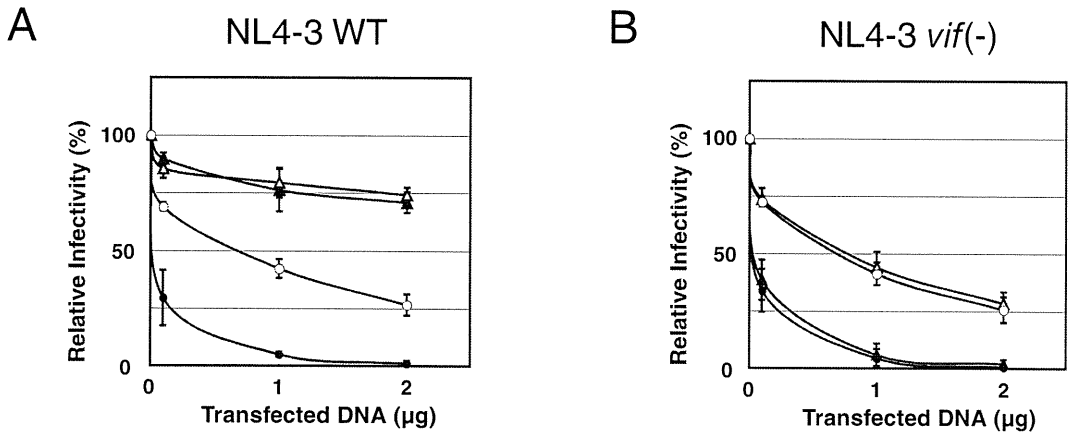

Fig. S6. Antiviral activity of A3G and S-A3G active-site mutants. 293T cells were cotransfected with (*A*) pNL4–3 and either A3G WT (▲), A3G E259Q (△), S-A3G (●), S-A3G E259Q (○) expression plasmids or (*B*) pNL4–3*vif*(-) and the indicated A3G expression plasmids, using 0.1, 1, or 2 μg of DNA. Amounts of added DNA for each of the expression plasmids were adjusted with empty vector pcDNA 3.1 (-) to a total of 2 μg. Infectivity is expressed relative to the values for 0 μg of the expression plasmids, set at 100%.

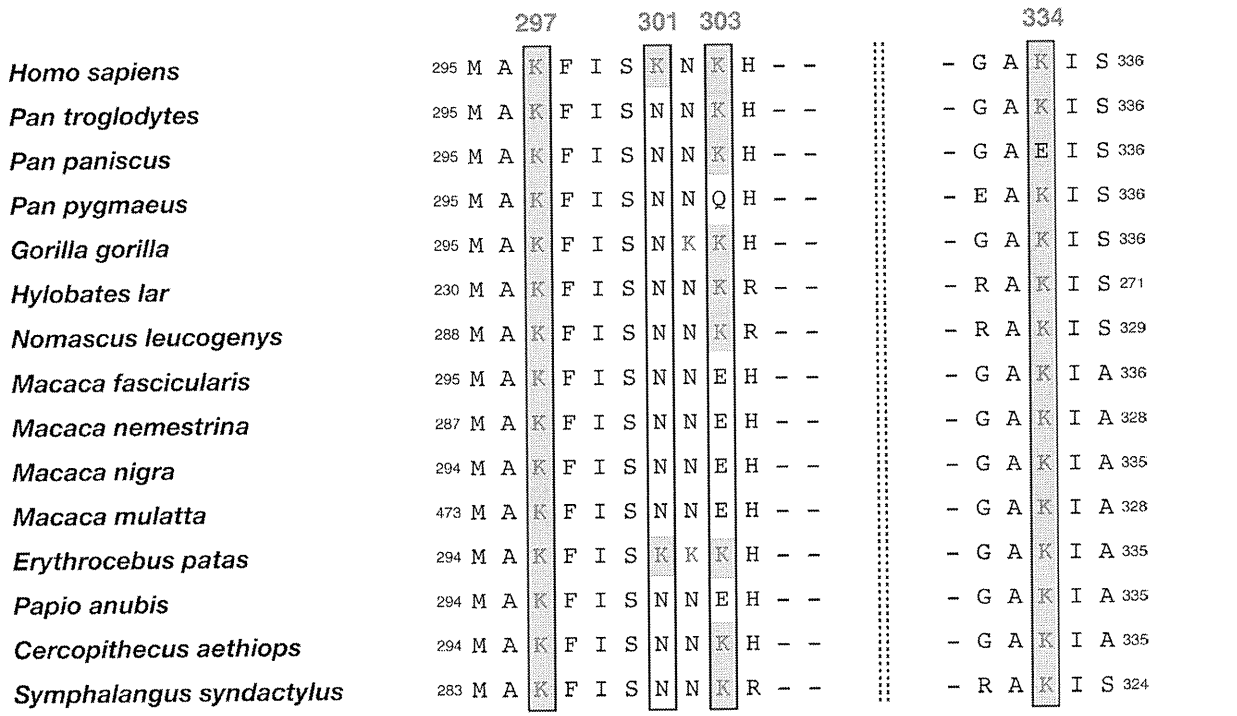

Fig. S7. Amino acid sequence comparison around the four C-terminal Lys residues of primate A3G proteins. Sequences were aligned by using MacVector 7.0 (MacVector, Inc.) software and residues at positions 297, 301 303, and 334 are enclosed in blue boxes. Lys residues at these positions are shown in red. The genbank accession numbers are: *Homo sapiens* (NP_068594); *Pan troglodytes* (AAT44392); *Pan paniscus* (AAT72156); *Pan pygmaeus* (AAT44395); *Gorilla gorilla* (AAT44394); *Hylobates lar* (ABB77894); *Nomascus leucogenys* (ABB77892); *Macaca fascicularis* (AAT44393); *Macaca nemestrina* (AAY99624); *Macaca nigra* (AAT44391); *Macaca mulatta* (AAP85256); *Erythrocebus patas* (AAT44390); *Papio anubis* (AAT44398); *Cercopithecus aethiops* (AAP85254); and *Symphalangus syndactylus* (ABB77893).

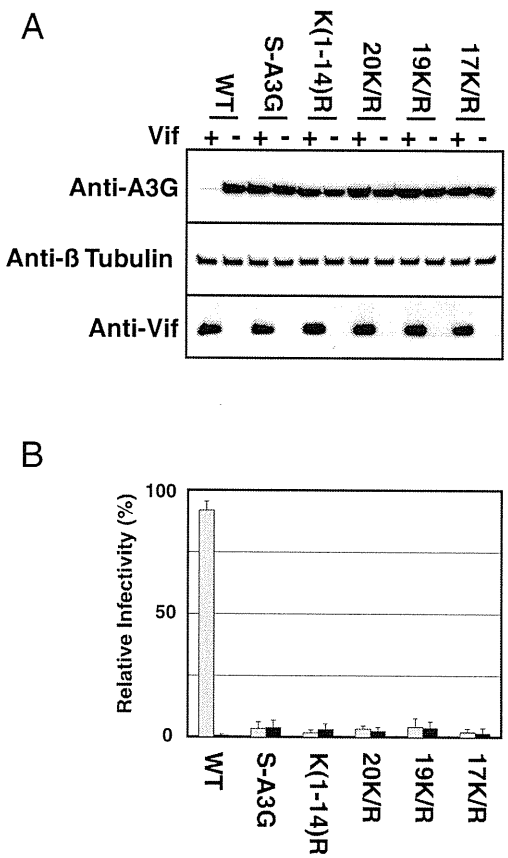

Fig. S8. Vif-dependent degradation (*A*) and antiviral activity (*B*) for various Lys to Arg substitution mutants of A3G. Designations for the different mutants are as follows: K(1–14)R at 63, 76, 79, 99, 113, 141, 180, 249, 270, 297, 301, 303, 334, and 344; 20K/R at 2, 40, 42, 52, 63, 76, 79, 99, 113, 141, 150, 163, 180, 249, 270, 297, 301, 303, 334, and 344; 19K/R at 40, 42, 52, 63, 76, 79, 99, 113, 141, 150, 163, 180, 249, 270, 297, 301, 303, 334, and 344; 17K/R at 40, 42, 52, 63, 76, 79, 99, 113, 141, 180, 249, 270, 297, 301, 303, 334, and 344. (*B*) 293T cells were cotransfected with 4 μg of either pNL4–3 (gray bar) or pNL4–3*vif*(-) (black bar), and 1 μg of the indicated A3G expression plasmid. Virus infectivity was measured using LuSIV cells, as described in the *SI Text*. Infectivity values are given relative to the value for the vector control, which was set at 100%.

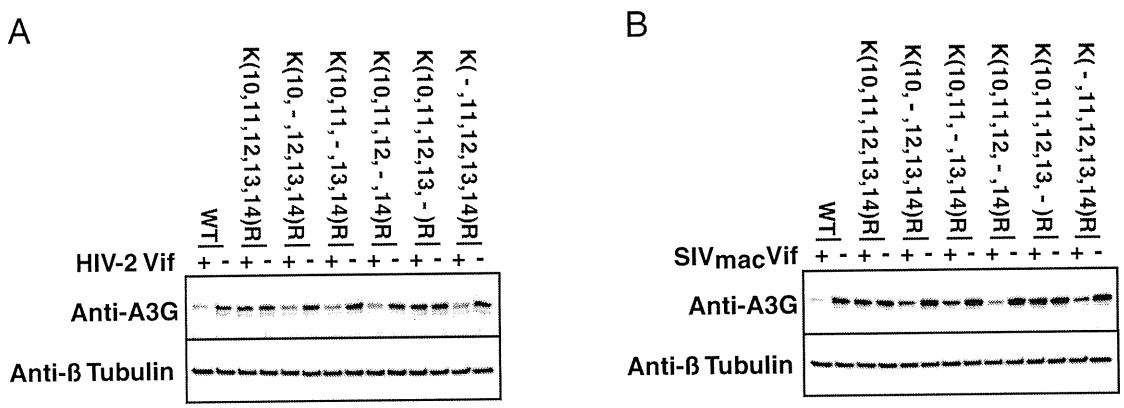

Fig. S9. A3G mutant sensitivity toward HIV-2 and SIV_{mac} Vif proteins. Assays for Vif-dependent degradation were performed using HIV-2 and SIV_{mac} Vif expression plasmids, pGH123 and pMA239, respectively. For the corresponding Vif-negative controls, pGH-Xb and pMA-Sc were used, respectively.

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第9回ECC山口メモリアルエイズ研究奨励賞受賞研究

宿主防御因子APOBEC3Gの抗HIV作用メカニズムに関する研究

Study on Molecular Mechanism of Host Defense Factor, APOBEC3G, against HIV

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はじめに

今回，第9回日本エイズ学会ECC山口メモリアルエイズ研究奨励賞受賞の背景となった研究について，概説する。

研究の背景

宿主防御因子APOBEC3G（A3G）は細胞内に発現するCytidine Deaminase（ssDNA中のCytidineを脱アミノ化し，dUに変換する酵素）で，レトロウイルスに対する宿主防御因子である。A3Gは，Vifを欠損したHIV-1（以下，HIV-1*vif*（−））の複製を強く阻害する。HIV-1*vif*（−）は，ウイルス産生細胞内で発現するA3Gを粒子内に取込む。そのため，新たな細胞に感染する時に，逆転写の過程がA3Gによって強く阻害される。一方，野生型のHIV-1（以下，HIV-1 WT）は，ウイルス産生細胞内でVifを発現し，ユビキチン/プロテアソーム系を介して選択的にA3Gを破壊し枯渇させてしまう。このため，HIV-1 WTはA3Gをウイルス粒子に取込まず，A3Gの防御システムから逃れることができる（図1）。A3Gの抗レトロウイルス作用の分子メカニズムについては，諸説あるが，Cytidine Deaminaseとしての酵素活性に依存的な機構と非依存的なメカニズムがあることまでは一致した見解である。その根拠は2つある。1）酵素活性を欠失したA3Gは，野生型より低いが，発現量依存的に抗HIV作用を示す。特に，過剰発現させた場合には，野生型の効果に匹敵する。2）A3Gは，B型肝炎ウイルスやパルボウイルス，いくつかのレトロトランスポゾンに対しても強い抗ウイルス作用（DNA合成阻害）を示すが，いずれもDeaminase非依存的に抑制する。

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実は，「細胞がHIV-1*vif*（−）の増殖を許容するか否かを決定する因子がA3Gである」という発見以前から，非許容細胞（A3Gを発現する細胞）から産生されるHIV-1*vif*（−）の感染では，感染細胞内における逆転写反応が抑制され，逆転写初期から後期にかけて逆転写産物が段階的に減少することが分かっていた。さらに，この抑制効果はターゲット細胞がどんな細胞種であれ同様に認められることが分かっていた。A3Gが広範な抗ウイルス作用スペクトルを示すことを考慮すると，A3Gの抗HIV作用機序は，A3Gがもつ何らかの分子生化学的特性が，細胞質でDNA合成をするウイルス（HBVやパルボウイルス，いくつかのレトロエレメント）に共通した分子機序に帰結することがもっともらしいと考えられる。

多くの研究者が感染細胞を用いた研究により，抗HIV作用機構の解明に取り組んでいる。しかし，図2に示したようなHIVの複雑な逆転写過程について各ステップを，感染細胞を用いて評価することは非常に困難である。そのため，逆転写がどのように抑制されるのかという分子レベルでの詳細な機序は明らかになっていなかった。そこで，筆者はA3Gタンパクを精製し，A3Gの生化学的，分子生物学的な性質を明らかにし[1]，*in vitro*再構築系を用いて逆転写の各過程に対する影響を解析し，抗HIV作用機序を分子レベルで明らかにする研究を行った。その結果，A3Gの抗HIV作用メカニズムの1つである酵素活性非依存的な分子機序を明らかにすることができた[2]。以下，その結果について概説する。

A3Gによる酵素活性非依存的なHIV-1逆転写阻害メカニズム

最初に，酵素活性をもつA3Gを，バキュロウイルスを用いた発現系を利用して発現・精製した[1]。筆者らが開発した*in vitro*の逆転写再構築系[3]を用いて，HIV-1の一連の

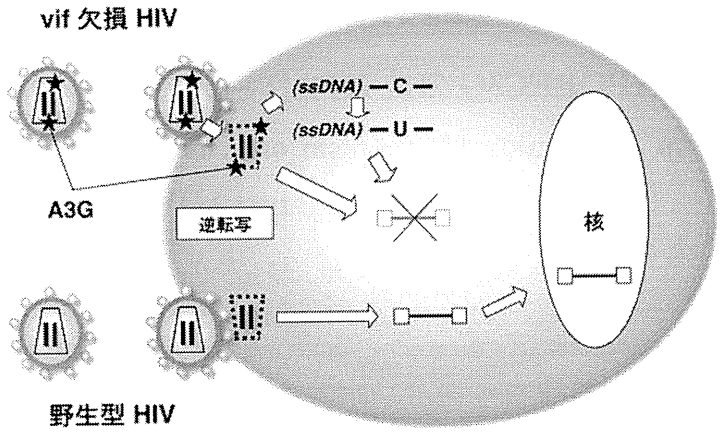

図 1 A3G による抗 HIV 作用
vif を欠損した HIV では，ウイルス産生細胞由来の A3G がウイルス粒子（コア）に取込まれ，新たに感染した細胞において逆転写産物が減少する。A3G による Cytidine Deaminase（酵素）活性依存的あるいは非依存的に阻害されるメカニズムがある。一方，野生型 HIV では，産生細胞内で Vif により A3G が枯渇させられるため，ウイルスは A3G を取込まず，A3G の抗ウイルス防御システムから逃れることができる。

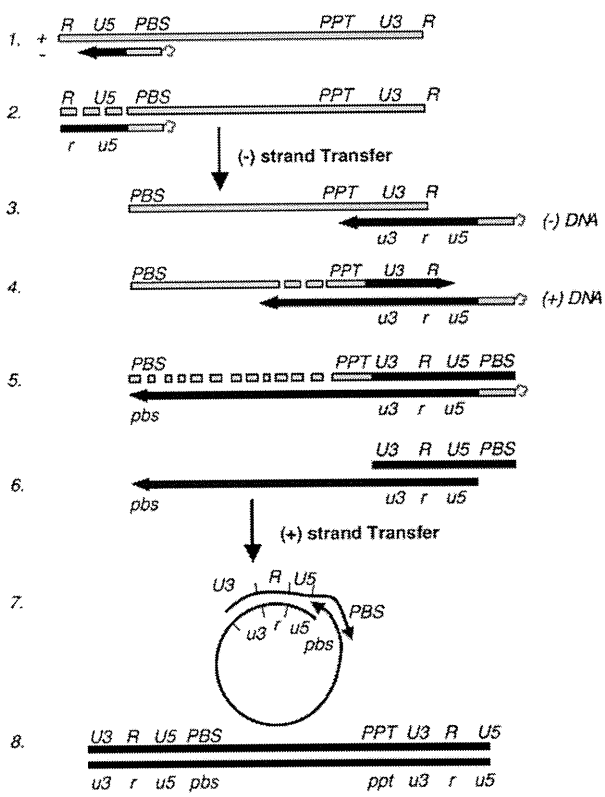

図 2 HIV の一連の逆転写複製過程
1；RT により，ゲノム RNA にアニーリングした tRNA から DNA 合成がスタートする（(−) SSDNA 合成）。2；RNaseH により，(−) SSDNA の鋳型 RNA が消化される（RNaseH Cleavage）。3；NC の Chaperone 活性により（−）SSDNA が下流の再アニーリングする（(−) Strand Transfer）。4；RNaseH による PPT 領域 RNA 以外の消化と，PPT をプライマーとした DNA 伸長反応がはじまる（PPT Processing と PPT Initiation）。5；(−) と（+）鎖合成ともに PBS 末端まで到達し，鋳型 RNA は消化される。さらに，tRNA が RNaseH により取り除かれ PBS 領域の DNA が一本鎖となる（tRNA removal）。6 & 7；PBS 領域がアニーリングし（(+) Strand Transfer），DNA 伸長反応が両方向に進む。8；RT の伸長反応が進み，完全長の 2 本鎖 DNA（プロウイルス DNA）がつくられる。

逆転写反応（図 2）において，A3G がどの過程に影響を与えるのか解析した。

まず，逆転写のプライマーである $tRNA_3^{Lys}$ と鋳型 RNA とのアニーリング反応に対する影響を検討した。ウイルス粒子内では，このアニーリングはウイルスタンパク NC（Nucleocapsid）の chaperone 機能によって行われる。図 3A

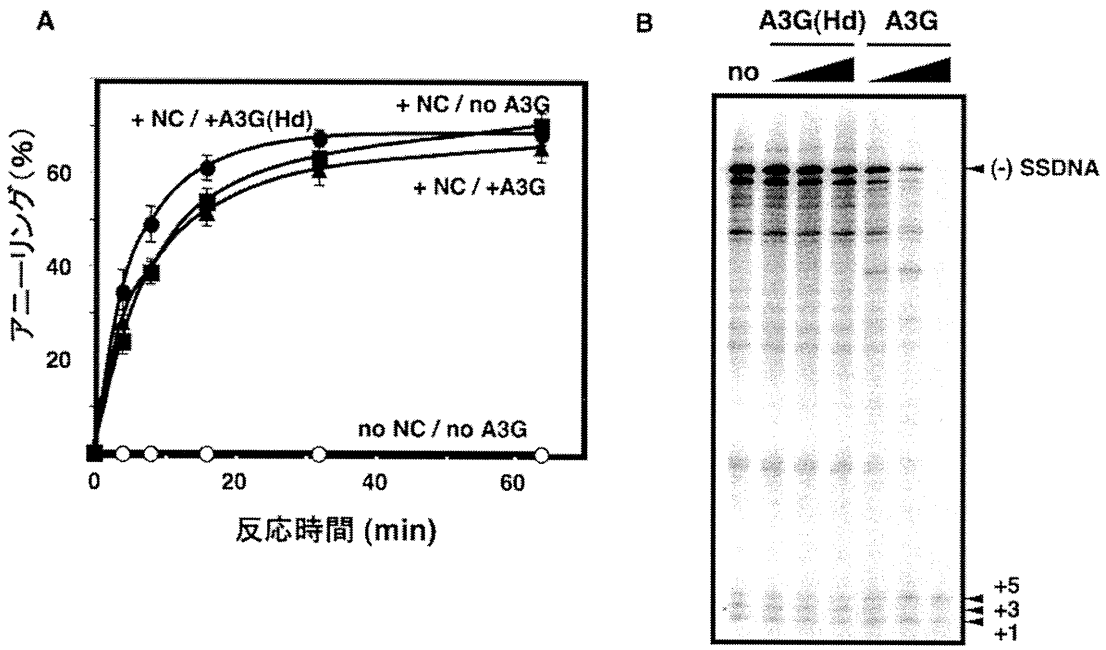

図 3 *in vitro* における A3G による tRNA アニーリングおよび tRNA からの（－）SSDNA 合成開始反応に対する影響
A；NC による tRNA アニーリング反応速度に対する A3G の影響を解析した。NC（核酸 7 base あたり 1 分子）量を，A3G（80 nM）を加えた。B；熱変性させた A3G と未変性 A3G を加え，（－）SSDNA 合成を比較した。1（0 nM），2 & 5（20 nM），3 & 6（40 nM），4 & 7（80 nM）。

に示したように，NC 非存在下（noNC/noA3G）では，60 分後でもアニーリングしない。NC 添加によってアニーリングが促進されるが（＋NC/noA3G），そこに A3G（＋NC/＋A3G）あるいは熱変性した A3G（＋NC/＋A3G（Hd））を加えても，アニーリング速度は変化しなかった。つまり，A3G は tRNA の Primer Placement の過程には影響を与えないことが示された。それに反して，$tRNA_3^{Lys}$ からはじまる逆転写伸長反応（（－）SSDNA 合成）（（－）SSDNA：minus-strong stop DNA）は，A3G の濃度依存的に強く抑制された（図 3B）。ちなみに，A3G を添加しない場合あるいは熱変性した A3G では，その抑制効果は認められなかった。さらに，（－）SSDNA 合成速度を調べた結果，RNA 鋳型 1 分子あたり 13 分子の A3G 存在比の場合では，約 1/24 に低下した。さらに，興味深いことに，A3G により，（－）SSDNA 合成の Pausing Products（停止した中間産物）が多く認められた。それらの Pausing Products を解析し鋳型 RNA の二次構造と照らし合わせた結果，A3G による（－）SSDNA 伸長停止が鋳型 RNA 二次構造上のループやバルジで起こっていることが分かった。このことは，一本鎖核酸（ssDNA と ssRNA）に特異的に結合する A3G が，RNA の一本鎖領域に結合することによるものであると考えられた。

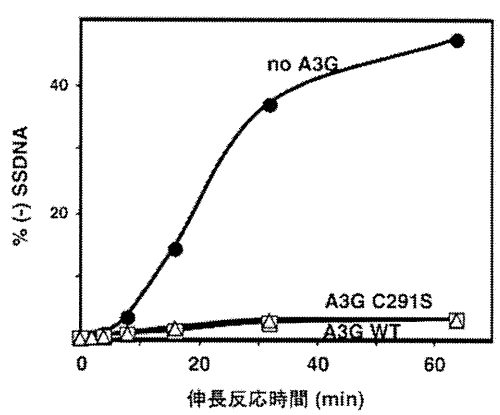

図 4 （－）SSDNA 合成阻害効果に対する A3G 酵素活性の影響
野生型 A3G（0 nM）（●），野生型 A3G（80 nM）（□），酵素活性欠損型 A3G C291S（80 nM）（△）存在下で，（－）SSDNA 合成量を経時的に測定した。

次に，A3G による（－）SSDNA 合成阻害が A3G の酵素活性に依存するのか調べてみた。酵素活性中心に変異を導入した A3G C291S タンパクを発現・精製し，（－）SSDNA

合成に対する抑制効果を解析した（図4）。その結果，A3G C291S も野生型と同等の抑制効果があることが分かった。これらの結果から，A3G の（－）SSDNA 合成抑制効果は，酵素活性非依存的におこることが明らかになった。さらに，RNaseH の切断反応に対する A3G の影響を調べた。その結果，RNaseH の切断速度かつ切断パターンは，A3G 添加によって影響を受けなかった。

（－）および（＋）Strand Transfer 反応においても，NC による Transfer アニーリング反応および伸長反応を含む全反応（Transfer 反応）に対する A3G の影響を解析した。これらの結果，A3G は，NC が介在するアニーリング反応自身には影響を与えずに，逆転写酵素（RT）が関わる伸長反応を含んだ Transfer 反応を阻害した。以上のことから，A3G は一連の逆転写の過程で，RT が関与するすべての伸長反応だけに抑制的に働くことが分かった。プルダウン法などによって A3G と RT の安定した直接的な結合は認められないことから，鋳型の核酸を介していると考えられた。

では，なぜ A3G は RT 伸長反応を阻害するが，同じく核酸結合タンパクである NC は抑制効果を示さないのか？この問いに答えるために，A3G と NC の核酸への結合特性の違いについて比較してみた。まず，蛍光ラベルした ssDNA（20 mer）を用いた蛍光偏光解消法により，HIV-1 NC と RT，A3G の解離定数（Kd）を測定した。その結果から，A3G（Kd：238 nM）と NC（84.1 nM）は，RT（Kd：1840 nM）を核酸から効率よく競合的に除外することができるが，A3G は NC を核酸から容易には排除できない可能性が示唆された。さらに，一分子 DNA の光学機械的な伸展測定（Single Molecule DNA Stretching 解析）を行い，NC と A3G の核酸への結合解離（On/Off Rate）状態および核酸のアニーリングへの影響を検討した（図5）。NC は S 曲線が低く，R 曲線との違いが少ない。つまり，二本鎖 DNA を簡単に解す反面，アニーリングの際（R 曲線）NC は早く一本鎖領域から解離する。一方，A3G では S と R 曲線の違いが大きく，A3G は一本鎖領域に長時間結合し続け解離しないことが示された。以上のことから，A3G は NC に比べ，核酸への On/Off Rate が非常に遅いことが明らかになった。

これらの一連の研究から，A3G による酵素活性非依存的な抗 HIV 作用メカニズムは，A3G が鋳型である核酸の一本鎖領域に結合し，物理的に RT の伸長反応を阻害してしまうことによると考えられた（図6）。この阻害メカニズムは，RT と NC，A3G の核酸への結合特性によって決定されることが示された。

おわりに

本研究では，A3G の酵素活性非依存的な分子メカニズムについて明らかにした。最近，筆者が提唱した分子モデルを支持する *in vivo* の報告がなされている[4,5]。しかし，酵素活性依存的な機序に関しては依然として不明である。筆者が用いた *in vitro* 再構築系では足りない他の因子が関与している可能性がある。細胞内には，本来 DNA 中に存

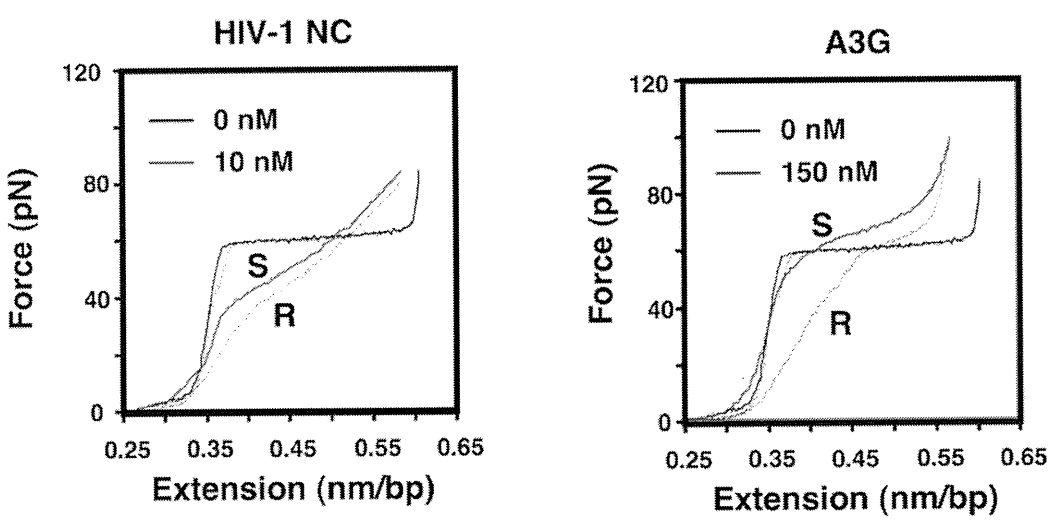

図 5　1分子 DNA ストレッチング法を用いた A3G の核酸結合特性の解析
λDNA（二本鎖）1分子を光学機械的に伸展させ，HIV-1NC（10 nM）あるいは A3G（150 nM）存在下（赤）で，伸長（Extension）対付加（Force）を計測した。伸展（S：Streched）を実線，回復（R：Relaxed）を点線で示す。

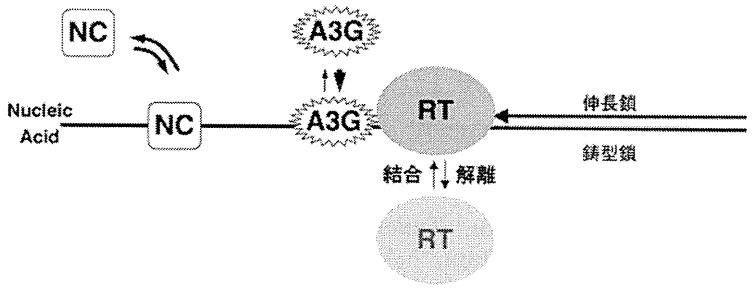

図 6　A3G による RT 伸長反応阻害機序のモデル図
A3G は，一本鎖核酸に対し結合親和性が高く解離速度が遅いため，見かけ上，逆転写の鋳型（一本鎖領域）にとどまり RT の伸長を物理的に止める。さらに，RT のプライマー部への再結合も抑制されてしまう。そのため，A3G は，酵素活性非依存的に逆転写伸長反応を抑制すると考えられる。一方，核酸結合タンパクである NC は，核酸に対して，結合・解離速度が速いため，RT の伸長には抑制的な影響を与えないと考えられる。

在すべきではない dU を除去する Uracil DNA Glycosilase（UDG）などの修復酵素が存在する。UDG などの関与も示唆されていたが，現在では直接的な関与はないと考えられている。今後，酵素活性依存的な阻害機序に関する研究も進み，A3G の抗 HIV 作用メカニズムの全容を分子レベルで解明されるであろう。

謝辞

この度，第 9 回 ECC 山口メモリアルエイズ研究奨励賞を受賞することになりましたことを深く感謝致します。これまで，ご指導いただきました杉浦亙先生をはじめとした先生方，受賞選考に関係された先生方に，厚く御礼申し上げます。

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Outbreak of Infections by Hepatitis B Virus Genotype A and Transmission of Genetic Drug Resistance in Patients Coinfected with HIV-1 in Japan▽

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The major routes of hepatitis B virus (HBV) infection in Japan has been mother-to-child transmission (MTCT) and blood transfusion. However, HBV cases transmitted through sexual contact are increasing, especially among HIV-1-seropositive patients. To understand the molecular epidemiology of HBV in HBV/HIV-1 coinfection, we analyzed HBV genotypes and HIV-1 subtypes in HBV/HIV-1-coinfected patients at Nagoya Medical Center from 2003 to 2007. Among 394 HIV-1-infected Japanese men having sex with men (MSM) who were newly diagnosed during the study period, 31 (7.9%) tested positive for the hepatitis B virus surface antigen. HBV sequence analyses were successful in 26 cases, with 21 (80.7%) and 5 (19.3%) cases determined as genotypes A and C, respectively. Our finding that HBV genotype A was dominant in HIV-1-seropositive patients alerts clinicians to an alternative outbreak of HBV genotype A in the HIV-1-infected MSM population and a shift in HBV genotype from C to A in Japan. The narrow genetic diversity in genotype A cases suggests that genotype A has been recently introduced into the MSM population and that sexual contacts among MSM were more active than speculated from HIV-1 tree analyses. In addition, we found a lamivudine resistance mutation in one naïve case, suggesting a risk of drug-resistant HBV transmission. As genotype A infection has a higher risk than infection with other genotypes for individuals to become HBV carriers, prevention programs are urgently needed for the target population.

The number of hepatitis B virus (HBV)-infected persons in Japan is estimated to be 1 million, or 0.8% of the total population (31). HBV is classified into eight genotypes, A to H, by their differences in genome sequences (11, 12, 22). Circulating genotypes in Japan differ according to geographical region, with the prevalent genotypes in 2001 being C (84.7%) and B (12.2%), while A (1.7%) and D (0.4%) were less frequent (17). HBV infection in Japan has been transmitted mainly by two routes, mother-to-child transmission (MTCT) and blood transfusion, which have been targeted by prevention programs still being operated today (13, 15–17, 25).

Regarding MTCT, all pregnant women are screened for HBV antigen and antibody. Mothers who are HBV infected are prohibited from breast-feeding, and their newborns are vaccinated against HBV. Regarding infection by blood transfusion, all donated blood is tested by anti-hepatitis B surface antibody (HBsAb) testing and PCR to exclude HBV-contaminated blood from the supply. These prevention programs have been successful, and the risks of HBV infection by these two routes have been reduced dramatically.

However, HBV infection by sexual contact has recently become a prevailing alternative transmission route of HBV in Japan (30, 36). In particular, coinfection with HBV and human immunodeficiency virus type 1 (HIV-1), the causative agent of AIDS, has been increasing among men who have sex with men (MSM), and the incidence of HBV infection associated with HIV-1-seropositive cases appeared to be 8.8%, which is higher than that in the general population (5). Thus, the epidemiology of HBV infection in Japan is quickly shifting. Here we report the most recent molecular epidemiologic status of HBV/HIV-1 coinfection.

MATERIALS AND METHODS

Sample. HIV/AIDS patients newly diagnosed at Nagoya Medical Center from 2003 to 2007 were tested for hepatitis B surface antigen (HBsAg), and HBsAg-positive patients were enrolled in the study. Clinical data (age, gender, suspected route of HIV-1 infection, aspartate aminotransferase [AST] and alanine aminotransferase [ALT] plasma levels, CD4-positive T cell count, and HIV viral load) were obtained from medical records. Plasma HBV viral load was measured with COBAS TaqMan (Roche Diagnostics, Basel, Switzerland), and plasma HBc IgM titer was measured with Lumipulse (Fujirebio, Tokyo, Japan). The time of HBV infection was estimated by patient interview and HBc IgM titer results. This study was conducted according to the principles expressed in the Declaration of Helsinki. The study was approved by the Institutional Review Boards of the National Institute of Infectious Diseases and Nagoya Medical Center. All pa-

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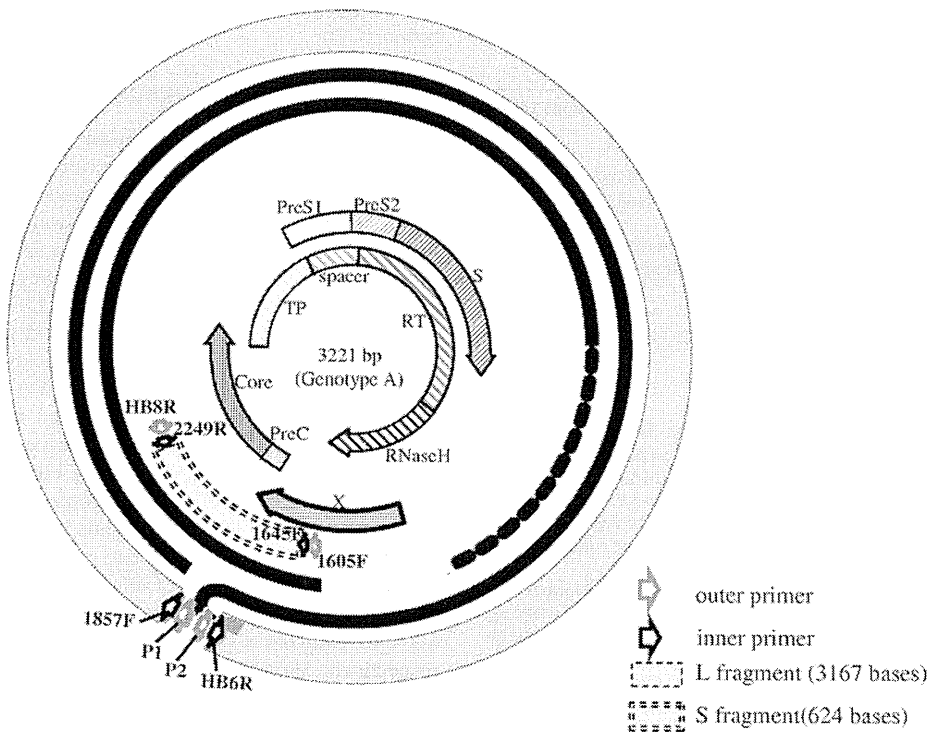

FIG. 1. Genetic regions of HBV and HIV-1 used for phylogenetic tree analyses. The whole HBV genome was amplified in two fragments, L and S, and assembled. L and S fragments are indicated by single and double dashed lines, respectively.

tients provided written informed consent for collection of samples and subsequent analysis.

Amplification of HBV and HIV DNA fragments and determination of DNA sequences. HBV nucleic acid was extracted from plasma using a MagNA Pure Compact Nucleic Acid Isolation Kit I (Roche Diagnostics). As shown in Fig. 1, the full-length HBV genome was amplified in two fragments, L (3,167 bases) and S (624 bases). The primers used for amplifying HBV DNA were both newly designed and have been published previously (27). Details of these primers are summarized in Table 1. The DNA polymerases used for the first and nested PCRs were LA *Taq* (Takara, Shiga, Japan) and Prime Star HS (Takara) polymerase, respectively. The HBV genotypes were also determined using a commercial kit (Institute of Immunology, Tokyo, Japan) based on enzyme immunoassay to confirm that the results did not differ from those based on phylogenetic tree analysis.

The HIV-1 *gag p17* (396 bp [bp 790 to 1185]), *pol* (1,117 bp [bp 2253 to 3369]), and *env C2V3* (222 bp [bp 6996 to 7217]) regions were amplified from extracted plasma HIV-1 RNA by reverse transcription-PCR (RT-PCR) using the SuperScript one-step RT-PCR system for long templates (Invitrogen, Carlsbad, CA)

TABLE 1. Primers for amplifying the HBV and HIV-1 genomes

Name	Direction[a]	Sequence (5′ → 3′)	Region
P1	F	TTTTCACCTCTGCCTAATCA	First PCR, HBV L fragment
P2	R	AAAAAGTTGCATGGTGCTGG	First PCR, HBV L fragment
1605F	F	CGCATGGAGACCACCGTGAA	First PCR, HBV S fragment
HB8R	R	ATAGGGGCATTTGGTGGTCT	First PCR, HBV S fragment
1857F	F	CTACTGTTCAAGCCTCCAAG	Nested PCR, HBV L fragment
HB6R	R	AACAGACCAATTTATGCCTA	Nested PCR, HBV L fragment
1645F	R	AGGTCTTGCATAAGAGGACT	Nested PCR, HBV S fragment
2249R	F	CCAAAAGACACCAAATAYTC	Nested PCR, HBV S fragment
172A	F	ATCTCTAGCAGTGGCGCCCGAACAG	RT-PCR, HIV-1 *gag* fragment
173B	R	CTGATAATGCTGAAAACATGGGTAT	RT-PCR, HIV-1 *gag* fragment
174A	F	CTCTCGACGCAGGACTCGGCTTGCT	Nested PCR, HIV-1 *gag* fragment
175B	R	CCCATGCATTCAAAGTTCTAGGTGA	Nested PCR, HIV-1 *gag* fragment
K1	F	AAGGGCTGTTGGAAATGTGG	RT-PCR, HIV-1 *pol* fragment
U13	R	CCCACTCAGGAATCCAGGT	RT-PCR, HIV-1 *pol* fragment
K4	F	GAAAGGAAGGACACCAAATGA	nested PCR, HIV-1 *pol* fragment
U12	R	CTCATTCTTGCATATTTTCCTGTT	Nested PCR, HIV-1 *pol* fragment
106A	F	CATACATTATTGTGCCCCGGCTGG	RT-PCR, HIV-1 *env* fragment
17B	R	AGAAAAATTCCCCTCTACAATTAA	RT-PCR, HIV-1 *env* fragment
14A	F	AATGTCAGCTCAGTACAATGCACAC	Nested PCR, HIV-1 *env* fragment
10B	R	ATTTCTGGGTCCCCTCCTGAGG	Nested PCR, HIV-1 *env* fragment

[a] F, forward; R, reverse.

TABLE 2. HBV genotype reference sequences collected from the DNA Database of Japan (DDBJ) for tMRCA analysis

Genotype	DDBJ accession no.
A	FJ692588, GQ325786, GQ477503, GQ477496, GQ486599, EU414132
B	FJ751547, GQ924611
C	GQ924615, GQ486096, EU939589, GQ486684
D	GQ486337, FJ349228, GQ924652, EU414124, GQ922001, GQ486586
E	GQ486756, GQ161830, FJ349237
F	GQ486537, GQ486515, GQ486570
G	GQ486843
H	GQ486592, AB266536

followed by a second PCR using LA *Taq* polymerase. The primers used for HIV-1 sequencing are also summarized in Table 1. The amplicons were purified using a MultiScreen PCR filter plate (Millipore, Billerica, MA), and the sequencing reaction was performed using the BigDye Terminator v3.1 cycle sequencing kit (Applied Biosystems, Carlsbad, CA) and analyzed with the ABI PRISM 3130 (Applied Biosystems) autosequencer. Electropherograms were edited and verified by SeqScape v2.5 software (Applied Biosystems).

Phylogenetic tree analyses and genotype determination. HBV genotypes were determined by phylogenetic tree analysis with reference sequences. HBV sequences were aligned with 23 reference sequences from the National Center for Biotechnology Information (NCBI) database by using the CLUSTAL W program and analyzed by Kimura two-parameter methods. Genetic distances were calculated by the maximum composite likelihood, and phylogenetic trees were constructed by the neighbor-joining method using MEGA version 4 software. The reliabilities of branches were evaluated by bootstrap analysis with 1,000 replicates.

Phylogenetic trees of the HIV-1 *gag*, *pol*, and *env* regions were also constructed with 62 HIV-1 reference sequences obtained from the HIV-1 sequence database (Los Alamos National Laboratory).

Estimated tMRCAs. Evolutionary rates, chronological phylogenies, and other evolutionary parameters of HBV genotypes were estimated from heterochronous data for the HBV genomic sequences collected in our study, together with reference sequences from public databases (Table 2), using the Bayesian Markov chain Monte Carlo (MCMC) method. The nucleotide substitution model was evaluated by the hierarchical likelihood ratio test using PAUP v4.0 (29) with MrModeltest (14) and the general time-reversible (GTR) model with both invariant site (I) and gamma-distributed site (G) heterogeneity for four rate categories showing maximum likelihood. Bayesian MCMC analyses were performed with BEAST v1.4.8 (4) using the substitution model of GTR + I + G, three partitions into codon positions, and a relaxed molecular clock model (the uncorrelated log normal-distributed model) (3). Four different population dynamic models (exponential growth, logistic growth, constant population, and Bayesian skyline plot [BSP]) were tested in the analyses. According to BSP properties, constant-growth models were adopted for the HBV genome sequences. Each Bayesian MCMC analysis was run for 40 million states and sampled every 10,000 states. Posterior probabilities were calculated with a burn-in of 4 million states and checked for convergence using Tracer v1.4 (21). The maximum clade credibility tree for analyzing the MCMC data set was annotated by TreeAnnotator in the BEAST package. The posterior distribution of the substitution rate obtained from the heterochronous sequences was subsequently incorporated as a prior distribution for the mean evolutionary rate of the HBV genome, thereby adding a time scale to the phylogenetic histories of the given viruses and enabling estimation of the time of the most recent common ancestor (tMRCA) (19).

Determination of HBV drug resistance mutations. HBV cases resistant to nucleoside analogue reverse transcriptase inhibitors (NRTI) were determined by analyzing amino acid sequences of the RT region. The approved anti-HBV drugs in Japan are lamivudine, adefovir, and entecavir. In cases of HBV/HIV-1 coinfection, tenofovir and emtricitabine are also used. We studied whether the viruses have drug resistance mutations against these antiretroviral drugs with or without a history of antiretroviral treatments and confirmed the following resistance mutations: lamivudine/emtricitabine resistance mutations V173L, L180M, and M204I/V; adefovir resistance mutations A181V, I233V, and N236T; entecavir resistance mutations I169T, L180M, T184G, S202I, M204I/V, and M250V; and tenofovir resistance mutation A194T (1, 2, 24, 32, 34, 35). Furthermore, major drug resistance mutations in HIV-1 were defined according to the criteria of the International AIDS Society (IAS)-USA and Stanford HIV drug resistance database (7, 23).

RESULTS

The major HBV genotype circulating among Japanese MSM is genotype A. During the study period, 394 cases were newly diagnosed as HIV/AIDS, and 31 cases were determined as HBsAg positive. Thus, the average prevalence of HBV/HIV-1 coinfection in our study population was 7.9%. Analysis of the coinfection prevalence in each year showed increases from 2.8 to 3.3% in 2003 to 2004 and from 7.4 to 13.2% in 2005 to 2007 (Fig. 2). As the suspected route of HIV-1 infections in all 31 cases was MSM, HBV appears to be quickly spreading among the MSM population. Of these HBV/HIV-1-coinfected cases, 26 isolates were successfully sequenced for both HBV and HIV-1, and their subtypes and genotypes were determined. Regarding the five cases for which the HBV genome could not be sequenced, plasma HBV DNA copies were undetectable in four cases, and low ($10^{3.3}$ copies/ml) in one case.

The median age of the patients was 34 years (interquartile range [IQR], 29.5 to 37.0) (Table 3). The median plasma viral loads of HBV and HIV-1 were 4.4×10^8 (IQR, 4.9×10^4 – 6.3×10^8) and 6.4×10^4 (IQR, 2.0×10^4 – 2.0×10^5) copies/ml, respectively. Hepatitis B core antigen (HBcAg) IgM was positive in nine patients, of which two were suspected to harbor acute HBV infection according to their HBsAg positivity, AST and ALT plasma levels, and patient interviews. The other 7 HBcAg-positive patients were categorized as having acute hepatitis or exacerbated chronic hepatitis, and 17 HBcAg-negative patients were determined as being in the chronic hepatitis stage.

According to phylogenetic tree analysis, 26 cases were classified into two genotypes, either A or C. As shown in Fig. 3, 21 and 5 cases were classified as genotypes A and C, respectively. The subgenotypes of the 21 genotype A cases were all A2, the predominant subgenotype in Europe and North America, whereas the subgenotypes of the 5 genotype C cases were all C1, the most prevalent subgenotype in eastern Asia, including Japan, South Korea, and northern China. Genotype B, the

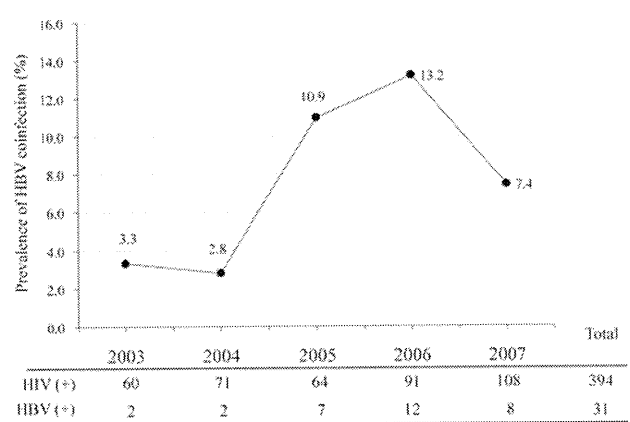

FIG. 2. Transitions in HBV infection rates in HBV/HIV-1-coinfected patients. HBV infection rates are plotted versus year, with the numbers of HIV-1-infected and HBV/HIV-1-coinfected patients shown below the *x* axis.

TABLE 3. Characteristics of HBV/HIV-1-coinfected patients

Characteristic	Value[a] for genotype:			P
	All (n = 26)	A (n = 21)	C (n = 5)	
Age (yr)	34 (30–37)	33 (29–37)	56 (46–57)	<0.01
Suspected route of HIV-1 infection	MSM	MSM	MSM	
AST (IU/liter)	31 (26–63)	29 (26–48)	54 (20–74)	
ALT (IU/liter)	43 (33–90)	42 (32–85)	44 (34–99)	
No. HBcAg IgM positive	9	9	0	<0.01
CD4 (/μl)	293 (91–492)	300 (94–484)	202 (9–494)	
HIV-1viral load (copies/ml)	6.4×10^4 (2.0×10^4–2.0×10^5)	6.8×10^4 (2.4×10^4–2.1×10^5)	2.4×10^4 (2.4×10^3–9.7×10^4)	
HBV viral load (copies/ml)	4.4×10^8 (4.9×10^4–6.3×10^8)	6.3×10^8 (4.7×10^4–6.3×10^8)	2.0×10^6 (4.7×10^5–6.3×10^8)	

[a] Median values are shown. Numbers in parentheses represent interquartile ranges.

second most predominant HBV genotype in Japan, was not detected in our study. Interestingly, the genotype A and C populations showed obvious differences in genetic diversity. The 21 group A2 samples (Fig. 3) formed a cluster with little

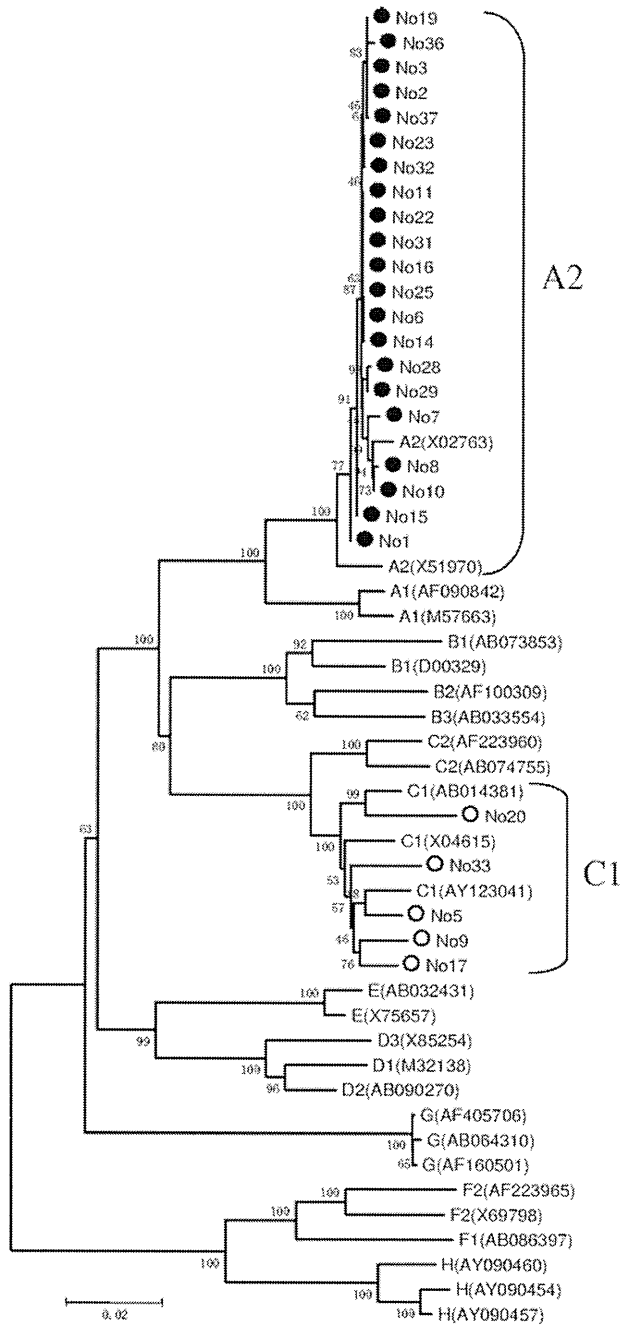

FIG. 3. Phylogenetic tree analyses of HBV isolated from HBV/HIV-1-coinfected patients. The phylogenetic tree was constructed using 26 full-length HBV genome sequences detected in HBV/HIV-1-coinfected patients in Nagoya (both solid and open circles) and 23 reference sequences from the NCBI database. Twenty-one and five cases were distributed in the clusters of genotype A (solid circles) and C (open circles), respectively.

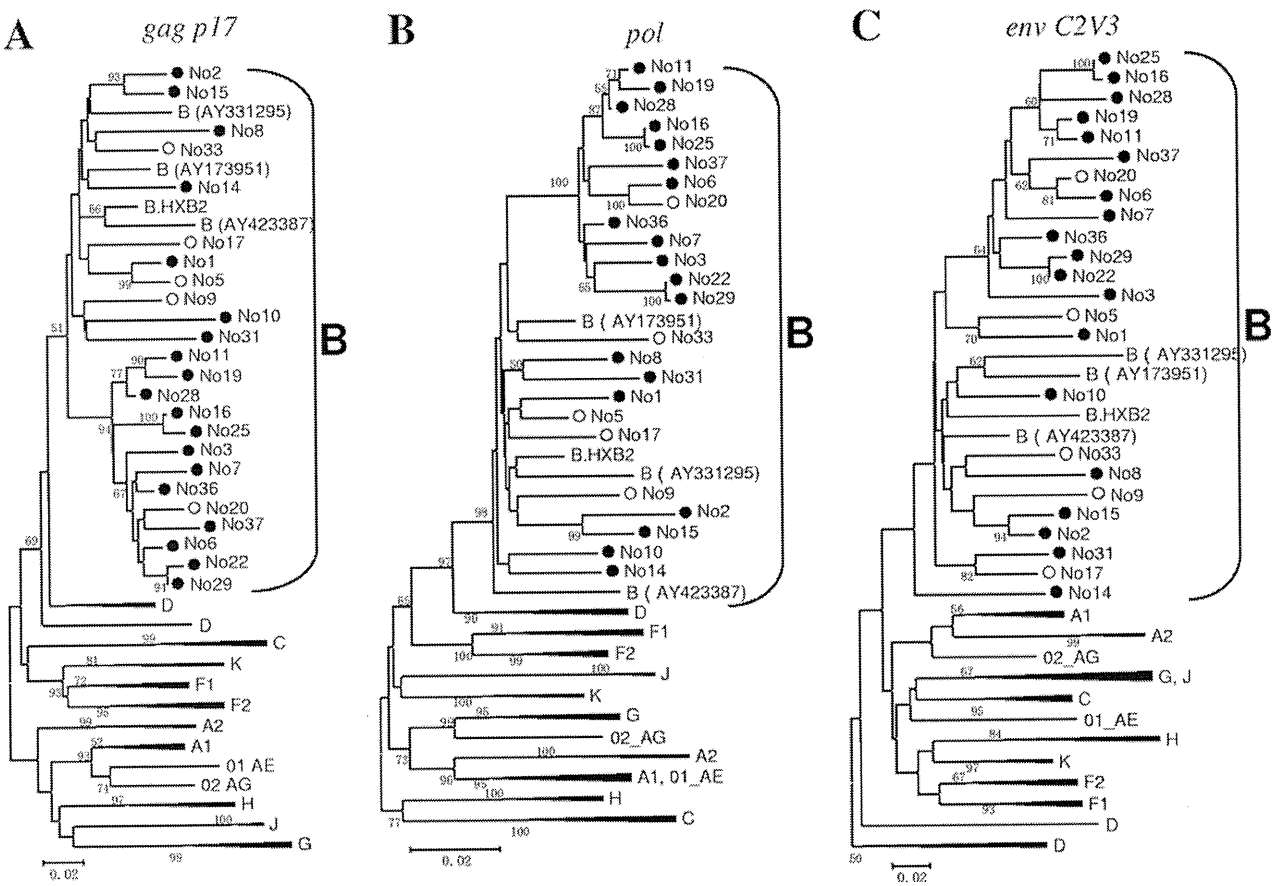

FIG. 4. Phylogenetic tree analyses of HIV-1 isolated from HBV/HIV-1-coinfected patients. Phylogenetic trees were constructed using the 25 HIV-1 sequences obtained in this study and 62 HIV-1 reference sequences from the Los Alamos National Laboratory database. The nucleotide base sequences of *gag p17* (A), *pol* PR to RT (B), and *env C2V3* (C) gene regions were analyzed. In all analyses, all the HIV-1-positive cases detected in Nagoya (both solid and open circles) were distributed in the subtype B cluster. Cases of coinfection with genotype C HBV are shown with open circles.

or no genetic distance between each other, indicating their extremely close genetic relationships. In contrast, the five group C1 cases did not form a single cluster and had longer branches than those of group A2.

Patients with genotypes A and C also differed significantly in age (Table 3). The median age of the genotype A patients was 33 years (IQR, 29 to 37), whereas that of the genotype C patients was 56 (IQR, 46 to 57) ($P < 0.01$). Furthermore, all nine HBcAg IgM-positive cases, including five suspected cases of acute infection, were categorized in genotype A2, suggesting ongoing active transmission of the virus among the Japanese MSM population. Thus, the genotype A2 population appeared to be younger, with more acute cases, and infected with an almost genetically identical HBV strain. These two genotypes did not differ significantly in regard other clinical data, such as AST and ALT levels, $CD4^+$ T cell count, and HBV and HIV-1 viral loads.

To clarify the detailed epidemiological features of HBV/HIV-1-coinfected patients, the HIV-1 subtypes and their genetic distances were determined by phylogenetic analyses of three genome regions, *gag p17*, *pol*, and *env C2V3*. All 26 samples were determined as subtype B (Fig. 4A, B, and C), and interestingly, branch patterns and relationships among cases were different from those for HBV. There were six paired cases, demonstrating a significantly close genetic relationship (>50% bootstrap value) in more than two regions. These paired cases were cases 1 and 5, 2 and 15, 6 and 20, 11 and 19, 16 and 25, and 22 and 29, and these connections were not evident in the HBV phylogeny, suggesting different origins of sexual partner between the two pathogens in each pair. An alternative explanation could be that little genetic variation in HBV made it difficult to clarify the genetic relationships between cases. However, there was one discordant pair (cases 1and 5); one case had HBV genotype A, and the other case had HBV genotype C2. Furthermore, the other four HBV genotype C2 cases (cases 9, 17, 20, and 33) were scattered among HIV-1 phylogenies within genotype B HIV-1-infected patients.

HBV strains detected in HIV-1-infected patients from Nagoya are the same viruses found in other parts of Japan. To clarify whether the dominance of genotype A HBV in HIV-1-infected MSM is a regional issue in the Nagoya urban area or a more nationwide epidemic, we reconstructed an HBV phylogenetic tree of 26 cases together with HBV sequences col-

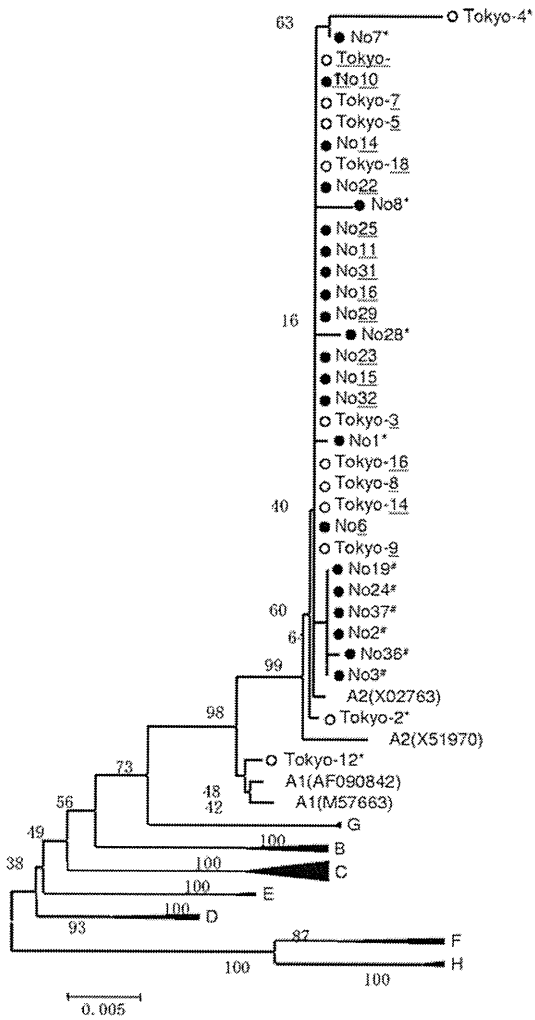

FIG. 5. Phylogenetic tree analysis of 35 HBV region S sequences, 22 from Nagoya (solid circles) and 13 from Tokyo (open circles). Three genetically different groups are indicated by asterisks, pound signs, and underlining.

lected at a different time and from a different area of Japan, i.e., 12 genotype A sequences from HBV/HIV-1-coinfected patients collected in Tokyo about 10 years before this study (8). As no full genome sequences were available for the Tokyo cases, only the S gene (681 bp [bp 155 to 835]) was analyzed. From the phylogenetic tree pattern, genotype A was classified into three groups (Fig. 5). The first is a group of 21 identical sequences (underlined in Fig. 5). As this group had the largest number of cases and included sequences from both Nagoya and Tokyo, this strain appears to be prevailing nationwide. The second group is a cluster of cases, i.e., cases 2, 3, 19, 24, 36, and 37. As all six cases were from Nagoya, this isolate still seems to be in an endemic status. The third group comprises isolates with longer branches (noted by asterisks), i.e., Tokyo-2, -4, and -12 and Nagoya-1, -7, -8, and -28. These isolates appear to be quite distinct from the others, suggesting that their origin may not be sexual contact but another route, such as MTCT or transfusions.

The prevailing HBV genotype A2 emerged more recently than most other genotypes. To estimate the emergence time of the prevailing genotype A2 strain, we estimated its mutation rate per year and tMRCA. First, the median mutation rate per year was calculated as 3.23×10^{-5} (5.62×10^{-8} to 9.01×10^{-5}), which is close to those previously reported (10, 18). Next, the median tMRCAs of all A strains, A1, A2, and C were determined to be 370.8, 88.9, 184.3, and 494.9 years ago, respectively (Table 4; Fig. 6). Thus, the A2 genotype is one of the youngest HBV genotypes.

A lamivudine resistance amino acid HBV mutation detected in an antiretroviral therapy-naïve patient. We clarified not only HBV genotypes but also the incidence of transmitted drug-resistant HBV among the study patients. Analysis of the amino acid sequence of the HBV RT region showed a combined triple amino acid mutation, rtV173L + rtL180M + rtM204V, which was a mutation causing resistance against lamivudine and its 5-fluoro analogue (2′,3′-dideoxy-3′-thia-5-fluorocytidine), in two patients (patients 5 and 8). However, one patient (patient 5) had been treated with stavudine-lamivudine-efavirenz at the time of sample collection, and thus only one case (case 8) was suspected to be a transmitted HBV drug-resistant case. No HIV-1 drug-resistant virus transmission was detected in the study sample.

DISCUSSION

This molecular epidemiological study of HBV infection in HIV-1-seropositive patients revealed epidemiological characteristics that were unique compared to those of the general population in Japan. All HBV/HIV-1-coinfected patients were MSM, they had a 10-fold-higher prevalence (7.9%) than that of the general population, and genotype A was the predominant HBV genotype (31). This distinct HBV epidemic in MSM was first reported in 2001 in other regions of the country (9, 36), a decade before our study. Furthermore, phylogenetic analysis of sequences from the two studies, collected in different regions and years, revealed that an identical genotype A strain prevails among the MSM population nationwide.

Considering the status of HBV epidemiology in the general population of Japan, genotypes C and B must have an equal or greater chance to disseminate among the HIV-1-seropositive

TABLE 4. Estimated times of the most recent ancestor (tMRCAs) for HBV genotypes

Genotype	Mean tMRCA (yr before)		95% HPD[a]	
	Mean	Median	L	H
A	1,294.2	370.8	27.1	4,046.4
A1	306.6	88.9	12.4	976.4
A2	597.4	184.3	18.8	1,886.2
B	345.8	88.5	4.2	1,069.3
C	1,655.3	494.9	36.6	5,124.7
C2	1,062.4	308.6	20.8	3,296.6
D	827.2	226.6	11.2	2,469.4
E	163.7	38.9	4.5	539.7
F	1,060.8	308.2	13.7	3,277.4
H	433.8	110.1	5.6	1,303.0

[a] HPD, highest posterior density.

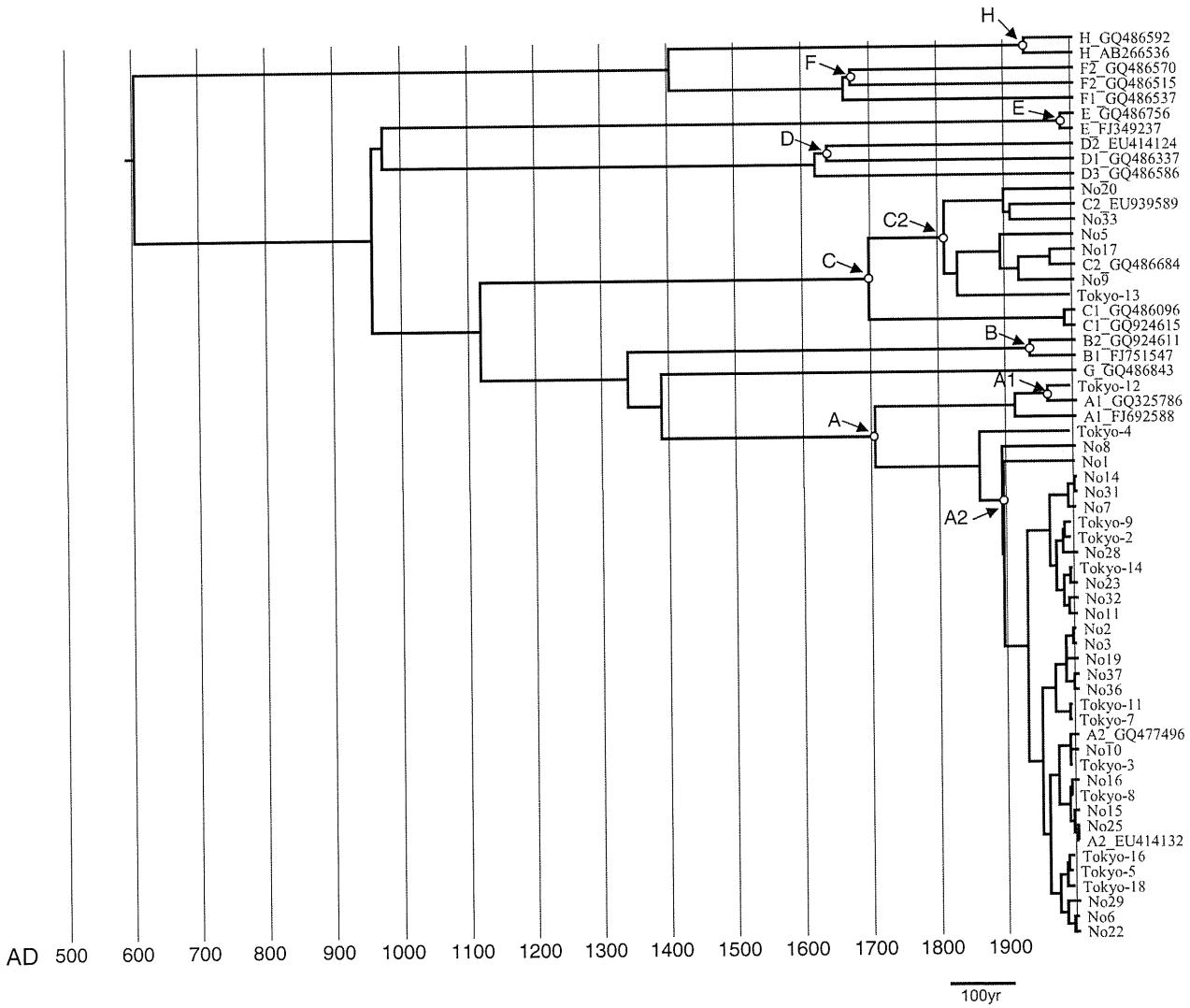

FIG. 6. Maximum clade credibility tree depicted according to median tMRCA. Nodes with open circles are evaluated points for each genotype summarized in Table 2.

MSM population. In fact, we found five genotype C patients in our study sample, and all five patients were MSM. However, because these five genotype C patients were older and their isolates had longer branches in phylogenetic analysis than the prevailing genotype A isolates, they appear to have been independently infected through either MTCT or blood transfusion events rather than sexual contact. Furthermore, as all five cases were singletons without any genetically close isolates among the samples analyzed, this genotype appeared to be less efficiently transmitted by sexual contact.

Interestingly, predominant genotype A HBV coinfection in HIV-seropositive MSM populations has also been reported in European and South American countries (20, 26, 33), suggesting that the prevailing genotype A in HIV-seropositive MSM has become a worldwide HBV epidemic. Regarding HBV genotypes in the HIV-negative population in Japan, genotype A has been increasing, but the major HBV genotype is still C, with genotype A remaining at 3.5% nationwide and 2.1% in the Tokai area, which includes Nagoya city (9). Therefore, the prevalence of genotype A HBV in the MSM population is significantly higher than in the rest of the population.

Thus, it is interesting to discuss the virological advantages disposing this genotype A isolate to become the major player in HBV/HIV-1 coinfection among MSM. One such advantage might be the higher progression rate (16 to 23%) to chronicity of genotype A than of genotype C (28, 30), enhancing its capacity to serve as a source of new infections. As 9 of 26 genotype A-infected patients (35%) were HBcAg IgM positive and 2 had acute hepatitis, it is obvious that genotype A infections are actively ongoing among the MSM population. Though further studies are needed, considering the tMRCA of the prevailing strain A2, the younger age of patients infected with this strain than of those infected with other genotypes,

and its high prevalence among MSM, this strain may have acquired higher infectivity and efficient transmission through sexual contact.

Another issue we wanted to clarify in this study was the transmission of antiviral drug resistance. We found no antiretroviral resistance in the 26 sequenced cases. On the other hand, we detected two cases with a mutation combination of rtV173L + rtL180M + rtM204V in HBV reverse transcriptase, demonstrating resistance against lamivudine-emtricitabine. One patient was antiretroviral therapy naïve; thus, transmission of drug-resistant HBV is strongly suspected. It is peculiar that the isolate harboring the drug-resistant mutations in HBV was a singleton, considering that genetically identical isolates were prevailing, that there were very low mutation rates that suggest few chances of reverting to wild type, and that there were actively ongoing *de novo* infections. This finding might be due to resistant viruses being masked by wild-type viruses under untreated conditions, as reported in the case of HIV-1 drug resistance (6). The possibility of minority resistance populations of HBV could be verified by detection with a highly sensitive method.

In conclusion, we clarified the molecular epidemiology of HBV/HIV-1 coinfection in Japan. Our data suggest that ongoing HBV infections lie outside prevention programs targeting the MTCT and blood transfusion infection routes, and they suggest the urgent need for new prevention strategies focusing on the high-risk group of the HIV-1-seropositive MSM population.

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HIV-2 CRF01_AB: First Circulating Recombinant Form of HIV-2

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Background: Five HIV-2–seropositive cases were recently identified in Japan, outside the HIV-2 endemic area of West Africa. To clarify the molecular epidemiology of HIV-2 in Japan, we analyzed sequences of these cases in detail.

Methods: HIV-2 genetic groups were determined by *gag* and *env* sequences. For suspected recombinant isolates, the genetic structure was determined by full-length genomic analyses. To understand the history and evolution of HIV-2 recombinant isolates, we estimated the time of most recent common ancestor by Bayesian Markov chain Monte Carlo method.

Results: Three isolates were determined as recombinants of groups A and B, and their mosaic genome structures were identical with that of 7312A, a recombinant isolate reported in 1990 from Côte d'Ivoire. Our 3 isolates and 7312A fulfilled the criteria for determining a circulating recombinant form (CRF). These isolates were verified by the Los Alamos HIV sequence database as the first CRF of HIV-2, HIV-2 CRF01_AB. The mean time of most recent common ancestor of CRF01_AB was estimated as between 1964 and 1973, several decades after the estimated emergence of HIV-2.

Conclusions: We recently identified HIV-2 CRF01_AB cases in Japan. This ectopic observation of the virus outside its original endemic area suggests an ongoing global spread of HIV-2 CRF01_AB.

Key Words: circulating recombinant form, CRF01_AB, HIV-2, molecular epidemiology

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INTRODUCTION

One million people worldwide are infected with HIV-2. The distribution of HIV-2, unlike the global epidemic of HIV-1, is still mainly restricted to West Africa and several European and Asian countries.[1–4] HIV-2 has been characterized as less pathogenic than HIV-1,[5–11] with more than 75% of HIV-2–infected cases remaining asymptomatic throughout their clinical course.[4] HIV-2 can be genetically classified into 8 groups, A to H, which have equivalent genetic distances to those of HIV-1 groups but not subtypes, with groups A and B circulating in the human population.[12–16] In addition, 2 different AB recombinants (7312A and 510-03) have been identified in West Africa,[12,13,17–19] but their circulation has not been identified to date.

In Japan, only 2 HIV-2–infected cases have been reported, but both were infected abroad.[20,21] Inside the country, there has been no evidence of HIV-2 transmission and circulation. Here we report 5 HIV-2–infected cases recently identified in Japan. Of these 5 cases, 3 were shown by full-length genomic analysis to be infected with the same type of recombinant virus determined to be the first circulating recombinant form (CRF) of HIV-2.

METHODS

HIV-2 Samples and Quantification of HIV Plasma Viral Loads

Among 843 HIV/AIDS cases registered at the Nagoya Medical Center (NMC), Japan from 1994 to 2008 (for demographic characteristics, see **Table, Supplemental Digital Content 1**, http://links.lww.com/QAI/A49), 5 cases (3 males and 2 females) were diagnosed serologically as HIV-2 infected. To better understand the molecular epidemiology of HIV-2 infection in Japan, we analyzed the HIV-2 genetic groups of the 5 cases.

Plasma HIV-1 viral loads were measured by the Cobas Amplicor HIV-1 monitor test v1.5 (Roche Diagnostics, Tokyo, Japan) or the Cobas TaqMan HIV-1 test (Roche Diagnostics),

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whereas plasma HIV-2 viral loads were measured by an in-house quantification assay, the Poisson quantification method described elsewhere.[22,23] In brief, total RNA was extracted from 500 μL of plasma sample using the QIAamp UltraSens Virus Kit (QIAGEN, Tokyo, Japan). Reverse transcription (RT) and nested polymerase chain reaction (PCR) (RT-nested PCR) were performed using serially diluted RNA samples, and HIV-2 viral loads were statistically calculated using results from samples diluted to near the endpoint. (For details of RT-nested PCR reaction mixtures and thermal programs, see **Table, Supplemental Digital Content 2**, http://links.lww.com/QAI/A50).

Genomic DNA Sequencing

HIV-2 proviral DNAs were purified from peripheral blood mononuclear cells using the DNA blood mini kit (QIAGEN). To determine HIV-2 genetic groups, *gag* (777 bps: 1163 to 1939 according to SIVmac239) and *env* (454 bps: 7300 to 7753) gene fragments were amplified by nested PCR using LA Taq polymerase (Takara Bio, Shiga, Japan) and previously reported[13,24] primers: gagA, gagB, gagC, and gagF for *gag*, and PFD1, LTR9574, EB2, and EB5 for *env*. To determine full-length genomic sequences, 4 DNA fragments containing (1) 5′ long terminal repeat (LTR) (915 bps: 31 to 945), (2) *gag* to *nef* genes (9122 bps: 899 to 10020), (3) 3′ LTR (791 bps: 9463 to 10252), and (4) the joining point of the circular 2 LTR form (597 bps: 10085 to 10279 and 1 to 402) were amplified by nested PCR using 8 primer pairs (see **Table, Supplemental Digital Content 3**, http://links.lww.com/QAI/A51). The following PCR program was used: denaturation (2 minutes at 94°C) followed by 40 cycles of PCR (94°C: 15 seconds, 60°C: 30 seconds, and 70°C: 1 minute/1000 bps). Sequencing was performed using a 3730 DNA Analyzer (Applied Biosystems, Tokyo, Japan).

Phylogenetic Tree Analysis and Determination of Recombinant Genome Structures

Multiple sequence alignment was performed using CLUSTAL W, and genetic distances were calculated based on the maximum composite likelihood model using MEGA software v4.[25] Phylogenetic trees were constructed using the neighbor-joining method.

Complete full-length genomic sequences of 4 HIV-2 group A strains (ALI, BEN, CAM2CG, and UC2), 3 HIV-2 group B strains (D205, EHO, and UC1), and SIVmac239, (a rhesus macaque-adapted simian immunodeficiency viral isolate) were used as reference sequences. After realigning the sequence set, recombinant breakpoints were determined by similarity plotting, bootscanning, and informative site analysis using SimPlot software, v3.5.1.[26]

Estimated Times of the Most Recent Common Ancestors

Evolutionary rates, chronological phylogenies, and other evolutionary parameters were estimated from 17 full-length or near full-length HIV-2/SIV genomic sequences (see **Table, Supplemental Digital Content 4**, http://links.lww.com/QAI/A52) using the Bayesian Markov chain Monte Carlo (MCMC) method implemented in BEAST v1.4.8.[27] The alignment data for the full-genome sequences were processed into 2 subsets consisting of sequences corresponding to the group A or B region of HIV-2 AB-recombinant virus. Bayesian MCMC analyses were performed using a relaxed molecular clock model.[28] The nucleotide substitution model was evaluated by the hierarchical likelihood ratio test using PAUP v4.0 beta[29] with MrModeltest (Nylander JAA. 2004. MrModeltest v2. Program distributed by the author. Evolutionary Biology Centre, Uppsala University), and the general time-reversible model[30] was adopted with both invariant sites and gamma-distributed site heterogeneity for 4 rate categories. The coalescent model used in the analyses was a logistically growing population because the population size of HIV-2 seemed constant in the early phase followed by exponential growth in the recent period.[31] Each Bayesian MCMC analysis was run for 40 million states and sampled every 10,000 states. Posterior probabilities were calculated with a burn-in of 4 million states and checked for convergence using Tracer v1.4. The posterior distribution of the substitution rate obtained from the heterochronous sequences was subsequently incorporated as a prior distribution for the evolutionary rate of HIV-2 genome regions A and B, thereby adding a timescale to the phylogenetic histories of the HIV-2 strains and enabling the times of most recent common ancestor (tMRCAs) to be estimated.[32]

Accession Numbers

Nucleotide sequences have been registered as #AB499685 to AB499695 in the DNA databank of Japan.

TABLE 1. Demographic and Clinical Characteristics of Patients Diagnosed as HIV-2 Infected

Patient #	Year	Sex	Age (Yrs)	Nationality	Risk Factor for Infection	$CD4^+$ Cell Count (Cells/μL)	HIV-1 Viral Load (Copies/mL)*	HIV-2 Viral Load (Copies/mL)	Western Blot†		Opportunistic Infections
									HIV-1	HIV-2	
NMC307	2004	M	28	Nigerian	Hetero	241	<50	350,000	I	P	Tuberculosis
NMC678	2007	F	28	Japanese	Hetero	883	<50	ND	I	P	—
NMC716	2007	M	36	Nigerian	Hetero	4	<50	680,000	I	P	Candidiasis
NMC786	2008	M	38	Ghanaian	Hetero	1	<40	60,000	N	I	Candidiasis, CMV infection
NMC842	2008	F	34	Japanese	Hetero	110	<40	25,000	N	P	—

*Detection limits of Cobas Amplicor HIV-1 monitor v1.5 and Cobas TaqMan HIV-1 tests were 50 and 40 copies/ml, respectively.
†New LAV Blot I and II kits (Bio-Rad Laboratories, Tokyo, Japan) were used.
CMV, cytomegalovirus; F, female; Hetero, heterosexual contact; I, intermediate; M, male; ND, not detected; N, negative; P, positive.

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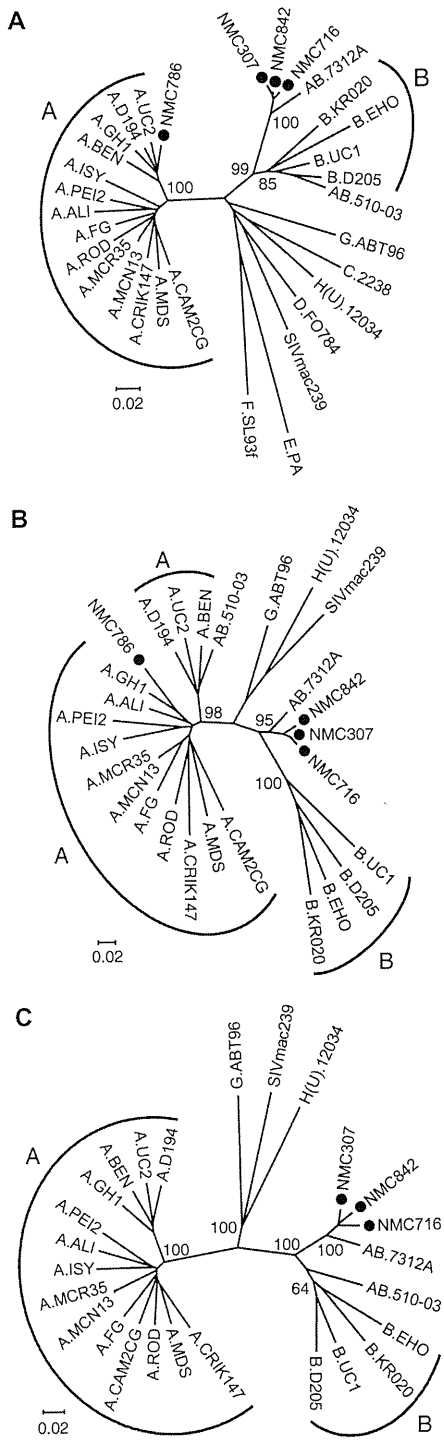

FIGURE 1. Phylogenetic tree analyses of HIV-2 isolates identified in this study. Phylogenetic tree analyses are shown using the following: A, HIV-2 *gag* gene sequences (bps: 1163 to 1939 in the reference SIVmac239 sequence); B, *env* gene sequences (bps: 7300 to 7753); and C, full-length or near full-length genomic sequences. Phylogenetic trees were constructed by the neighbor-joining method. Bootstrap values were calculated by 1000 analyses and are shown at the major tree nodes. Scale bar represents 0.02 nucleotide substitutions per site. Each reference HIV-2 strain is represented by its genetic group and name. HIV-2 isolates identified in this study (NMC307, NMC716, NMC786, and NMC842) are shown by filled circles.

RESULTS

HIV-2 Infection Confirmed by Nucleotide Amplification in Four AIDS Cases

Profiles of 5 HIV-2–seropositive cases are summarized in Table 1. The 3 males were from West African countries, a major endemic area for HIV-2, and suspected as seropositive before arriving in Japan. However, 2 females, both Japanese, were suspected to be recently infected within Japan based on their interviews. All their risk factors were heterosexual contacts, and no personal connection was confirmed among any of these cases. Thus, these 5 cases were independently infected with HIV-2 on different occasions. Notably, 4 cases (NMC307, NMC716, NMC786, and NMC842) were found at advanced stage AIDS with low $CD4^+$ cell counts and high HIV-2 viral loads, accompanied by opportunistic infections (Table 1). One case (NMC678) was found at an asymptomatic stage with high $CD4^+$ cell count and undetectable viremia. HIV-1 RNAs were undetectable in all 5 cases, indicating that they were infected by HIV-2 alone.

The First Circulating Recombinant Form Discovered in HIV-2: HIV-2 CRF01_AB

HIV-2 genetic groups were determined by both *gag* and *env* sequences. We were successful in analyzing 4 AIDS cases, however, we failed to amplify these 2 genes and analyze in asymptomatic case NMC678. One isolate (NMC786) was clearly classified into group A in phylogenetic tree analysis (Fig. 1A, B). On the other hand, isolates NMC307, NMC716, and NMC842 formed an independent cluster with a reference AB recombinant isolate 7312A (Fig. 1A, B). To better understand the detailed genomic structures of the 3 suspected AB recombinants, full-length genomic sequences of the 3 cases were analyzed. In the phylogenetic tree with full-length or near full-length reference sequences (Fig. 1C), NMC307, NMC716, NMC842, and 7312A formed an independent cluster with a high bootstrap value of 100%, suggesting these 4 isolates are the same type of AB-recombinant virus.

We next compared their genomic structures. As shown in Fig. 2A, similarity plotting and bootscanning analyses revealed that the recombinant breakpoints of our 3 isolates perfectly matched those of 7312A. This finding was supported by subregion phylogenetic analyses (Fig. 2B). In conclusion, NMC307, NMC716, and NMC842 are AB-recombinant forms with a mosaic genome structure identical to that of 7312A, demonstrating that they are the same type of HIV-2 AB-recombinant form.

The minimum requirement for declaring a new CRF, as proposed by the Los Alamos HIV sequence database in 1999, is at least 3 cases with no direct linkage, accompanied with near full-length sequences.[33,34] These CRF nomenclature

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