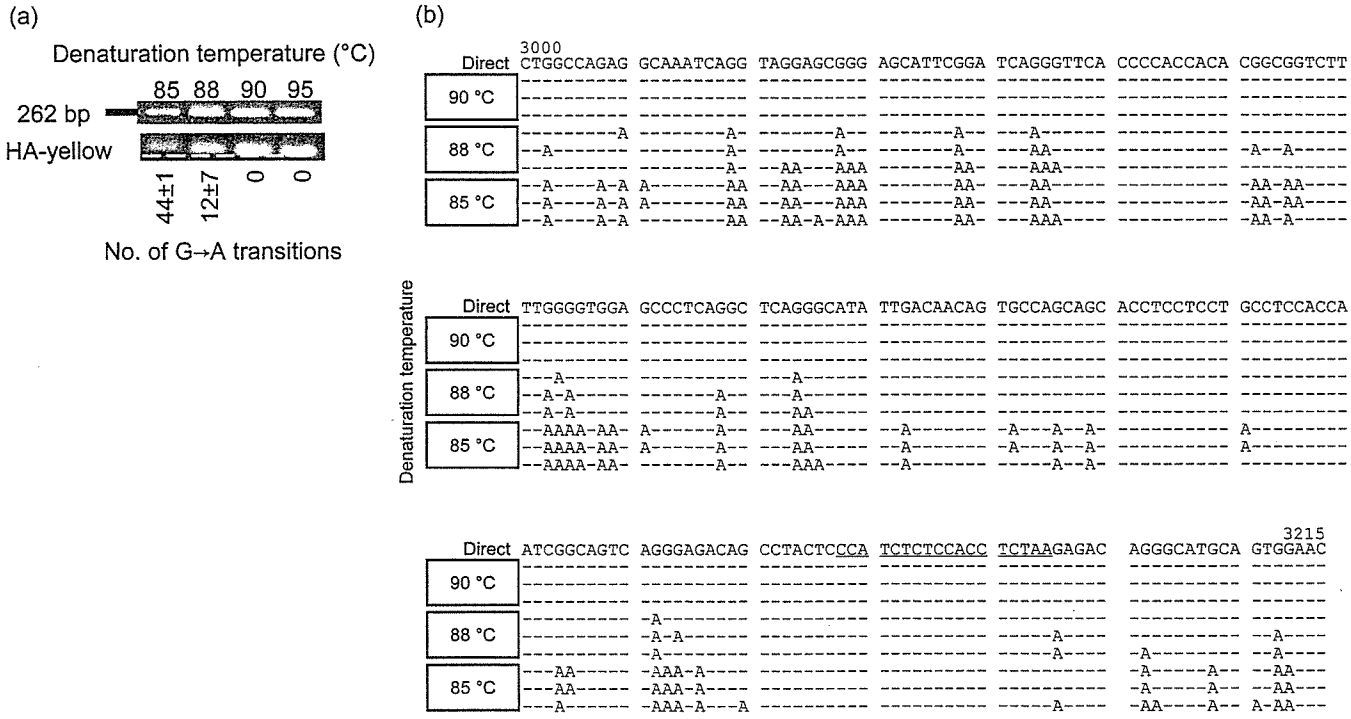

Fig. 1. Amplification of HBV DNA by 3D-PCR. (a) Detection of hypermutated genomes by HA-yellow agarose gel electrophoresis. The numbers of G to A transitions are expressed as means ± SD generated from the sequence analysis of five independent clones from PCR products. The white dotted line was added to help visualize the retardation of AT-rich DNA in HA-yellow agarose gel. (b) Nucleotide sequences of HBV amplified by 3D-PCR. The nucleotide sequences obtained by direct sequencing are used as a reference sequence. The nucleotide sequences where the probe hybridizes are underlined. Note that the number of G to A mutations correlates with denaturation temperature.

and 0.625 U AmpliTaq Gold DNA polymerase (Applied Biosystems) in a final volume of 25 µl. A standard curve was constructed by the simultaneous amplification of serial dilutions of the 3D-PCR products.

Western blot analysis. Cell lysates were prepared as described above, resolved on 10 % (w/v) SDS-polyacrylamide gels and transferred to nitrocellulose membranes (Whatman) via electro-blotting. The membranes were incubated with anti-haemagglutinin fusion epitope monoclonal antibody (Roche) or with anti-β-actin monoclonal antibody (Sigma-Aldrich) followed by incubation with horseradish peroxidase-conjugated donkey anti-rabbit antibody or sheep anti-mouse immunoglobulin (Amersham Biosciences). Proteins were visualized via the ECL system (Amersham Biosciences).

Quantification of mRNA of APOBEC3G or APOBEC3F by reverse transcription and real-time PCR. Total RNA was extracted from HepG2 cell lines by using an RNeasy Mini kit (Qiagen). The RNA was reverse transcribed with random primers and Moloney murine leukemia virus reverse transcriptase (ReverTra Ace, TOYOBO Co.) at 42 °C for 60 min according to the instructions provided by the manufacturer. Quantitative analysis of APOBEC3G and APOBEC3F cDNA was performed by real-time PCR using TaqMan Gene Expression assays (Applied Biosystems). To confirm that the APOBEC3G and -3F PCR primers specifically amplify the target genes, quantitative PCR on the expression plasmids encoding human APOBEC3G and -3F, used as templates, was performed. No cross amplification was observed, even when we used 10^7 copies of APOBEC3G plasmid in the amplification reaction of APOBEC3F and vice versa. A standard curve was constructed by the amplification of serial dilutions of the known number of plasmids containing human APOBEC3G and APOBEC3F. The target cDNA was normalized to the endogenous RNA level of the housekeeping reference gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH). The primers and FAM-labelled probe used to quantify GAPDH were purchased from Applied Biosystems.

Infectivity of luciferase reporter viruses produced from HepG2 and Huh7 cell lines. Luciferase reporter viruses with or without viral infectivity factor (Vif) were prepared by co-transfection of pNL43/ΔEnv-Luc (wild-type) or pNL43/ΔEnvΔvif-Luc (ΔVif) plus pVSV-G together with a mock vector or expression vectors for A3G by Lipofectamine (Invitrogen) as described previously (Janini *et al.*, 2001; Shindo *et al.*, 2003). Productive infection was measured by luciferase activity. Values were presented as percentage of infectivity relative to the value of each virus without expression of APOBEC3G proteins.

RESULTS

Quantitative analysis of hypermutated genome by real-time PCR

Using serum samples from a patient with a high viral load, we amplified a large number of hypermutated genomes by 3D-PCR and detected them by HA-yellow agarose gel electrophoresis (Fig. 1a). Nucleotide sequence analysis

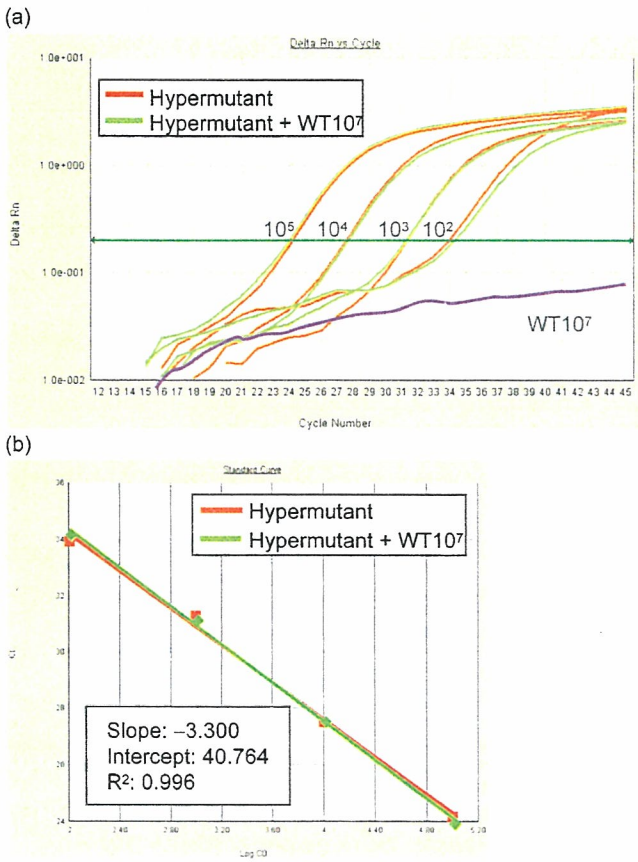

Fig. 2. Quantitative measurement of hypermutated HBV DNA using 3D-PCR combined with real-time PCR. The indicated numbers (10^2–10^5) of hypermutated genomes alone (orange lines) and a mixture of wild-type plus hypermutated genomes (green lines) were amplified by 3D-PCR. 3D-PCR did not result in amplification of wild-type sequence (purple line). Denaturation temperature was 88 °C.

showed detection of more heavily hypermutated genomes at lower denaturation temperatures (Fig. 1b). To develop quantitative measurement, we selected sequences with many G residues, designed primers that contained only a small number of G residues and used degenerate primers. A probe sequence was designed without a G residue. Using this primer and probe set, we could amplify only hypermutated genomes (Fig. 2). When hypermutated and non-mutated genomes were co-amplified, only hypermutated genomes were successfully amplified using the above primer and probe set (Fig. 2b). Non-hypermutated genomes (10^7 copies) were not amplified, although conventional PCR amplified both mutated and non-mutated genomes equally (data not shown). We also tried to detect only slightly (four of the 58 G residues) mutated genomes by 3D-PCR, but could not detect such genomes. It should thus be noted that the quantitative measurement we developed in this study detects only hypermutated genomes.

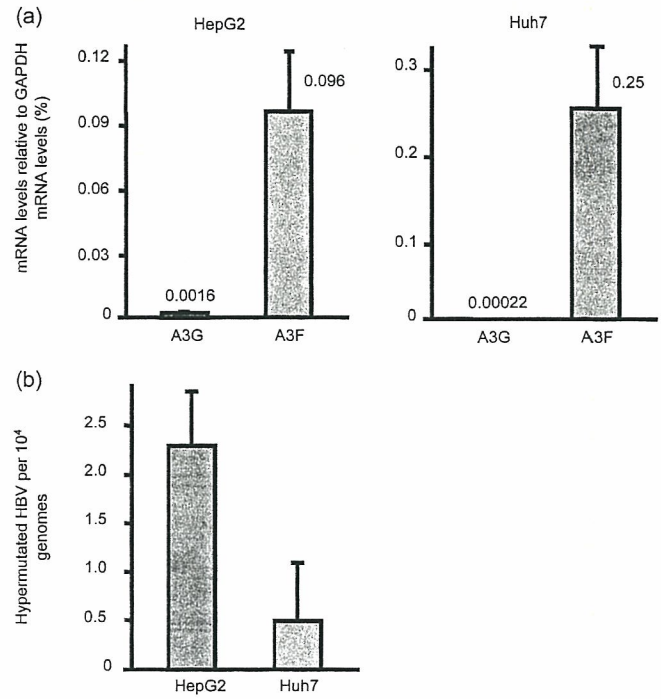

Fig. 3. Expression levels of APOBEC3G and -3F protein mRNAs in HepG2 and Huh7 cell lines. (a) mRNAs were extracted from cultured cell lines and the number of mRNA was quantified by real-time PCR with a probe for APOBEC3G and -3F. The expression levels were expressed as a percentage of GAPDH mRNA. (b) Number of hypermutated HBV genomes measured by real-time 3D-PCR in HepG2 and Huh7 cell lines transiently transfected with pTRE-HBV-wt. Results are means ± SD values of three independent experiments.

Detection of APOBEC3G mRNA and hypermutated genomes in semi-permissive and permissive cell lines

In retrovirus studies, it is known that some cell lines allow production of infectious retrovirus virions with Vif deficiency (permissive cells) while others do not. The difference between semi-permissive and permissive cell lines is the expression of APOBEC3G (Mangeat *et al.*, 2003; Zhang *et al.*, 2003; Lecossier *et al.*, 2003; Harris *et al.*, 2003; Shirakawa *et al.*, 2006). Thus, we examined the expression of APOBEC3G in both HepG2 and Huh7 cell lines. The APOBEC3G mRNA level detected by real-time PCR was very low (approx. 0.002 % relative to GAPDH mRNA) and about ten times greater in HepG2 cells than in Huh7 cells (Fig. 3a).

The number of hypermutated genomes in HepG2 cells transiently transfected with pTRE-HB-wt was about five times that in Huh7 cells (Fig. 3b). Vif-deficient HIV-1 virions produced from HepG2 cell exhibited very low infectivity compared with wild-type (Fig. 4a). In contrast, the infectivity of HIV-1 virions produced by Huh7 was