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Within-host co-evolution of Gag P453L and protease D30N/N88D demonstrates virological advantage in a highly protease inhibitor-exposed HIV-1 case

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ABSTRACT

To better understand the mechanism of HIV group-specific antigen (Gag) and protease (PR) co-evolution in drug-resistance acquisition, we analyzed a drug-resistance case by both bioinformatics and virological methods. We especially considered the quality of sequence data and analytical accuracy by introducing single-genome sequencing (SGS) and Spidermonkey/Bayesian graphical models (BGM) analysis, respectively. We analyzed 129 HIV-1 Gag–PR linkage sequences obtained from 8 time points, and the resulting sequences were applied to the Spidermonkey co-evolution analysis program, which identified ten mutation pairs as significantly co-evolving. Among these, we focused on associations between Gag-P453L, the P5′ position of the p1/p6 cleavage-site mutation, and PR-D30N/N88D nelfinavir-resistant mutations, and attempted to clarify their virological significance *in vitro* by constructing recombinant clones. The results showed that $P453L^{Gag}$ has the potential to improve replication capacity and the Gag processing efficiency of viruses with $D30N^{PR}/N88D^{PR}$ but has little effect on nelfinavir susceptibility. Homology modeling analysis suggested that hydrogen bonds between the 30th PR residue and the $R452^{Gag}$ are disturbed by the $D30N^{PR}$ mutation, but the impaired interaction is compensated by $P453L^{Gag}$ generating new hydrophobic interactions. Furthermore, database analysis indicated that the $P453L^{Gag}/D30N^{PR}/N88D^{PR}$ association was not specific only to our clinical case, but was common among AIDS patients.

1. Introduction

Major mutations in the human immunodeficiency virus-1 (HIV-1) protease (PR)-coding region selected by protease inhibitors (PIs) are mainly located within the active sites of the PR, and these mutations significantly reduce PR activity and viral replication capacity, i.e., viral fitness, compared to that of wild-type strains (Mahalingam et al., 1999). However, PI-resistant viruses have the potential to undergo further selection and evolution to recover their impaired PR activity and viral fitness by acquiring additional mutations not only in the PR but also in its natural substrate, the Gag protein (Nijhuis et al., 1999). In particular, mutations in Gag cleavage sites can improve the replication capacity of PI-resistant viruses. Indeed, tight associations have been demonstrated between PI-resistant mutations and Gag cleavage-site mutations, such as $S373Q^{Gag}$ (Malet et al., 2007) and $I376V^{Gag}$ (Ho et al., 2008) at p2/NC, $A431V^{Gag}$ (Bally et al., 2000; Doyon et al., 1996; Gallego et al., 2003; Koch et al., 2001; Maguire et al., 2002; Malet et al., 2007; Verheyen et al., 2006; Zhang et al., 1997) at NC/p1, $L449F^{Gag}$ (Doyon et al., 1996), and $P453L^{Gag}$ (Bally et al., 2000; Maguire et al., 2002; Verheyen et al., 2006) at p1/p6. Gag mutations at non-cleavage sites have also been reported to improve the fitness of PI-resistant viruses (Myint et al., 2004). Thus, the selection and evolution of Gag and PR are accepted to significantly interfere with each other. This phenomenon is known as "Gag–PR co-evolution."

However, previous reports of Gag–PR co-evolution appear to have two technical limitations related to data quality and analytical method. First, the standard population-based genotyping method commonly used for determining HIV-1 variants has limited accuracy for technological reasons. To sequence the viral genome,

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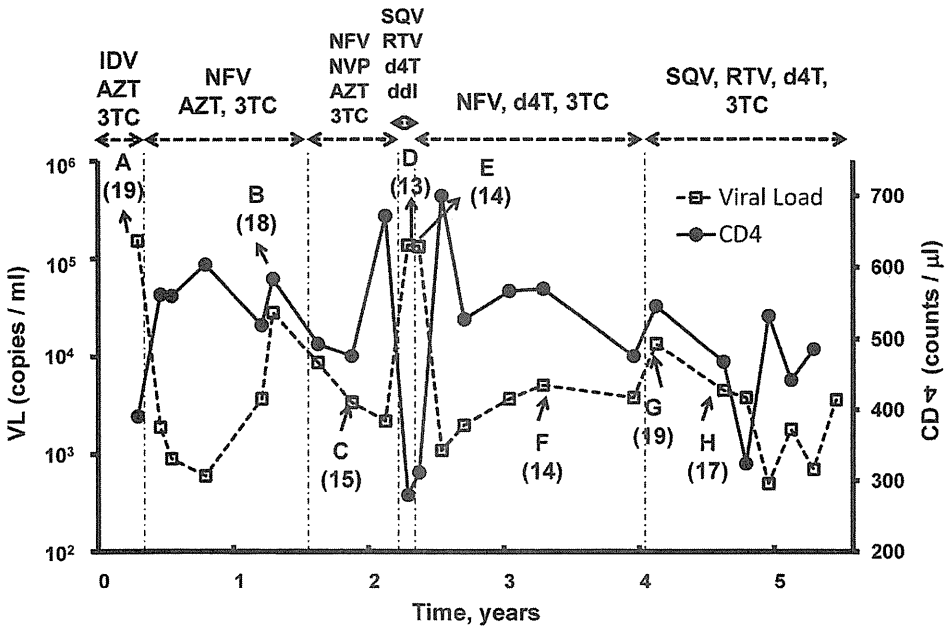

Fig. 1. Clinical course of patient-treatment protocols. Open squares and solid circles indicate plasma viral load and CD4+ cell count, respectively. Each treatment regimen is indicated in the upper part of the graph by horizontal dashed lines between arrows. Sample collection points are labelled A to H, and numbers of sequences analyzed at each point are shown in parentheses. AZT, zidovudine; d4T, stavudine; ddI, didanosine; 3TC, lamivudine; NVP, nevirapine; NFV, nelfinavir; RTV, ritonavir; SQV, saquinavir; IDV, indinavir.

viral RNA from patient plasma must be reverse-transcribed to cDNA, and the target regions are then amplified by PCR. Since viral cDNA includes multiple viral populations, using such samples as a template for the PCR step followed by sequencing results in amplification of predominantly the major variants (Gunthard et al., 1998; Hance et al., 2001), which may not represent the diversity of the original population. Furthermore, there is always a risk of artificial recombination of viral cDNAs, which are estimated to occur at rates of 4–70% during the PCR step (Meyerhans et al., 1990) and may disturb the linkage of mutation sites. Thus, this artificial linkage information may significantly affect the results of co-evolution analyses. To circumvent this technical problem, we employed the single-genome sequencing (SGS) technique based on limiting-dilution assays (Palmer et al., 2005). With SGS, artificial recombination can be avoided during PCR because cDNA samples are diluted after the reverse transcriptase reaction to a single cDNA molecule, which is used as the PCR template. Therefore, the viral sequence information obtained by SGS is not only more sensitive for analyzing HIV population diversity and mutation linkages than that obtained by standard genotype analysis, but also more precise for identifying co-evolving sites.

Second, many analytical methods have been developed for detecting co-evolving sites in molecular sequences. However, early methods did not accommodate the evolutionary history among sequences, and risked generating false-positive predictions (Altschuh et al., 1987; Gutell et al., 1992; Martin et al., 2005; Neher, 1994; Pollock and Taylor, 1997; Tillier and Lui, 2003). In recent years, the accuracy of estimating co-evolving sites has greatly improved due to several computational algorithms that incorporate the phylogenetic relationships among molecular data (Bhattacharya et al., 2007; Dutheil et al., 2005; Poon et al., 2008; Tuff and Darlu, 2000; Wollenberg and Atchley, 2000; Yeang and Haussler, 2007). In particular, Spidermonkey analysis (Poon et al., 2007a,b, 2008) not only considered the phylogenetic relationships but also implemented two empirical HIV-1 subtype B amino acid-substitution models for describing between- and within-host HIV evolution (Nickle et al., 2007).

In this study, we clarified the impact of Gag and PR co-evolution in the acquisition of drug resistance by inferring co-evolving sites between Gag and PR in a dataset of HIV-1 Gag–PR linkage sequences from a single patient who had undergone highly active antiretroviral therapy (HAART) for a long period. We used the SGS method for sequencing viral samples to avoid artificial recombination, and applied Spidermonkey analysis to our data by using its option for the within-host HIV substitution model. Virological significance of the estimated co-evolving sites was analyzed *in vitro* by constructing recombinant viruses on a pNL4-3 backbone. The replication kinetics, susceptibility to anti-HIV drugs, and the Gag processing efficiency were evaluated for each clone. We also conducted homology modeling analysis to examine Gag–PR interactions and database analysis to confirm the universality of the co-evolving sites.

2. Materials and methods

2.1. Sample collection and gag-PR-coding region sequencing by single-genome sequencing

From all HIV/AIDS patients monitored from April 1998 to August 2002 at the National Institute of Infectious Diseases in Japan, we selected a virological failure case with a history of multiple antiretroviral treatments. Of all cases, this one had been followed for the longest period and had enough detailed clinical information to perform our analysis. For the selected case, we collected plasma samples and clinical information, such as treatment regimens, changes in viral load, and CD4 counts (Fig. 1).

The single-genome sequencing (SGS) method was used as described (Palmer et al., 2005). Briefly, HIV-1 RNA was extracted from plasma samples (containing a minimum of 1000 copies of HIV-1 RNA) by guanidinium isothiocyanate treatment. cDNA was synthesized using Superscript II RT kit (Invitrogen, Carlsbad, CA) and a random hexamer. cDNA was serially diluted and amplified by PCR and nested PCR using Platinum Taq DNA Polymerase High Fidelity (Invitrogen). The endpoint of reverse-transcribed cDNA was determined as a single clone by the Poisson distribution, with cDNA dilutions yielding PCR products in 3 out of 10 reactions. The following primer sets were used in the first and nested PCR amplifications: first PCR-WGPF1 (5′-CTCTCTCGACGCAGGACTCG-

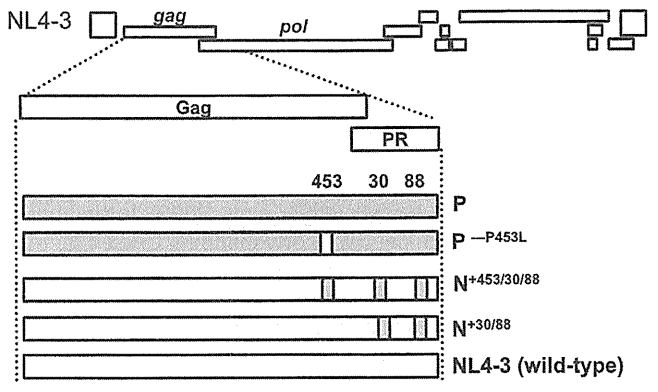

Fig. 2. Construction of recombinant viruses. Four different types of recombinant viruses were constructed according to their mutation patterns: (1) P, NL4-3 backbone with patient-derived Gag and protease (PR); (2) P^{-453}, NL4-3 backbone with patient-derived Gag and PR but $P453L^{Gag}$ was converted to wild-type; (3) $N^{+453/30/88}$, NL4-3 backbone including the substitutions $P453L^{Gag}$, $D30N^{PR}$, and $N88D^{PR}$; (4) $N^{+30/88}$, NL4-3 backbone including the substitutions $D30N^{PR}$and $N88D^{PR}$.

3′) and 3500- (5′-CTATTAAGTATTTTGATGGGTCATAA-3′), nested PCR-WGPF2 (5′-TTGCTGAAGCGCGCACGGCAAGA-3′) and 3410- (5′-CAGTTAGTGGTATTACTTCTGTTAGTGCTT-3′). These primer sets amplified a 2.7 kbps fragment containing *gag*, the PR-coding region of *pol*, and the first 900 nucleotides of the reverse transcriptase-coding region of *pol*. Positive PCR products were determined by agarose gel electrophoresis and sequenced using ABI Prism BigDye Terminator version 3.1 dideoxyterminator cycle sequencing (Applied Biosystems, Carlsbad, CA). Sequences, including the entire *gag* gene (1500 bp) and PR-coding region (297 bp), were aligned on the HXB2 reference using Sequence Navigator software (Applied Biosystems). Sequences containing mixtures at any position were excluded from analysis. PI-resistant mutations and other PR mutations in our data set were determined using the Calibrated Population Resistance tool Version 4.3 beta (http://cpr.stanford.edu/cpr/).

2.2. Detecting co-evolution using Spidermonkey analysis

Co-evolving sites between Gag and PR were inferred using Spidermonkey analysis (Poon et al., 2007a,b, 2008) to analyze all sequences (data type = protein), including the entire Gag (500 amino acids) and PR (99 amino acids) sequences. A phylogenetic tree was first reconstructed using the neighbor-joining method (Saitou and Nei, 1987). To infer co-evolving sites, we selected the within-host HIV protein-substitution model (frequencies: model-defined), based on the maximum likelihood method (Nickle et al., 2007). Amino acid sequences at MA/CA, CA/p2, p2/NC, NC/p1, and p1/p6 Gag cleavage-sites (residues P5 to P5′) and the complete PR sequence were selected for BGM analysis. We used the default options for calculations and inferred co-evolving sites if the estimated posterior probability for a pair of positions exceeded the cutoff value of 0.5.

2.3. Construction of recombinant HIV with a patient-derived gag-PR-coding region

To confirm the contributions of $P453L^{Gag}$ in the context of patient-derived *gag*-PR-coding region, three types of recombinant viruses were constructed (Fig. 2). Recombinant viruses were constructed using the pNL4-3 molecular clone of HIV-1 (GenBank accession No. AF324493). A patient-derived *gag*-PR-coding region containing $P453L^{Gag}$, $D30N^{PR}$, and $N88D^{PR}$ was chosen from sampling point F. The recombinants were (1) P, NL4-3 backbone with patient-derived *gag*-PR-coding region; (2) P^{-P453L}, NL4-3 backbone with patient-derived *gag*-PR-coding region but $P453L^{Gag}$ was converted to wild-type.

Details of the recombinant virus construction were as follows. The patient-derived 1.9 kbp *gag*-PR-coding region was subcloned into pCR4-TOPO (Invitrogen) (designated $pCR4\text{-}TOPO^{Patient}$). Using $pCR4\text{-}TOPO^{Patient}$ as a template, $P453L^{Gag}$ was substituted with wild-type P453. Each substitution was introduced using the following primer sets: For the Gag-P453 substitution, forward: 5′-TTCAGAACAGACCAGAGCCATCAGCT-3′, reverse: 5′-AGCTGATGGCTCTGGTCTGTTCTGAA-3′. Subsequently, the substituted *gag*-PR-coding region was amplified using the following primers: WGPF2: 5′-TTGCTGAAGCGCGCACGGCAAGA-3′ and DRPRO6: 5′-ACTTTTGGGCCATCCATTCCTGGCTTT-3′. The NL4-3-derived RT-coding region was amplified using the following primers: RT-63F: 5′-TAAACAATGGCCATTGACAGAAG-3′ and RT-898R: 5′-CTGCTTCTTCTGTTAGTGGTACTAC-3′. Primers DRPRO6 and RT-63F are phosphorylated at their 5′ ends. Both resulting fragments were ligated and digested with BssHII and SbfI, and cloned back into pNL4-3. All pNL4-3-based recombinant DNAs (3.75 μg) were transfected into 2×10^5 HeLa cells using Fugene6 (Roche, Indianapolis, IN), and culture supernatants were harvested at 72 h after transfection, filtered through a 0.45 μm membrane, assayed for reverse transcriptase (RT) activity (Willey et al., 1988), and kept as virus stocks at −80 °C until use. Each virus stock (5×10^6 ^{32}P cpm of RT activity) was used for replication kinetics analyses.

2.4. Construction of pNL43 with $P453L^{Gag}$, $D30N^{PR}$, and $N88D^{PR}$ by site-directed mutagenesis

In addition to constructing patient-derived *gag*-PR-coding region recombinant viruses, we evaluated the effect of $P453L^{Gag}$, $D30N^{PR}$, and $N88D^{PR}$ interference by constructing three types of pNL4-3-based recombinant viruses (Fig. 2). These were (1) $N^{+453/30/88}$, NL4-3 including $P453L^{Gag}/D30N^{PR}/N88D^{PR}$; and (2) $N^{+30/88}$, NL4-3 including $D30N^{PR}/N88D^{PR}$. These recombinant viruses were constructed as follows. pCR4-TOPO (Invitrogen) including the ApaI-SbfI fragment of pNL4-3, which contained the complete p1/p6 of *gag* and PR-coding region, was constructed as a template for further mutagenesis. This construct was designated as $pCR4\text{-}TOPO/NL^{ApaI\text{-}SbfI}$. Using $pCR4\text{-}TOPO/NL^{ApaI\text{-}SbfI}$ as a template, we introduced three mutations ($P453L^{Gag}$, $D30N^{PR}$, and $N88D^{PR}$) by site-directed mutagenesis. The following primer sets were used to introduce each mutation: For $P453L^{Gag}$, forward: 5′-TTCAGAGCAGACTAGAGCCAACAGCC-3′ and reverse: 5′-GGCTGTTGGCTCTAGTCTGCTCTGAA-3′. For $D30N^{PR}$, forward: 5′-CAGGAGCAGATAATACAGTATTAGAAG-3′ and reverse: 5′-CTTCTAATACTGTATTATCTGCTCCTG-3′. For $N88D^{PR}$, forward: 5′-CATAATTGGAAGAGATCTGTTGACTC-3′ and reverse: 5′-GAGTCAACAGATCTCTTCCAATTATG-3′. After each mutagenesis reaction, the entire sequences were verified and cloned back into pNL4-3 using ApaI and SbfI restriction enzymes. All pNL4-3-based recombinant DNAs (3.75 μg) were transfected into 2×10^5 HeLa cells using Fugene6 (Roche), and culture supernatants were harvested at 72 h after transfection, filtered through a 0.45 μm membrane, assayed for RT activity (Willey et al., 1988), and kept as virus stocks at −80 °C until use. Each virus stock (4×10^5 ^{32}P cpm of RT activity) was used for replication kinetics analysis.

2.5. Replication kinetics of recombinant viruses

Replication kinetics of recombinant viruses was evaluated as described (Matsuoka-Aizawa et al., 2003). Briefly, 5×10^4 MT-2 cells were infected with each virus stock in the absence or presence of 0.1 μM nelfinavir at 37 °C for 16 h. Cells were then washed once and resuspended in 0.5 ml culture medium with the same concen-

tration of nelfinavir, and cultures were maintained for 12–22 days, changing half of the medium every 2 or 3 days. The titer of each virus was evaluated by both RT activity and p24 amount; as both measures demonstrated good correlation, p24 amount was used to adjust the virus inoculum. Culture supernatants were collected, and residual supernatants were kept at −80 °C until use. Replication kinetics was independently analyzed two times.

2.6. Evaluation of nelfinavir susceptibilities of recombinant viruses

Recombinant viruses were evaluated for nelfinavir susceptibility using an in-house drug susceptibility assay with the MaRBLE cell line (MaRBLE assay) as described (Chiba-Mizutani et al., 2007). Briefly, 1×10^5 MaRBLE cells were infected with 100 $CCID_{50}$ of each recombinant virus, and virus replication was monitored in serial dilutions of nelfinavir from 1.28×10^{-13} to 1.0×10^{-6} M for 7 days in triplicate. At day 7, cells were harvested and lysed in luciferase assay reagent. Firefly (FF) and renilla luciferase (RF) activities produced by the cells were quantified using a Dual-luciferase Reporter Assay System (Promega, Madison, WI). The relative virus replication rate (% replication) at each drug concentration was calculated by the following formula: % replication = (observed FF luciferase activity with drug − background [mock] FF luciferase activity)/(observed FF luciferase activity without drug − background [mock] FF luciferase activity) × 100. IC_{50} values were calculated with 95% confidence intervals using GraphPad Prism software and nonlinear regression analysis fitted with a sigmoidal dose–response curve with variable slope.

2.7. Analysis of Gag processing patterns in recombinant viruses

The Gag processing patterns of recombinant viruses were analyzed as described (Sugiura et al., 2002) with minor modifications. In brief, NL4-3-based recombinant DNAs (18 μg) was transfected into 5×10^6 HeLa cells using Fugene6 (Roche). The culture supernatants were harvested at 48 h after transfection with a culture medium change at 12 h post transfection to the absence or presence of 0.1 μM nelfinavir. For pelleting virus through a sucrose cushion, 25 ml of cell culture medium was layered onto 10 ml of 20% sucrose (wt/vol, in PBS) before centrifugation at 30,000 rpm in a swing rotor for 1.5 h. The medium and cushion were discarded, and the virus pellet was dissolved in 200 μl Laemmli sample buffer (Bio-Rad, Hercules, CA). Viral supernatants were normalized using PETRO-TEK HIV-1 p24 Antigen ELISA (ZeptoMetrix Corporation, Buffalo, NY) and subjected to sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS–PAGE). Proteins in the gels were passively transferred to PVDF membranes (Bio-Rad). The membranes were incubated with a chicken anti-p6 polyclonal antibody (Sigma–Aldrich Corporation, St. Louis, MO) and a murine anti-p24 monoclonal antibody (ZeptoMetrix Corporation) for 2 h, followed by incubation with secondary antibody, a chicken HRP-conjugated, IgG antibody (Bethyl Laboratories Inc., Montgomery, TX), a mouse HRP-conjugated, IgG antibody (Thermo Fisher Scientific, Yokohama, JP), respectively. Finally, proteins were visualized using SuperSignal West Dura Extended Duration Substrate (Thermo Fisher Scientific).

2.8. Molecular modeling of Gag p1/p6 peptide–PR complexes

We constructed three-dimensional models of PR in complex with peptide representing Gag-p1/p6 substrate by a homology modeling method (Baker and Sali, 2001; Marti-Renom et al., 2000; Shirakawa et al., 2008) using Molecular Operating Environment (MOE) ver. 2008.10 (http://www.chemcomp.com/, Chemical Computing Group Inc., Montreal, Quebec, Canada). For the modeling template, we used an X-ray crystal structure of inactive D25N PR in complex with the p1/p6 substrate at 2 Å resolution (PDB code: 1KJF) (Prabu-Jeyabalan et al., 2002), as it had the highest similarity (94.2% identity) to the HIV-1 NL4-3 strain among HIV-1 protease-p1/p6 peptide structures in the protein data bank, even though this is an inactive model. Furthermore, the active-site $D25N^{PR}$ mutation has been reported to hardly influence the structure of the protease in complex with ligands (Sayer et al., 2008). In the models, nine-amino-acid-length peptides corresponding to the Gag p1/p6 of the NL4-3, $N^{+30/88}$, and $N^{+453/30/88}$ strains were bound to the catalytic sites of PRs of the same strains. We considered effects of a water molecule (HOH11) that mediates important hydrogen bonds between the Gag p1/p6 peptide and the PR. AMBER ff99 force field (Wang et al., 2000) and generalized Born/volume integral (GB/VI) implicit solvent model (Labute, 2008) were applied for intra- and inter-molecular energy calculations.

2.9. Database analysis

To confirm the universality of $P453L^{Gag}/D30N^{PR}/N88D^{PR}$, we obtained 3249 sequences of HIV-1 subtype B *gag*-PR-coding region (positions 2146–2516) from the Los Alamos National Laboratory HIV sequence database (http://www.hiv.lanl.gov/). For the dataset, we applied Fisher's exact test and investigated associations between the D30N/N88D mutations in PR and the P453L mutation in Gag and between the 30/88 mutations in PR and the P453L mutation in Gag, respectively. Fisher's exact test was implemented using the R 2.6.1 statistical package.

3. Results

3.1. The majority of patient-derived Gag cleavage-site mutations are in p2/NC and p1/p6

Twenty-three plasma samples were serially collected from a patient who had received HAART for 52 months. The patient's clinical history, changes in viral load, and CD4 counts are depicted in Fig. 1. At 8 sampling points (A–H), 129 *gag*-PR-coding region sequences were obtained, with p2/NC and p1/p6 Gag cleavage-site mutations observed at each point in more than 60% of clones (Table 1). PI-resistant mutations and other PR mutations are also summarized in Table 1. None of the clones had CA/p2 cleavage-site mutations, and only a few clones had MA/CA or NC/p1 cleavage-site mutations. The $Y132F^{Gag}$ mutation in MA/CA was found in 1.6% of the clones, and $E428D^{Gag}$ and $R429K^{Gag}$ within NC/p1 had prevalences of 1.6% and 5.4%, respectively. On the other hand, cleavage-site mutations were frequently observed within p2/NC and p1/p6. In the p2/NC site, we observed 11 mutations: $S373Q^{Gag}$ (83.7%), $S373P^{Gag}$ (15.5%), $A374G^{Gag}$ (79.8%), $A374V^{Gag}$ (16.3%), $A374S^{Gag}$ (2.3%), $A374R^{Gag}$ (0.8%), $T375N^{Gag}$ (100%), $I376V^{Gag}$ (3.9%), $M378L^{Gag}$ (0.8%), $G381S^{Gag}$ (0.8%), and $N382Y^{Gag}$ (0.8%). In the p1/p6 site, we observed three mutations: $L449F^{Gag}$ (0.8%), $S451N^{Gag}$ (100%), and $P453L^{Gag}$ (65.1%).

3.2. Ten Gag–protease co-evolving sites are inferred by Spidermonkey analysis

Among the 129 *gag*-PR-coding region sequences analyzed by Spidermonkey analysis (Poon et al., 2007a,b, 2008), ten co-evolving sites were inferred. These sites were identified using the default cutoff posterior probability (pp) value of 0.5. The ten co-evolving pairs identified with pp > 0.5 are shown in Table 2. Four pairs, $R429K^{Gag}/M36V^{PR}$ (pp = 0.87), $S373Q^{Gag}/T12A^{PR}$ (pp = 0.83), $P453L^{Gag}/D30N^{PR}$ (pp = 0.63), and $P453L^{Gag}/N88D^{PR}$ (pp = 0.61), represented Gag/PR inter-molecular co-evolution;

Table 1
Gag cleavage-site mutations and PR mutations from a HAART-treated case.

Sampling point	Gag cleavage-site mutation (%)[a]		PR mutation (%)[a]	
	p2/NC	p1/p6	PI-resistant mutations	Other PR mutations
A to H (n = 129)	T375N	S451N	–	I62V, L63P,A71T, I93L
A (n = 19)	S373Q(100), A374G (89),	–	–	M36I(95), I72V(11), V77I(100)
B (n = 18)	S373Q(100), A374G (94)	P453L(100)	D30N(100), M46I(6), N88D(100)	E35D(94), M36I(100), V77I(100),
C (n = 15)	S373Q(100), A374G(100)	P453L(100)	D30N(100), N88D(100)	L10F(80), E35D(100), M36I(100), K45R(27), I72T(7), V77I (100),
D (n = 13)	S373P (85), A374V (85)	P453L (8)	D30N (8), N88D (8)	L10F(8), V11I (8), T12A(8), K20R(8), E35D(8), M36I(100), H69Y(8), V77I(8)
E (n = 14)	S373P (64), A374V(71)	–	–	T12A(7), M36I(100), K55N (7), V77I(7)
F (n = 14)	S373Q(100), A374G(100)	P453L(100)	D30N(100), N88D(100)	L10F(100), I13V(79), E34G(7), E35D(100), M36I (100), N37T (86), K45R (14), Q58E (86), I72T (7), V77I (100)
G (n = 19)	S373Q(100), A374G(100)	P453L(100)	D30N(100), N88D(100)	L10F(100), I13V (68), E35D(100), M36I(68), M36V(32), N37T(100), Q58E(100), V77I(100)
H (n = 17)	S373Q(100), A374G(100)	P453L(100)	D30N(100), I54V(76), N88D(100), L90M (47)	L10F(100), I13V (94), K20R (76), E35D (100), M36I (94), M36V (6), N37T (100), Q58E (100), I72T (53), V77I (100), G78R (6)

Gag mutations refer to HXB2 and PI-resistant mutations and other PR mutations were determined using the Calibrated Population Resistance tool Version 4.3 beta.
[a] Numbers in parentheses are the percentages of mutations at each sampling point.

one pair, $S373Q^{Gag}/A374G^{Gag}$, represented Gag/Gag intra-molecular co-evolution; and the other five pairs, $N37T^{PR}/Q58E^{PR}$ (pp = 0.94), $E35D^{PR}/M46I^{PR}$ (pp = 0.89), $K20R^{PR}/I54V^{PR}$ (pp = 0.88), $V11I^{PR}/K20R^{PR}$ (pp = 0.86), and $D30N^{PR}/N88D^{PR}$ (pp = 0.62), represented PR/PR intra-molecular co-evolution.

We focused on three pairs, $P453L^{Gag}/N88D^{PR}$, $P453L^{Gag}/D30N^{PR}$, and $D30N^{PR}/N88D^{PR}$, because $D30N^{PR}$ and $N88D^{PR}$ are well-known major and minor nelfinavir-resistant mutations, respectively (Johnson et al., 2008), and $P453L^{Gag}$ is the P5′ position of the p1/p6 cleavage-site mutation. Although $D30N^{PR}$ has been associated with $N88D^{PR}$ (Rhee et al., 2007; Wu et al., 2003), the interactions among $P453L^{Gag}$, $D30N^{PR}$, and $N88D^{PR}$ have not been investigated. Since $P453L^{Gag}/D30N^{PR}/N88D^{PR}$ was frequently observed in the presence of nelfinavir (Fig. 1 and Table 1), we conducted *in vitro* experiments to confirm whether the co-existence of $P453L^{Gag}/D30N^{PR}/N88D^{PR}$ has a virological advantage in the presence of nelfinavir.

3.3. $P453L^{Gag}$ improves the replication capacity of viruses with $D30N^{PR}/N88D^{PR}$ in both patient- and NL4-3-derived genetic backgrounds

To evaluate the virological impact of $P453L^{Gag}$ in the patient-derived genetic background, we constructed two types of patient-derived *gag*-PR-coding region viruses, P and P^{-P453L} (Fig. 2). These two recombinant viruses and the wild-type virus (NL4-3) were cultured independently in the absence or presence of nelfinavir, and their replication kinetics was monitored by measuring RT activity in culture supernatants. Assays for replication kinetics were independently performed twice, confirming identical orders of replication kinetics.

Table 2
Positions of coevolving pairs inferred by Spidermonkey analysis.

Position	Position	Expected posterior probability	Total number of sequences
429^{Gag}	36^{PR}	0.866	7
373^{Gag}	12^{PR}	0.825	2
453^{Gag}	30^{PR}	0.632	84
453^{Gag}	88^{PR}	0.607	84
373^{Gag}	374^{Gag}	0.895	22
37^{PR}	58^{PR}	0.938	48
35^{PR}	46^{PR}	0.887	1
20^{PR}	54^{PR}	0.877	13
11^{PR}	20^{PR}	0.858	1
30^{PR}	88^{PR}	0.618	84

Significant coevolving sites (pp value > 0.5) are shown.

In the absence of nelfinavir (Fig. 3A), the RT activities of the NL4-3 and P viruses peaked at 6 days after infection, and the RT activity of NL4-3 was higher than that of the P virus, whereas viral replication was delayed in the P^{-P453L} virus (i.e., P virus without $P453L^{Gag}$), and its RT activity peaked at 10 days after infection. The order of replication kinetics in the absence of nelfinavir was wild-type (NL4-3) > P > P^{-P453L}. On the other hand, in the presence of nelfinavir (Fig. 3B), replication of the wild-type virus was completely suppressed, and replication of P virus was the most active, demonstrating peak RT activity at 6 days after infection. The P^{-P453L} virus showed lower replication capacity than the P virus. Thus, the order of replication kinetics in the presence of nelfinavir was P > P^{-P453L} > wild-type (NL4-3).

To assess whether the complex $P453L^{Gag}/D30N^{PR}/N88D^{PR}$ conferred an advantage not only in the patient-derived genetic background but also in the HIV-1 molecular clone (NL4-3)-derived genetic background, we constructed two types of NL4-3-based *gag*-PR-coding region recombinant viruses, $N^{+453/30/88}$ and $N^{+30/88}$ (Fig. 2). The results of independent culture studies are shown in Fig. 3C and D. In the absence of nelfinavir, the RT activities of NL4-3, NL4-3 with $P453L^{Gag}/D30N^{PR}/N88D^{PR}$ ($N^{+453/30/88}$) and NL4-3 with $D30N^{PR}/N88D^{PR}$ ($N^{+30/88}$) viruses peaked at 11 days, 14 days, and 16 days after infection, respectively, and the order of replication kinetics was NL4-3 > $N^{+453/30/88}$ > $N^{+30/88}$. On the other hand, in the presence of nelfinavir, the $N^{+453/30/88}$ virus grew the most actively with its peak RT activity at 14 days after infection. The $N^{+30/88}$ virus was the second most actively replicating, and the wild-type did not replicate. Thus, the order of replication kinetics in the presence of nelfinavir was $N^{+453/30/88}$ > $N^{+30/88}$ > NL4-3.

In both studies, not only in the patient-derived genetic background but also in the NL4-3-derived genetic background, the virus with $D30N^{PR}/N88D^{PR}$ showed lower replication capacity than the virus with $P453L^{Gag}/D30N^{PR}/N88D^{PR}$, suggesting that $P453L^{Gag}$ significantly contributes to the fitness recovery of virus with $D30N^{PR}/N88D^{PR}$.

3.4. $P453L^{Gag}$ does not influence susceptibility to nelfinavir

To clarify whether $P453L^{Gag}$ affects nelfinavir susceptibility, the IC_{50}s of nelfinavir for NL4-3-based *gag*-PR-coding region recombinants were determined using MaRBLE cells. Both $N^{+453/30/88}$ and $N^{+30/88}$ recombinants showed 14.4 and 15.8-fold greater resistance to nelfinavir than wild-type virus, respectively (Table 3). However, the difference in IC_{50} between the two recombinants was

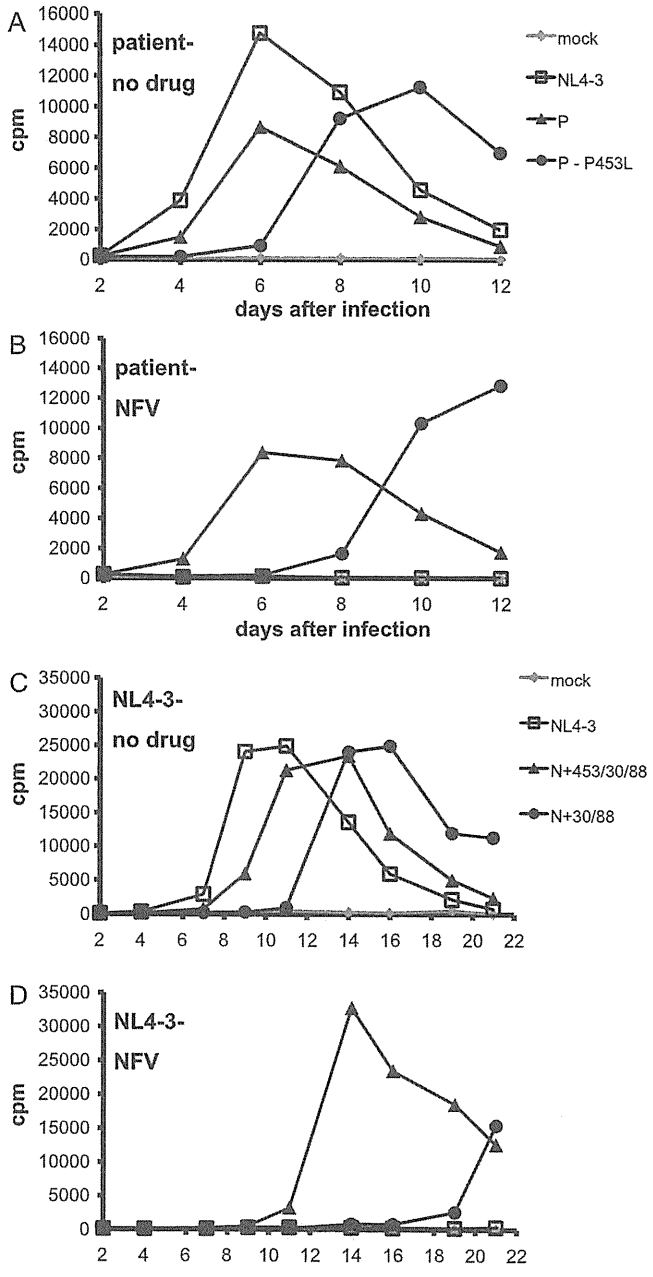

Fig. 3. Replication kinetics of recombinants. MT-2 cells were infected with patient-derived *gag*-PR-coding region, (A) in the absence of, and (B) in the presence of 0.1 μM nelfinavir. Open squares, solid triangles, and solid circles indicate wild-type NL4-3, NL4-3 with patient *gag*-PR-coding region insert, and NL4-3 with patient insert without P453L substitution, respectively. MT-2 cells were also infected with NL4-3-based recombinant, (C) in the absence of, and (D) in the presence of 0.1 μM nelfinavir. Open squares, solid triangles, and solid circles indicate wild type NL4-3; NL4-3 with P453L, D30N, and N88D; and NL4-3 with D30N and N88D, respectively. Diamonds indicate mock-infected controls. Assays were independently performed twice, and one representative set of results is shown.

not significant, suggesting that P453L^{Gag} had no effect on nelfinavir susceptibility.

3.5. P453L^{Gag} improves Gag p1/p6 processing in virus with D30N^{PR}/N88D^{PR}

To gain further insights into the virological effects of P453L^{Gag}, we examined Gag processing patterns in the absence and presence of 0.1 μM nelfinavir by Western blot analysis with an anti-p6 polyclonal antibody and an anti-p24 monoclonal antibody. The amount of sample loaded in each lane was normalized by p24 antigen content (600 ng for each lane as determined by ELISA) (Fig. 4A). In the absence of nelfinavir, the partially cleaved Gag intermediate p15 (including NC, p1, and p6) of NL4-3 was efficiently cleaved to the p6 peptide (Fig. 4B, lane 3). In contrast, the processing of NL4-3 was less efficient in the presence of nelfinavir, as indicated by the accumulation of p15 (Fig. 4B, lane 4). N$^{+30/88}$ showed defects in cleavage at the p1/p6 site, as demonstrated by the accumulation of p15 and p7 (p1/p6) (Fig. 4B, lanes 5 and 6). On the other hand, lesser p15 and p7 accumulated in N$^{+453/30/88}$ than in N$^{+30/88}$ (Fig. 4, lanes 7 and 8). Interestingly, a 8–9 kDa band, which is neither p6 nor p7, was observed in N$^{+30/88}$ (Fig. 4B, lanes 5 and 6).

Table 3
Nelfinavir susceptibilities of recombinant viruses.

HIV-1	IC_{50} (nM)	95% confidence interval	Fold-resistance
NL4-3	1.3	0.8–2.4	1.0
NL$^{+453/30/88}$	18.7	9.2–37.8	14.4
N$^{+30/88}$	20.6	12.6–33.6	15.8

3.6. Impaired Gag–PR affinity in the N$^{+30/88}$ strain is recovered by new L453^{Gag} interactions with M46′^{PR} and F53′^{PR}

To elucidate the structural impact of the mutations described above on interactions between Gag-p1/p6 substrate and PR, we generated three-dimensional models of the PR in complex with peptide representing Gag-p1/p6 substrate by homology modeling (Baker and Sali, 2001; Marti-Renom et al., 2000; Shirakawa et al., 2008) using Gag and PR sequences of the NL4-3, N$^{+30/88}$, and N$^{+453/30/88}$ strains. Comparison of the thermodynamically optimized models showed obvious differences in interactions between side chains of PR and p1/p6 (Fig. 5). First, D30N^{PR} mutation resulted in fewer hydrophilic interactions between side chains of the 30th PR and 452nd p1/p6 residues; NL4-3 had two hydrogen bonds between the side chains of D30^{PR} and R452^{Gag} (Fig. 5A), while N$^{+30/88}$ and N$^{+453/30/88}$ had only a single hydrogen bond between the side chains of N30^{PR} and R452^{Gag} (Fig. 5B and C). Second, the P453L^{Gag} mutation in the p1/p6 substrate of the N$^{+453/30/88}$ strain led to new hydrophobic interactions between side chains of the 46th PR and 453rd p1/p6 residues, as well as of the PR 53F and p1/p6 P453L^{Gag} residues (Fig. 5C).

To examine whether these changes in interactions influenced the binding affinity of the p1/p6 substrate to PR, we analyzed

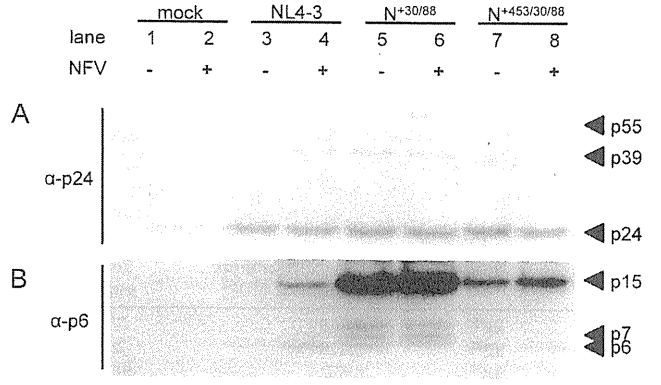

Fig. 4. Western blot analysis of Gag processing in the absence or presence of nelfinavir. Western blot analyses in the absence or presence of nelfinavir. HeLa cells were transfected by each recombinant clones and cultured in the absence or presence of NFV (0.1 μM). At 48 h post-transfection, virions in culture supernatants were harvested and subjected to Western blot analysis with anti-p24 monoclonal antibody (A) and an anti-p6 polyclonal antibody (B). Each lane was normalized by p24 antigen content (5 ng for each lane as determined by ELISA). Lanes 1 and 2, mock, lanes 3 and 4, NL4-3, lanes 5 and 6, N$^{+453/30/88}$, lanes 7 and 8, N$^{+30/88}$.

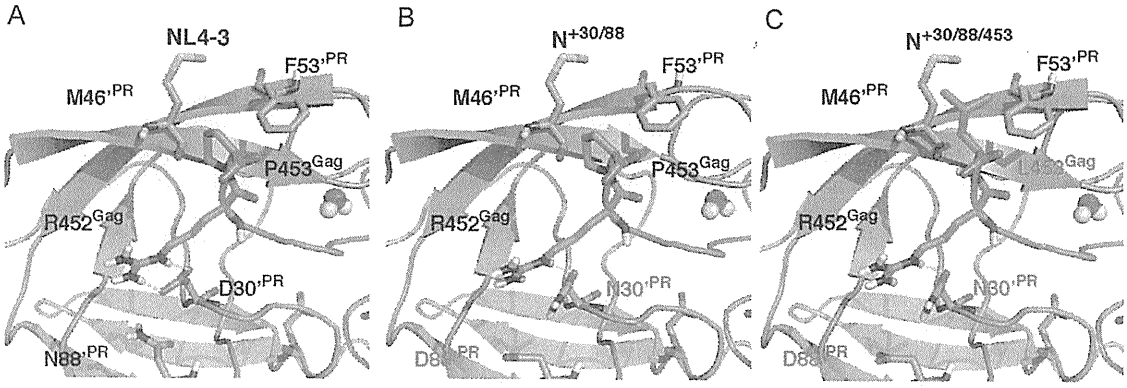

Fig. 5. Structural models of the Gag–PR complexes. Catalytic sites of the NL4-3 (A), $N^{+30/88}$ models (B), and $N^{+453/30/88}$ models (C) are highlighted. Green cartoons and sticks represent main and side chains of PR, respectively. Cyan sticks represent 452nd and 453rd residues in Gag corresponding to the p1/p6 region. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

their binding energies using the PR–p1/p6 peptide complex models. The predicted binding energies of the NL4-3, $N^{+30/88}$ and $N^{+453/30/88}$ models were −137.6 kcal/mol, −133.3 kcal/mol, and −137.1 kcal/mol, respectively, suggesting that p1/p6 substrate has lower affinity with $N^{+30/88}$ PR than with NL4-3 and $N^{+453/30/88}$ PRs. Taken together, these data suggest that PR mutations in the $N^{+30/88}$ strain can reduce Gag–PR affinity primarily via loss of the hydrogen bond between $N30^{PR}$ and $R452^{Gag}$ and that the Gag p1/p6 mutation in the $N^{+453/30/88}$ strain ($P453L^{Gag}$) can recover affinity by generating new hydrophobic interactions of $L453^{Gag}$ with $M46^{PR}$ and $F53^{PR}$.

3.7. $P453L^{Gag}/D30N^{PR}/N88D^{PR}$ association is commonly observed in a large database

To confirm the prevalence of the $P453L^{Gag}/D30N^{PR}/N88D^{PR}$ association in other HIV-infected individuals, we investigated 3249 sequences of HIV-1 subtype B *gag*-PR-coding region from the Los Alamos National Laboratory HIV sequence database (http://www.hiv.lanl.gov/). We found that the $P453L^{Gag}$ mutation was significantly associated with $D30N^{PR}/N88D^{PR}$ (Table 4; $p < 0.001$, Fisher's exact test). These data support the virological advantage of the $P453L^{Gag}/D30N^{PR}/N88D^{PR}$ association.

4. Discussion

In this study, we analyzed mechanisms of anti-HIV drug-resistant mutation acquisition by investigating crosstalk between Gag and PR mutations. We traced the clinical course and sequence changes in *gag* and the PR-coding region of a virological-failure case heavily treated with multiple regimens, including different protease inhibitors. To focus on the quality of sequence data and accuracy of analysis, we used SGS and Spidermonkey analysis, respectively.

Among the ten co-evolving Gag–Protease pairs inferred by Spidermonkey analysis, we confirmed a linkage between $P453L^{Gag}$ and $D30N^{PR}/N88D^{PR}$. $D30N^{PR}$ has been reported to associate with $N88D^{PR}$ (Rhee et al., 2007; Wu et al., 2003), P453L (Verheyen et al., 2006), and positively correlate with p1/p6 cleavage-site mutations (Kolli et al., 2006). We were interested in the association between $P453L^{Gag}$ and $D30N^{PR}/N88D^{PR}$ nelfinavir-resistant mutations, as $P453L^{Gag}$ is located at the P5′ position of the p1/p6 cleavage site, and expected to physically interact with the protease. Thus, we sought to clarify the biological advantage of interference among $P453L^{Gag}$, $D30N^{PR}$, and $N88D^{PR}$ in recombinant viruses with patient- and NL4-3-derived genetic backgrounds. Virological advantage was evaluated in three aspects: (1) virological superiority in replication competency, (2) resistance to antiretroviral selective pressure, and (3) Gag processing pattern in virions. Our results indicated that the $P453L^{Gag}$ cleavage-site mutation has the potential to improve the replication capacity and Gag processing of viruses with $D30N^{PR}/N88D^{PR}$, but has little effect on nelfinavir susceptibility. This latter finding is of interest since Gag cleavage-site mutations have been suggested as a mechanism for protease to develop drug resistance (Dam et al., 2009; Kolli et al., 2009). We also need to consider not only antiretroviral selective pressure but also immune selective pressure. Several of the positions we noted have been described as associated with human leukocyte antigen (HLA) escape mutations. For example, PR codons 12, 35, and 36, and Gag codons 373 and 374 are all potentially HLA-related (Brumme et al., 2009). To confirm the contributions of HLA and immune pressure, further study is required.

Although samples were collected chronologically at multiple times, the order of $P453L^{Gag}$, $D30N^{PR}$ and $N88D^{PR}$ acquisitions was unclear as all three mutations were detected at the same time. However, a plausible order seems to be the selection of $D30N^{PR}/N88D^{PR}$ followed by $P453L^{Gag}$ acquisition. Nelfinavir appears to select $D30N^{PR}/N88D^{PR}$ mutations for resistance because these mutations obviously increase drug resistance to nelfinavir (Johnson et al., 2009), whereas the $P453L^{Gag}$ mutation without any PR mutations has been reported to have almost no effect on susceptibility to PIs and on viral replication capacity (Maguire et al., 2002). Although $D30N^{PR}$ is known as one of the most unstable PI-resistant mutations (Martinez-Picado et al., 1999) and viruses with this mutation have lower PR activity than the wild-type, the impaired replication caused by $D30N^{PR}$ has been reported to be compensated by $N88D^{PR}$ (Mitsuya et al., 2006; Sugiura et al., 2002). Furthermore, the fitness of virus with $D30N^{PR}/N88D^{PR}$ was recovered in our study by an additional $P453L^{Gag}$ mutation (Fig. 3). However, the $P453L^{Gag}$ mutation was not introduced into NL4-3 carrying $D30N^{PR}/N88D^{PR}$ ($N^{+30/88}$) and patient $D30N^{PR}/N88D^{PR}$ clones (P^{-P453L}) during *in vitro* culture with nelfinavir, suggesting that the $P453L^{Gag}$ mutation is a sufficient condition for D30N/N88D clones to replicate efficiently.

Virus with $D30N^{PR}/N88D^{PR}$ was suggested by results of our Western blot analyses to process p1/p6 cleavage inefficiently, as

Table 4
Los Alamos National Laboratory HIV sequence database analysis.

453^{Gag}	$30^{PR}/88^{PR}$	
	D30/N88	N30N/D88D
P ($n = 2801$)	$n = 2743$	$n = 8$
L ($n = 237$)	$n = 223$	$n = 8$
p-value		<0.001

demonstrated by the accumulation of p15 and p7 non-cleaved precursors, but addition of P453L^{Gag} improved the processing (Fig. 4B). Accumulation of p15 and p7 products has been reported in previous studies using different PR mutant viruses (Doyon et al., 1996; Maguire et al., 2002). In these studies, the additional mutations at NC/p1 or p1/p6 cleavage sites also resulted in efficient processing of these precursors. Interestingly, an aberrant band, which did not match either p6 or p7, was observed in N$^{+30/88}$ in our study (Fig. 4, lanes 5 and 6). Although further analyses will be required to determine the exact mechanisms, the band suggests inaccurate or alternative recognition of the cleavage site by virus with D30N^{PR}/N88D^{PR}, and P453L^{Gag} may confer an advantage by adjusting the protease to recognize and cleave the right site. As the HIV-1 p6 protein is important for efficient particle budding (von Schwedler et al., 2003), the defect in p1/p6 cleavage may affect viral maturation, which in turn may reduce viral infectivity and replication capacity.

To understand the relevance of P453L^{Gag} from a structural viewpoint, we used homology modeling with the published X-ray crystal structure of the PR–p1/p6 substrate complex as a template (Fig. 5). Although P453L^{Gag} is located at the P5′ position and does not directly interfere with the protease active site or subsites, the modeling demonstrated that P453L^{Gag} can compensate for the binding affinity of PR and p1/p6. This mechanism is interesting because it suggests that a mutation outside the cleavage site interferes with the PR–Gag interaction. Indeed, Prabu-Jeyabalan et al. (2004) documented that a Gag mutation (A431V^{Gag}) compensates for a PR mutation (V82A^{PR}), which is not in direct contact with A431V^{Gag}. Thus, our data confirm the virological and structural advantages of P453L^{Gag} in viruses possessing D30N^{PR}/N88D^{PR}. Furthermore, this association appeared to be quite common as the frequency of P453L^{Gag} is 7.3% in the Los Alamos National Laboratory HIV sequence database. Though nelfinavir is no longer recommended as a first-line antiretroviral in the guidelines of developed countries, many cases previously exposed to nelfinavir have acquired D30N/N88D mutations. Indeed, the prevalence of the D30N mutation in PI-treated persons infected with subtype B viruses (n = 7396) and in nelfinavir-treated persons (n = 1128) is 7.9% and 28.1%, respectively, in the Stanford HIV drug resistance database (http://hivdb.stanford.edu/).

Regarding the bioinformatics analysis strategy, we selected the within-host substitution model in Spidermonkey analysis to infer the co-evolving sites (Nickle et al., 2007) as the data were sequences serially collected over 5 years from a single patient under anti-HIV treatment. One disadvantage of this program is that it does not account for the number of descendant clones. Often, mutation pairs on few viral clones might be determined as co-evolving pairs. In our study, 46^{PR} and 35^{PR} mutations were determined as a co-evolving pair with high posterior probability, but only one clone with this pair was observed among 129 sequences (Table 2), suggesting this co-mutation pair could not become "fixed" in a viral population. Thus, it is important to confirm the significance of the program output.

5. Conclusions

In conclusion, we successfully determined the Gag-protease associated sites P453L^{Gag}/D30N^{PR}/N88D^{PR} by applying single-genome sequencing, suggesting the usefulness of this method. However, as SGS is a more expensive method than direct sequencing, researchers need to consider which method has the best advantage for their samples. Extrapolating from our data, the relationships between the major mutations found by SGS may not differ significantly from direct sequencing results, but we might have a greater chance of seeing a variety of minority clones with minor mutations. In addition, our observation of higher variation at later sampling points suggests that cases with longer treatment histories are good sample candidates.

We found that the viruses acquiring P453L^{Gag}/D30N^{PR}/N88D^{PR} distinctly showed biological advantages. From results obtained using both viral experiments and bioinformatics, we speculate that the P453L^{Gag} mutation does not necessarily occur in the presence of nelfinavir, but if it occurs with D30N^{PR}/N88D^{PR} mutations, viral fitness can be improved, which may eventually lead to worse clinical outcomes of anti-HIV therapy. We believe that the findings of this study provide new insight into the mechanism of within-patient HIV-1 co-evolution and into the acquisition of resistance to anti-HIV drugs.

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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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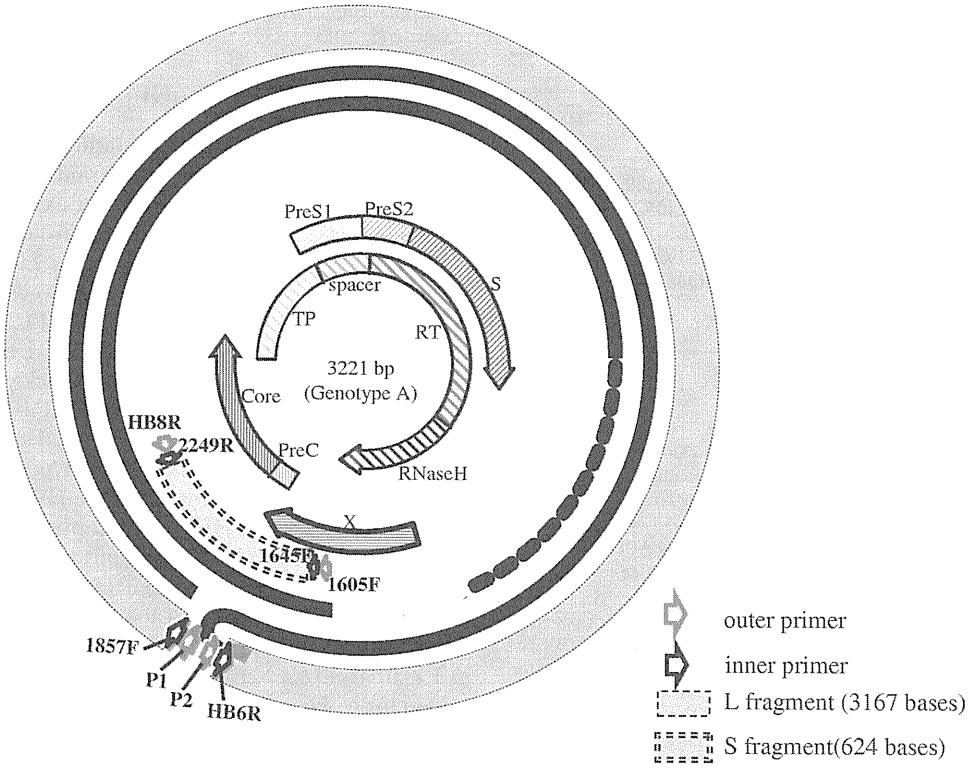

FIG. 1. Genetic regions of HBV and HIV-1 used for phylogenetic tree analyses. The whole HBV genome was amplified in two fragments, L and S, and assembled. L and S fragments are indicated by single and double dashed lines, respectively.

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

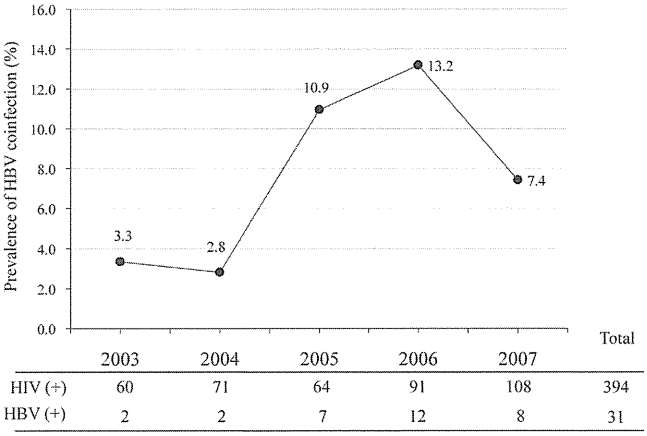

FIG. 2. Transitions in HBV infection rates in HBV/HIV-1-coinfected patients. HBV infection rates are plotted versus year, with the numbers of HIV-1-infected and HBV/HIV-1-coinfected patients shown below the *x* axis.

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)	2.0×10^8 (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.

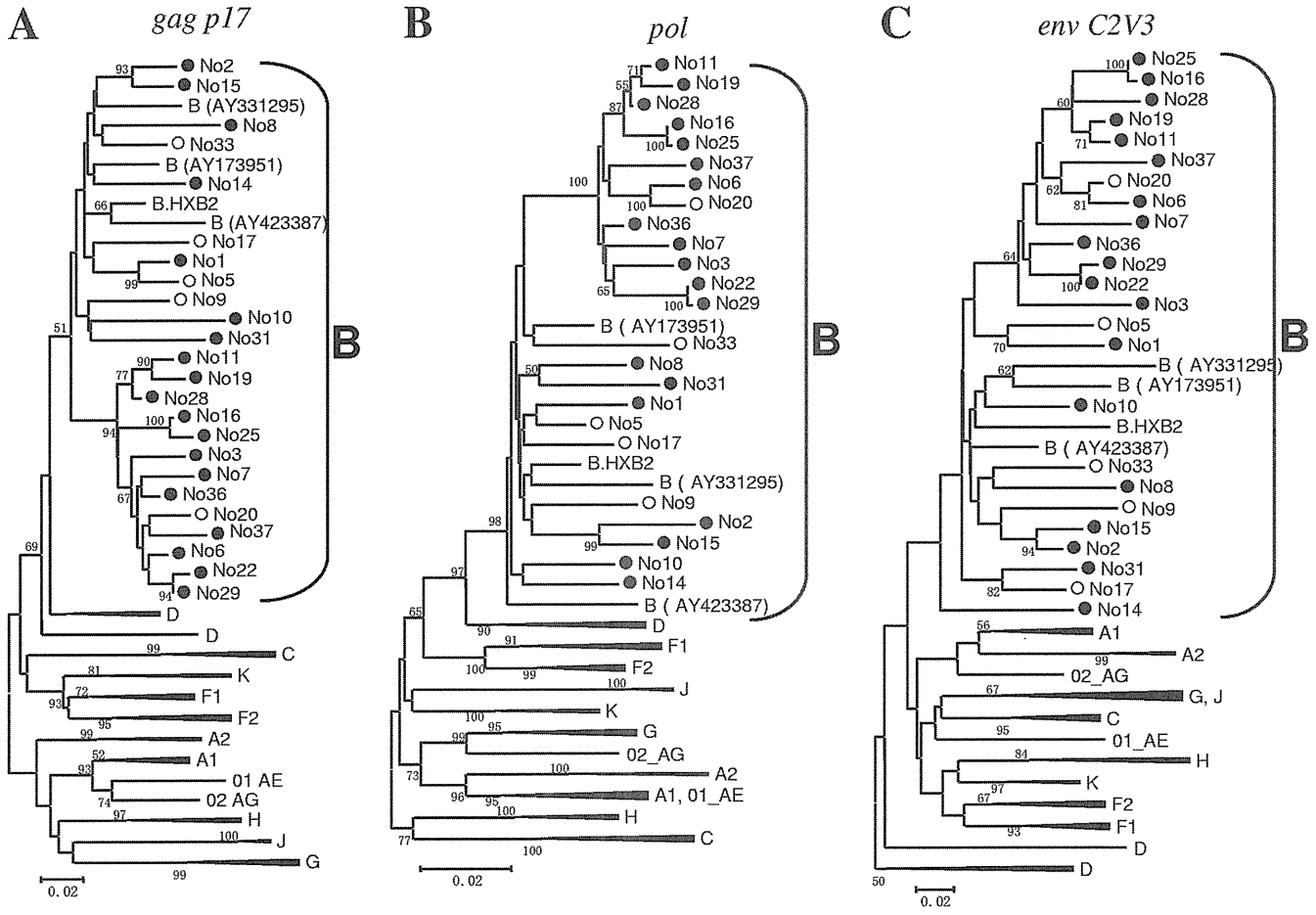

FIG. 4. Phylogenetic tree analyses of HIV-1 isolated from HBV/HIV-1-coinfected patients. Phylogenetic trees were constructed using the 25 HIV-1 sequences obtained in this study and 62 HIV-1 reference sequences from the Los Alamos National Laboratory database. The nucleotide base sequences of *gag p17* (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-

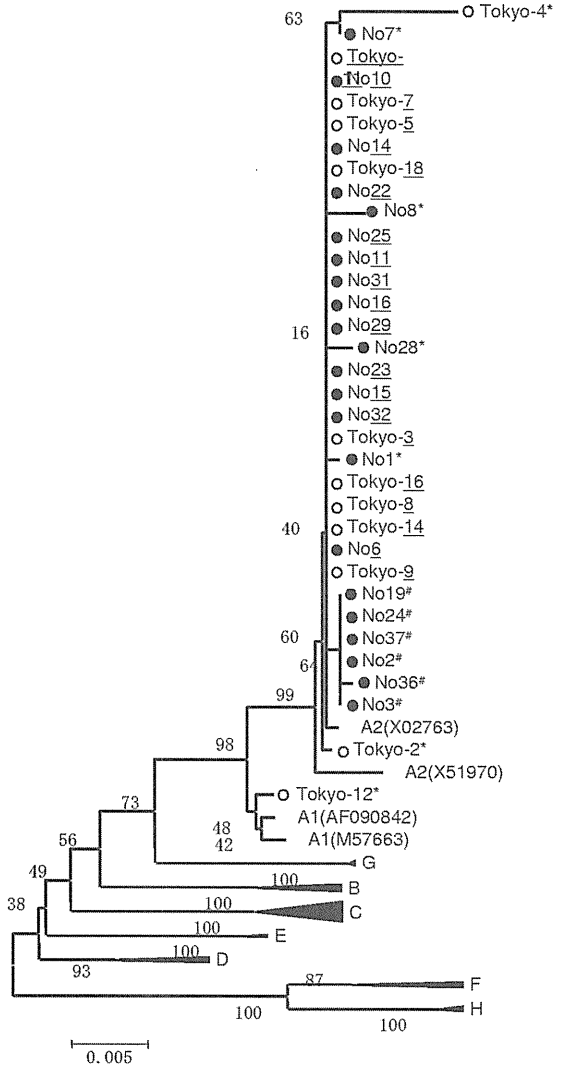

FIG. 5. Phylogenetic tree analysis of 35 HBV region S sequences, 22 from Nagoya (solid circles) and 13 from Tokyo (open circles). Three genetically different groups are indicated by asterisks, pound signs, and underlining.

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,