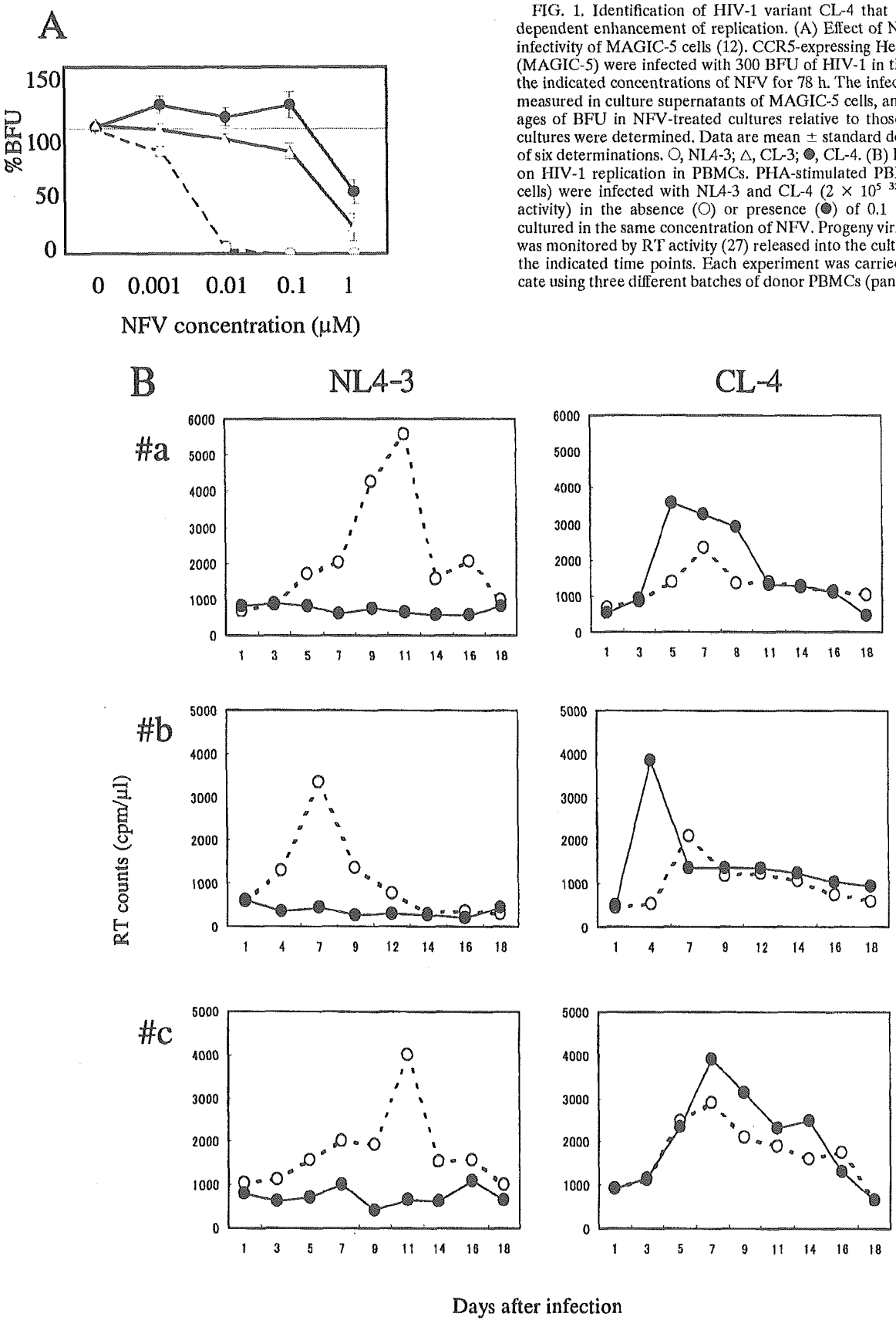

FIG. 1. Identification of HIV-1 variant CL-4 that exhibits NFV-dependent enhancement of replication. (A) Effect of NFV on HIV-1 infectivity of MAGIC-5 cells (12). CCR5-expressing HeLa/CD4$^+$ cells (MAGIC-5) were infected with 300 BFU of HIV-1 in the presence of the indicated concentrations of NFV for 78 h. The infectious titer was measured in culture supernatants of MAGIC-5 cells, and the percentages of BFU in NFV-treated cultures relative to those in NFV-free cultures were determined. Data are mean ± standard deviation values of six determinations. ○, NL4-3; △, CL-3; ●, CL-4. (B) Effects of NFV on HIV-1 replication in PBMCs. PHA-stimulated PBMCs (2×10^5 cells) were infected with NL4-3 and CL-4 (2×10^5 ^{32}P cpm of RT activity) in the absence (○) or presence (●) of 0.1 μM NFV and cultured in the same concentration of NFV. Progeny virion production was monitored by RT activity (27) released into the culture medium at the indicated time points. Each experiment was carried out in duplicate using three different batches of donor PBMCs (panels a, b, and c).

TABLE 2. Amino acid substitutions in PR[a]

Isolate	Resistance-associated mutations					Other mutations					
	L10	M36	G48	I54	V82	E35	N37	K43	I62	I72	T74
CL-1	I	—	—	—	—	—	—	—	—	—	—
CL-2	I	—	V	V	A	—	—	—	—	V	S
CL-3	I	I	V	V	A	—	—	—	V	V	S
CL-4	I	I	V	V	A	D	S	T	V	V	S

[a] For each amino acid residue, the letter in the top lane indicates the amino acid associated with NL4-3. A dash indicates identity with NL4-3, and a single letter indicates the amino acid substitution. For example, CL-1 had an amino acid substitution at Leu10→Ile (L10I).

tested. The replication kinetics in PBMCs were comparable among parental and each recombinant NL4-3, whereas those in PM-1 and MT-2 were significantly slower with the recombinant NL4-3 carrying the p17-PR segment of CL-3 or CL-4 than with others (Fig. 3). These data suggest the presence of intrinsic impairment of the Gag-PR segment of CL-3 and CL-4 that becomes apparent only on replication in transformed T-cell lines.

As expected, the parental NL4-3 did not grow at all in the presence of NFV, whereas all CL-4 recombinants retained replicative capacity under such an environment. The recombinant carrying the C-terminal portion of Gag and the entire PR (NL4-3PRmt of CL-4) grew efficiently in the presence of 0.1 μM NFV in all cells tested (Fig. 3A, PR), suggesting that mutations in these segments alone are sufficient to confer NFV resistance on NL4-3.

Of note was the recombinant carrying the entire *gag* and PR genes of CL-4 (NL4-3p17PRmt of CL-4). In the presence of 0.1 μM NFV, the recombinant replicated with significantly faster kinetics to higher titers than the same amount of virus did in the absence of the drug (Fig. 3A, p17PR). The NFV-dependent replication enhancement was observed in all cells tested in three repeated experiments. In contrast, the phenomenon was not seen in other recombinants of CL-4 (Fig. 3A, PR and p24PR), or in a recombinant carrying the entire *gag-pro* genes of CL-3 (Fig. 3B, p17PR). These data suggest that mutations in the Gag p17 segment of CL-4 are indispensable for generating the phenotype of the original CL-4 virus isolate.

Western blot analyses of the Gag processing pattern in the presence of NFV. To obtain further insight into the role of NFV in modulating viral infectivity, the Gag processing pattern was assessed in the absence or presence of NFV by Western blot analysis (Fig. 4). After transfection of equal amounts of the parental and recombinant NL4-3 DNAs into HeLa cells, the cells were cultured with or without NFV for 48 h. Virions corresponding to 6×10^5 cpm of RT activity (Fig. 4A) and cell lysate containing 25 μg of protein (Fig. 4B) were loaded in each lane for electrophoresis.

In the absence of NFV in virions (Fig. 4A), the Gag p55 precursor of the NL4-3 control was efficiently cleaved into the Gag p24 CA peptide (Fig. 4A, lane 1). In contrast, processing of the recombinant carrying the PR of CL4 was less efficient, as evidenced by the presence of p55 and p41 Gag uncleaved products (Fig. 4A, lane 3), suggesting that the CL-4 PR mutant altered the substrate specificity and that the NL4-3 Gag precursor was not an efficient substrate. Interestingly, the processing of the recombinant carrying the entire Gag and PR of CL4 was almost as efficient as that of the parental NL4-3 (Fig. 4A, lane 5), suggesting that mutations in CL-4 Gag compensate for the impairment of the PR mutant.

In the presence of NFV (0.1 μM), NL4-3 virions only had the p55 precursor (Fig. 4A, lane 2), confirming that the concentration of NFV used in the present replication experiment could completely block the PR function of the PI-sensitive clone. In contrast, processing of the recombinant carrying the PR of CL4 was not significantly affected by NFV (Fig. 4A, lane 4). The amount of p55 Gag precursor was completely cleaved in the recombinant virus carrying p17PR4 (Fig. 4A, lane 6).

The Western blot analysis of virions failed to reveal cleavage enhancement by NFV in p17PR4 carrying recombinant virus (Fig. 4A, lanes 5 and 6). Therefore, we further analyzed the cleavage pattern in the transfected HeLa cells. As expected, p41 Gag was efficiently cleaved in NL4-3 in the absence of NFV (Fig. 4B, lane 1). In contrast, such a cleavage was inhibited by NFV (Fig. 4B, lane 2). In PR4 carrying recombinant virus, p41 Gag was visible both in the absence and presence of NFV (Fig. 4B, lanes 3 and 4), suggesting that the cleavage efficiency was partially complicated but not affected by NFV. In the absence of NFV, the cleavage efficiency of the p17PR4-carrying recombinant virus was still impaired, as suggested by the presence of visible p41 Gag (Fig. 4B, lane 5). However, in the presence of NFV, p41 Gag was cleaved as efficiently as NL4-3 (wild type) (Fig. 4B, lane 6).

TABLE 3. Amino acid substitutions in Gag p17

Isolate	Amino acid substitution in Gag p17[a]									
CL-1	N47	K55	M61	G62	F66	V82	S109	Q117	N129	N130
CL-2	—	E	I	—	S	—	—	—	—	D
CL-3	D	E	I	—	S	—	—	—	—	D
CL-4	D	Q	I	R	S	I	N	E	D	—

[a] The letter and number in the top lane indicate the amino acid residue and its position in the Gag region associated with CL-1. A dash indicates identity with CL-1, and a single letter indicates the amino acid substitution. For example, CL-3 had an amino acid substitution at Asn47→Asp (N47D).

DISCUSSION

In the present study, (i) we identified a unique NFV-resistant variant of HIV-1 (CL-4) by applying our simple drug resistance assay system (12); (ii) we classified, based on sequence comparisons, the mutations potentially involved in conferring the CL-4 phenotype, which were determined by using the pNL4-3-based chimeric viruses harboring the gene segment responsible for conferring the CL-4 phenotype; and (iii) we assessed the roles of CL-4 Gag mutations in compensating Gag cleavage defects of the PR mutant. Based on the above analyses, we showed in the present study (i) the existence of an HIV-1 variant that replicated more efficiently under NFV than in the absence of this drug (Fig. 1); (ii) gradual accumulation of point mutations in the PR and Gag p17 during treatment with an NFV-containing regimen, but lack of any such change in most cleavage sites of the Gag precursor (Tables 2, 3, and 4); (iii) mutations in the CL4 Gag p6-PR segment alone are sufficient to confer NFV resistance, while those in the CL-4 Gag p17 are indispensable for generating the CL-4 phenotype (Fig.

TABLE 4. Amino acid substitutions at cleavage sites of Gag precursor

Isolate	Amino acid substitutions at cleavage sites of Gag precursor[a]				
	MA/CA	CA/p2	p2/NC	NC/p1	p1/p6
CL-1	SQNF/PIVQ	ARVL/AEAM	GAIM/MQRG	RQAN/FLGK	PGNF/LQSR
CL-2	----/----	----/----	----/----	----/----	----/----
CL-3	----/----	----/----	----/----	----/----	----/----
CL-4	----/----	--I-/----	----/----	----/----	----/----

[a] Flanking amino acid residues at cleavage sites of Gag precursor are listed. Each amino acid is associated with CL-1. A dash indicates identity with CL-1. MA, matrix (p17); CA, capsid (p24); NC, nucleocapsid (p7).

3); and (iv) mutations in the CL-4 Gag can compensate for Gag cleavage defects caused by PR mutations (Fig. 4).

During acquisition of the CL-4 phenotype, three amino acid substitutions (E35D, N37S, and K43T), a single substitution (V to I), and seven substitutions (E55Q, G62R, V82I, S109N, Q117E, N129D, and D130N) accumulated in a stepwise fashion in the PR, the Gag CA/p2 cleavage site, and the Gag p17, respectively (Tables 2, 3, and 4). In contrast, other regions remained highly conserved. Our data suggest that some or all of these mutations, in concert with preexisting mutations, culminated in the formation of the CL-4 phenotype of HIV-1 during the 9-month NFV-based therapy. In particular, the substitutions in Gag p17 are essential, because only the p17PR segment of CL-4, but not of CL-3, or the p24PR segment or PR segment could confer the CL-4 phenotype of the drug-sensitive virus (Fig. 3). It is possible, however, that substitutions localized to the α-helix of the C-terminal domain of Gag p17 might interact with a p24 mutation and alter the exposure of the MA-CA cleavage site in the Gag precursor. Further studies involving site-directed mutagenesis are necessary to determine the precise set of mutations conferring the NFV-dependent replication enhancement phenotype.

The underlying molecular mechanism(s) of the NFV-dependent replication enhancement was not identified in the present study. It is possible that in the case of the mutant PR of CL-4, NFV acts as an allosteric effector and regulator of the enzyme function, instead of acting as a competitive inhibitor. Modulation of binding affinity to a substrate by binding of different

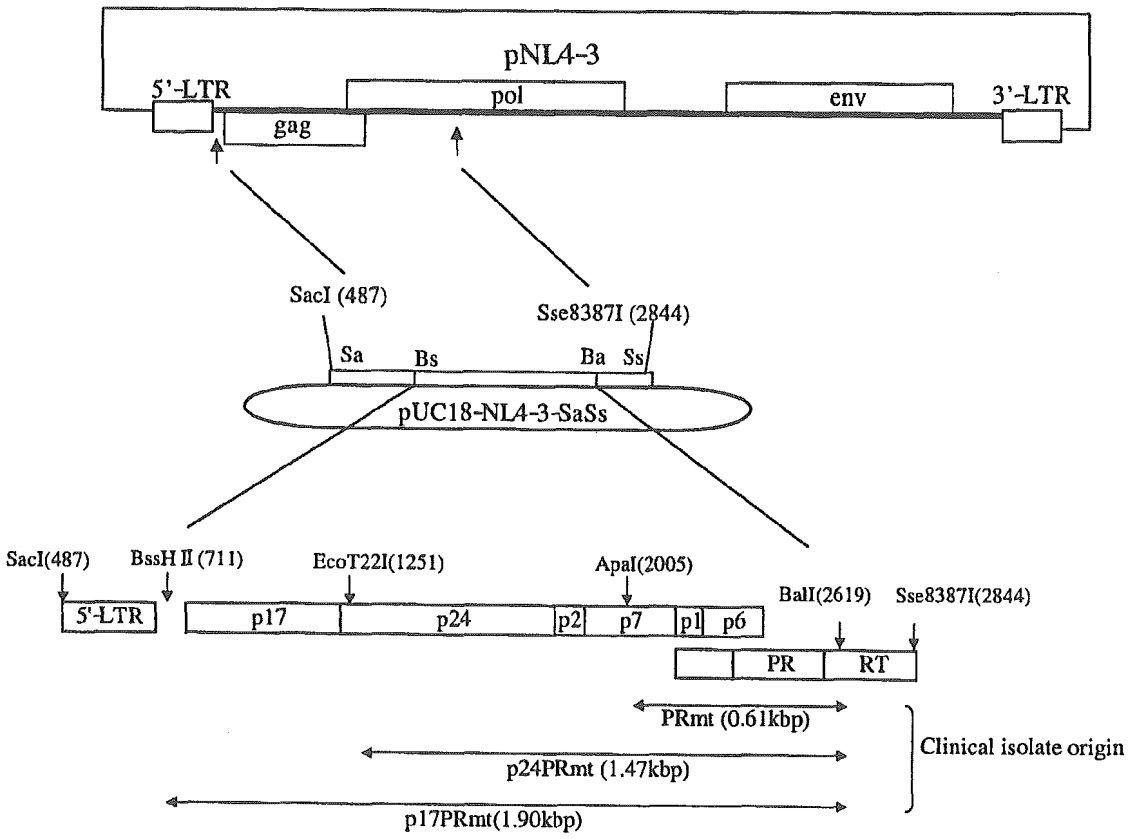

FIG. 2. Construction of pNL4-3-based *gag-pro* recombinants. The HIV-1 *gag-pro* DNA segment was amplified by RT-PCR from the CL-3 or CL-4 virus isolate and replaced with the *Bss*HII-*Bal*I fragment of pUC18-NL4-3-SaSs. Subsequently, the *Bss*HII-*Sse*8387I fragment of pUC18-NL4-3-SaSs was cloned into pNL4-3 to reconstitute full-length HIV-1 molecular clones.

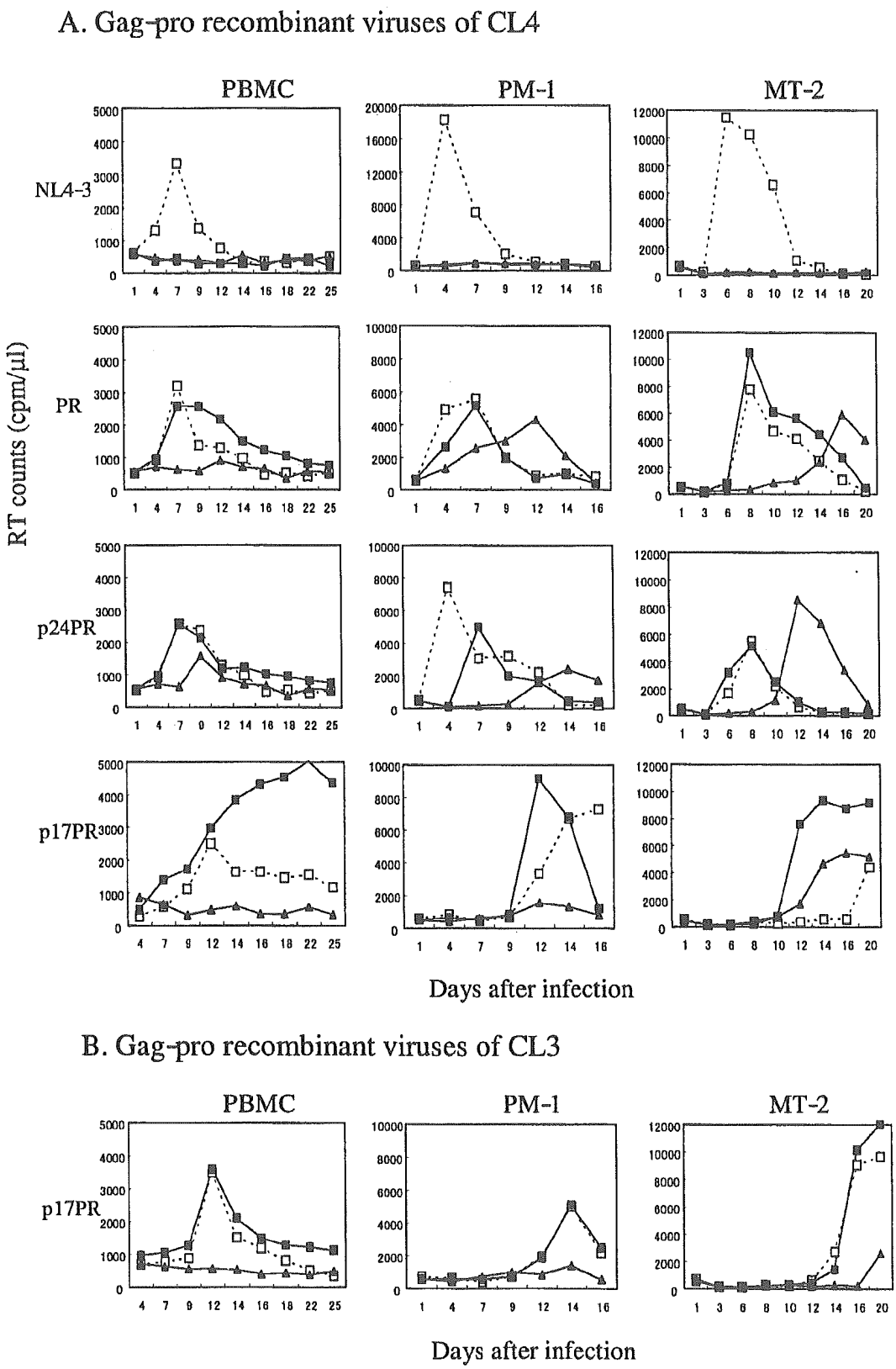

FIG. 3. Effects of NFV on replication of *gag-pro* recombinant viruses in PBMCs, PM-1, and MT-2 cells. (A) Replication kinetics of NL4-3 and NL4-3PRmt, NL4-3p24PRmt, and NL4-3p17PRmt of CL-4 were examined with PBMCs, PM-1, and MT-2 in the absence (□) or presence of 0.1 μM (■) and 1 μM (▲) NFV. (B) Replication kinetics of NL4-3p17PRmt of CL-3 were examined under the same conditions.

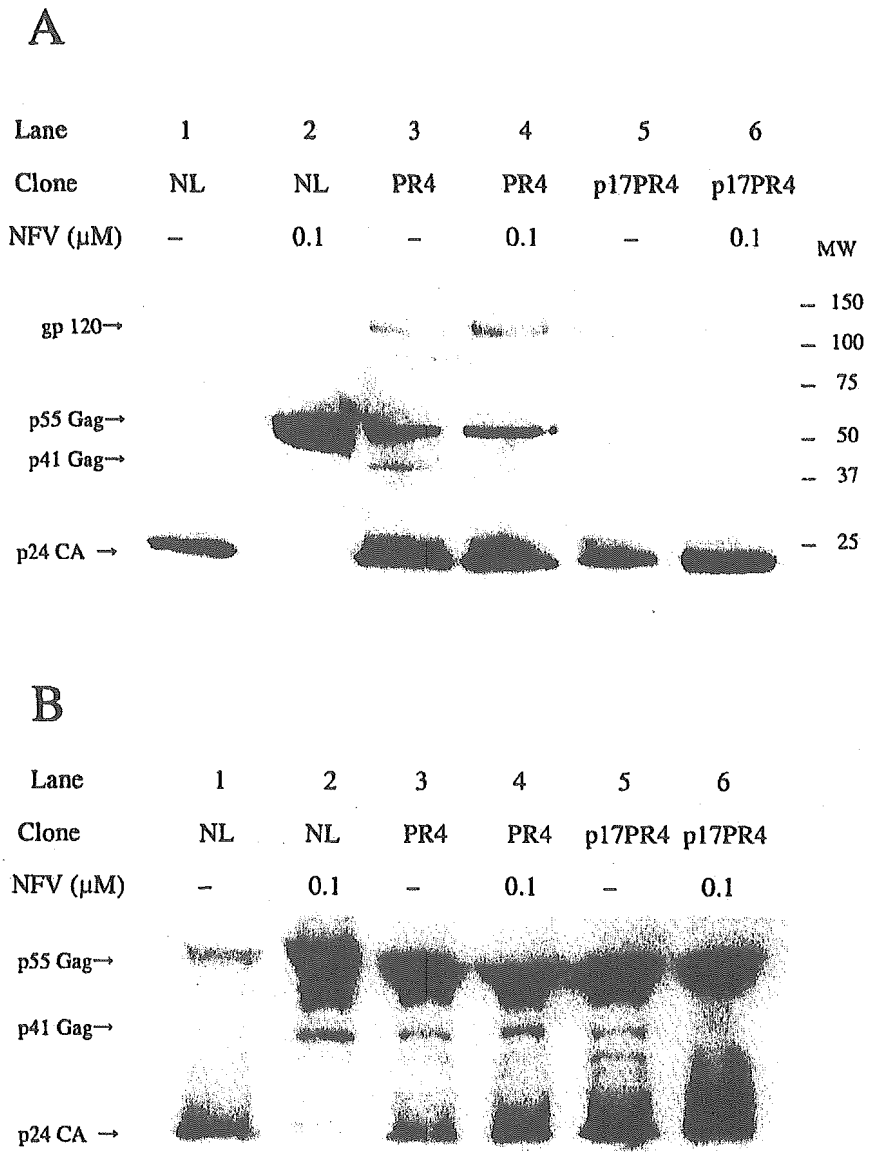

FIG. 4. Western blot analyses in the absence or presence of NFV. HeLa cells were transfected by full-length molecular clones and cultured in the absence or presence of NFV (0.1 μM). At 48 h posttransfection, virions in culture supernatants (A) and cells (B) were harvested and subjected to Western blot analysis. HIV Gag protein was visualized using serum from an HIV-1-seropositive patient. Lanes 1 and 2, pNL4-3; lanes 3 and 4, pNL4-3PRmt; lanes 5 and 6, pNL4-p17PRmt.

low-molecular-weight ligands is commonly seen in multisubunit proteins for metabolic control and is an important mechanism for regulating enzyme activity. Although no study has reported such an allosteric feature for the HIV-1 PR homodimer, prolonged selective pressures of NFV during HAART, combined with a high level of tolerance of the HIV-1 PR to the sequence variation, might have generated the PR mutant possessing an allosteric binding site to NFV. In this context, mutations of p17 on the Gag-pol precursor of CL-4 could be critical in enhancing the cleavage of the Gag-pol polypeptide by the NFV-bound PR.

In this regard, Western blot analyses suggested that the cleavage efficiency of the Gag C-terminus p6 of this mutant can be enhanced in the presence of NFV, which is consistent with the above possibility. The possible allosteric effects of NFV on the CL-4 PR should be most effective for CL-4 Gag substrates, because only the chimera possessing the entire Gag of CL-4 exhibited a detectable NFV effect on virus replication (Fig. 3). Biochemical and structural studies of the CL-4 PR are necessary in order to assess each of these issues.

Several studies have suggested coevolution of *gag* and *pro* genes during treatment with PI-containing regimens (6, 16, 21, 29). It is conceivable that the HIV-1 PR and its substrates evolve coordinately to generate the correct processing products, thereby assuring production of infectious progeny virions. Most of the *gag* mutations for PI resistance reported so far are

located around the cleavage sites of the Gag p55 precursor (6, 16, 21, 29), whereas the roles of mutations at the Gag non-cleavage sites have been poorly addressed. Because highly PI-resistant viruses often lack cleavage site mutations and their compensatory effects on the impaired PR function appear to be partial (16), and because mutations apart from the cleavage sites can affect the cleavability of the precursor in the three-dimensional structure, it is important to evaluate the roles of non-cleavage site mutations in PI resistance.

The present findings evoke an argument regarding continuation of incomplete HAART. When HAART results in incomplete suppression of virus replication due to emergence of variants resistant to the available drugs, some clinical trials currently recommend continuation of therapy, because any interruption could result in increased plasma viral load and low CD4 cell counts (3, 9, 19). In general, it is believed that continuation of HAART under such circumstances can still produce some clinical benefits because of the reduced replication capacity of the PI-resistant virus (9). The present findings, however, suggest that in some cases continuation of incomplete HAART may allow viral replication, resulting in the generation of variants of phenotypes similar to those described in the present study. Thus, phenotypic drug resistance assays appear to deserve more attention, particularly for patients who fail to respond to HAART but must continue treatment using the same regimen.

In conclusion, we have described in the present study a novel mechanism, NFV-dependent replication enhancement, for HIV-1 adaptive changes. Our results suggested that coevolution of Gag and PR genes was a key event for adaptation of HIV-1 to survive the strong pressure of NFV-containing therapy in this particular patient. The present findings have clinical implications that may have an impact on HAART.

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Identification and Characterization of a New Class of Human Immunodeficiency Virus Type 1 Recombinants Comprised of Two Circulating Recombinant Forms, CRF07_BC and CRF08_BC, in China

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We identified a new class of human immunodeficiency virus type 1 (HIV-1) recombinants (00CN-HH069 and 00CN-HH086) in which further recombination occurred between two established circulating recombinant forms (CRFs). These two isolates were found among 57 HIV-1 samples from a cohort of injecting drug users in eastern Yunnan Province of China. Informative-site analysis in conjunction with bootscanning plots and exploratory tree analysis revealed that these two strains were closely related mosaics comprised of CRF07_BC and CRF08_BC, which are found in China. The genotype screening based on *gag*-reverse transcriptase sequences of 57 samples from eastern Yunnan identified 47 CRF08_BC specimens (82.5%), 5 CRF07_BC specimens (8.8%), and 3 additional specimens with the novel recombinant structure. These new "second-generation" recombinants thus constitute a substantial proportion (5 of 57; 8.8%) of HIV-1 strains in this population and may belong to a new but yet-undefined class of CRF. This might be the first example of CRFs recombining with each other, leading to the evolution of second-generation inter-CRF recombinants.

One of the major characteristics of the human immunodeficiency viruses (HIVs) is their extremely high genetic diversity. Phylogenetic analyses of globally circulating viral strains have identified three distinct groups of HIV type 1 (HIV-1) (M, N, and O), and nine genetic subtypes (A to D, F to H, J, and K) within the major group (M) (D. L. Robertson, J. P. Anderson, J. A. Bradac, J. K. Carr, B. Foley, R. K. Funkhouser, F. Gao, B. H. Hahn, M. L. Kalish, C. Kuiken, G. H. Learn, T. Leitner, F. McCutchan, S. Osmanov, M. Peeters, D. Pieniazek, M. Salminen, P. M. Sharp, S. Wolinsky, and B. Korber, Letter, Science **288**:55-56, 2000). Moreover, certain isolates cluster with different subtypes in different regions of their genomes. Some of these mosaic HIV-1 strains disseminate widely in populations, becoming circulating recombinant forms (CRFs) (2) that play a major role in global or regional HIV epidemics. According to the Los Alamos HIV database, 14 CRFs are currently recognized (http://hiv-web.lanl.gov/CRFs/CRFs.html).

Three CRFs play a critical role in the HIV-1 epidemic in Asia. CRF01_AE was originally identified in Thailand (13, 15) and is being spread throughout Southeast Asia (22). Two closely related CRFs, CRF07_BC and CRF08_BC, have been identified among injecting drug users (IDUs) in China (16, 19). CRF07_BC (prototype strains, 97CN54 and 97CN001) was distributed among IDUs in Xinjiang Province in northwest China (19), while CRF08_BC (prototype strain, 97CNGX6F) was circulating widely among IDUs in Guangxi Province in southeast China (16). Each CRF appears to be associated with a different overland heroin trafficking route (1): CRF07_BC has spread northwestward to Xinjiang Province, while CRF08_BC has spread eastward to Guangxi Province, from their common origin, presumably in Yunnan, where subtypes B′ and C are cocirculating (16, 19). Yunnan Province in southwestern China is thus thought to be an epicenter of the HIV-1 epidemic in China, where the cumulative number of HIV cases would be expected to reach 10 million by 2010 with the current rate of increase (30%) (20).

It has been reported that the HIV-1 epidemic among IDUs in Yunnan was initiated with both HIV-1 subtype B (same lineage of subtype B strains isolated in the United States and Europe) and subtype B′ (Thailand variant of subtype B, also referred to as Thai-B) strains (8, 21, 22) in the late 1980s. Subtype B′ appeared to replace subtype B of U.S.-European lineage, increasing from 20% of all subtype B in strains 1990 to 90% in 1996 (8, 15, 21, 22). Subsequently, HIV-1 subtype C strains were identified among IDUs in China by the early 1990s (11). A recent study revealed unique geographical differences in distribution of HIV-1 subtypes among IDUs in Yunnan Province (24). HIV-1 subtype B′ and various forms of unique HIV-1 intersubtype B′-C recombinants were distributed in western Yunnan (Dehong Prefecture). In contrast, CRF08_BC predominated and CRF07_BC was detected at low prevalence in eastern Yunnan (Wenshan and Honghe Prefectures) near the border with Guangxi Province. Moreover, unique recombinant forms that showed structural similarity to CRF07_BC were detected in eastern Yunnan (24).

We report here the identification and characterization of a

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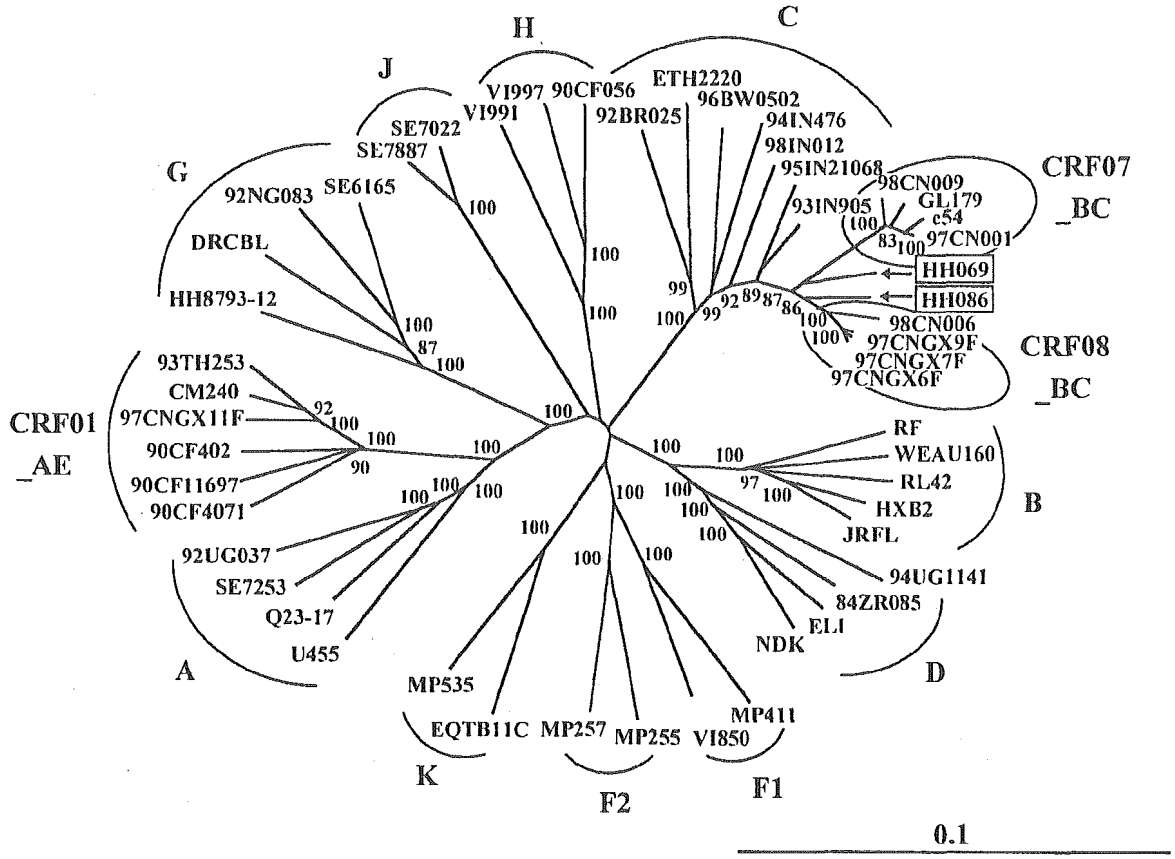


FIG. 1. Neighbor-joining tree analysis based on near-full-length nucleotide sequences of 00CN-HH069 and 00CN-HH086 with HIV-1 group M (subtypes A to D, F to H, J, and K) and CRF reference sequences (http://hiv-web.lanl.gov/content/hiv-db/SUBTYPE_REF/Table1.html). $SIV_{CPZ}GAB$ was used as an outgroup but is not shown for simplicity. Bootstrap values (>80) are shown at the corresponding nodes. Subtypes and CRF clades are shown outside the tree. The clusters of CRF07_BC and CRF08_BC are circled. Boxed are 00CN-HH069 (HH069) and 00CN-HH086 (HH086).

new class of HIV-1 recombinants comprised of two previously established CRFs (CRF07_BC and CRF08_BC) among unique recombinant forms detected in eastern Yunnan Province and discuss the spread of these novel recombinants and their biological implications.

As a part of cohort study of HIV-hepatitis C virus coinfection in Yunnan Province (27), 57 consenting HIV-1-positive IDUs were recruited from drug detoxification and rehabilitation centers in Honghe (44 IDUs) and Wenshan (13 IDUs) Prefectures during the period August 2000 to March 2001. The subjects included 43 males and 14 females with an age range of 17 to 43 years (mean, 29.1 years). The mean duration of drug use was 5 years (range, 1 to 11 years). Fourteen additional specimens from IDUs in Dehong Prefecture in west Yunnan Province were analyzed in parallel (24). EDTA-treated blood samples were drawn from HIV-1-positive individuals, and the peripheral blood mononuclear cells (PBMCs) were separated by Ficoll-Hypaque (Pharmacia, Piscataway, N.J.) density gradient centrifugation. For virus isolation, PBMCs from HIV-1-positive individuals were cocultured with phytohemagglutinin (2 μg/ml)-stimulated $CD8^+$-T cell-depleted PBMCs from HIV-negative healthy donors in RPMI 1640 containing 10% fetal calf serum and interleukin-2 (20 U/ml). Virus production was detected by virion-associated reverse transcriptase (RT) assay as described previously (9). Plasma specimens were saved for genotype screening based on the nucleotide sequence determination of virion HIV-1 RNAs (24).

A total of 25 HIV-1 strains were isolated from 57 HIV-1-positive specimens from eastern Yunnan, and their genotypes were screened on the basis of the nucleotide sequences of the *gag*-RT regions (24), where CRF07_BC and CRF08_BC, which are circulating widely among IDUs in China, display distinct profiles of recombination between subtypes B′ and C (16, 19, 24). The phylogenetic analyses revealed that the majority of HIV-1 strains circulating in eastern Yunnan province belonged to CRF08_BC (21 of 25; 84%) and that CRF07_BC was found only at a low prevalence (2 of 25; 8%). However, the remaining two isolates (00CN-HH069 and 00CN-HH086) were classified as outliers placed outside the clusters of CRF07_BC and CRF08_BC (24) (data not shown). They were isolated from 24- and 29-year-old asymptomatic male IDUs in Honghe Prefecture in eastern Yunnan Province who started their injecting drug use relatively recently (from October 1998 and July 1999, respectively). The specimens for the present study were collected in August 2000. The interperson distance of 21 CRF08_BC strains identified in eastern Yunnan was remark-

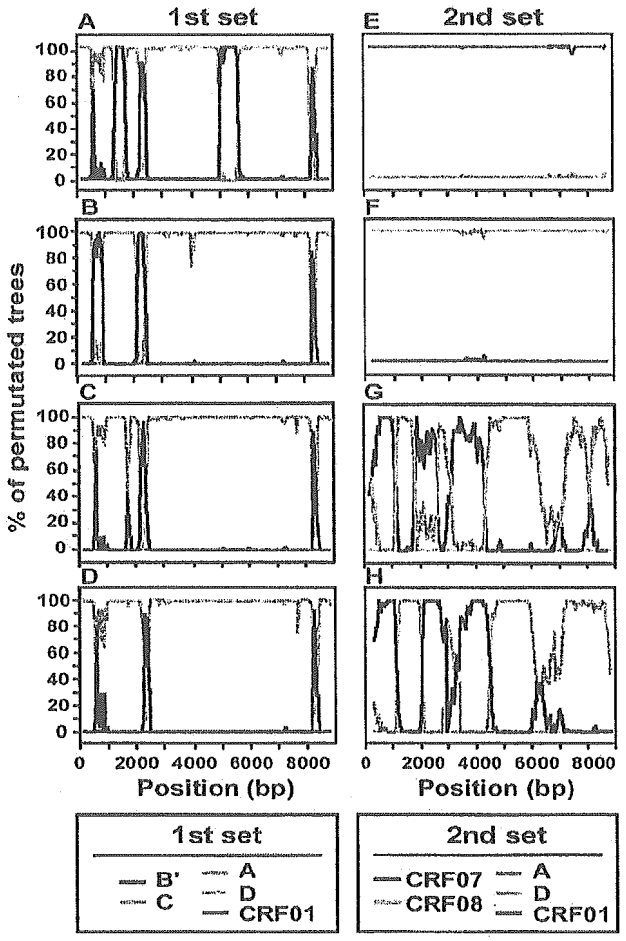


FIG. 2. Bootscanning analyses of near-full-length HIV-1 nucleotide sequences of 00CN-HH069 and 00CN-HH086. Two sets of bootscanning plots were performed. (A to D) Bootscanning plots (17), depicting the relationship of CRF07_BC (97CN001) (A), CRF08_BC (97CNGX6F) (B), 00CN-HH069 (C), and 00CN-HH086 (D) to the representative strains of HIV-1 subtypes (A, B', C, and D) and CRF01_AE. (G and H) The second set of bootscanning plots, delineating the relationship of 00CN-HH069 (G) and 00CN-HH086 (H) to the reference strains for CRF07_BC (97CN001) and CRF08_BC (97CNGX6F) with the respective representatives of HIV-1 subtypes A and D and CRF01_AE. (E and F) Control plots for the reference strains of CRF07_BC (97CN001) and CRF08_BC (97CNGX6F), respectively. The bootstrap values are plotted for a window of 500 bp moving in increments of 50 bp along the alignment. The reference strains used are shown at the bottom.

ably low: 1.4% ± 0.4% (mean ± standard deviation) (range, 0.4 to 2.4%) for the 1.4-kb *pol* region and 1.5% ± 0.6% (range, 0.7 to 2.4%) for the 2.6-kb *gag*-RT region (n = 21). According to a study based on specimens collected from IDUs in neighboring Guangxi Province in July 1996 and July 1997, CRF08_BC prevailing in this region was highly homogeneous, with an interpatient diversity of 0.4% (for all structural genes) and 0.9% (for regulatory genes), compared with the reference strains of subtype C from Botswana and subtype A from Uganda, Somalia, and Tanzania (7.6 and 8.1%, respectively) (16). These results suggest that a common ancestor of CRF08_BC has been introduced into IDU populations in eastern Yunnan and Guangxi Provinces very recently (16). It is plausible that this ancestor might have been arisen in Yunnan, since subtypes B' and C circulated among IDUs in Yunnan in the early 1990s (11, 22, 23), while subtype C was first introduced into IDUs in Guangxi around 1996 and 1997 (3, 25, 26), as suggested by Piyasirisilp et al. (16).

To elucidate the complete genome structures of the two Honghe isolates with outlier genotypes (00CN-HH069 and 00CN-HH086), we cloned and determined the nucleotide sequences of their near-full-length HIV-1 proviral genomes amplified by PCR as described previously (10, 18). Briefly, DNAs were extracted from CD8-depleted phytohemagglutinin-stimulated PBMCs infected with the HIV-1 strains of interest by use of a blood and cell culture DNA midi kit (Qiagen, Hilden, Germany). The near-full-length HIV-1 genomes were amplified by using the Expand long-template PCR system (Boehringer GmbH, Manheim, Germany) with the primer set of pbs-496A (sense, 5'-AGTGGCGCCCGAACAGG-3' [the *Nar*I site is underlined]) (7) and U5-497B (antisense, 5'-GGTCTG AGGGATCTCTAGTTACCAG-3'). Purified PCR fragments were ligated with a pBR322-based vector carrying an *Xcm* I site for TA cloning (12) (for 00CN-HH069) or with the pCR TOPO XL vector (Invitrogen, Carlsbad, Calif.) (for 00CN-HH086). Positive clones with 9-kb near-full-length inserts were selected, and the nucleotide sequences of near-full-length HIV-1 genomes were determined on both strands by the direct sequencing method with fluorescent dye terminators in an automated ABI PRISM310 DNA sequencer (Applied Biosystems, Inc., Foster City, Calif.), using the primer-walking approach. The resulting near-full-length DNA clones were designated 00CN-HH069.1 and 00CN-HH086.1. Both clones had intact open reading frames for all nine HIV-1 genes. Phylogenetic tree analysis based on the near-full-length nucleotide sequences revealed that they were placed between the monophyletic clusters of CRF07_BC and CRF08_BC, suggesting their close relationship to these two CRFs (Fig. 1).

To search for the possible recombination breakpoints, near-full-length nucleotide sequences were subjected to recombination breakpoint analyses (17), in comparison with the prototype strains of CRF07_BC (97CN001) and CRF08_BC (97CNGX6F) (Fig. 2). Bootscanning plots using the representative reference strains of HIV-1 subtypes (Fig. 2A to D) revealed that they were comprised of subtype C with two (00CN-HH086.1) or three (00CN-HH069.1) small segments of subtype B' (Fig. 2C and D). These two strains appeared to show structural similarity to both CRF07_BC (Fig. 2A) and CRF08_BC (Fig. 2B) in different regions of the HIV-1 genome. The short subtype B' segments in the 5' parts of their genomes (corresponding to 5' part of the *gag* region) in 00CN-HH086.1 and 00CN-HH069.1 appeared to be identical to those in CRF07_BC, while the subtype B' segments in the middle parts of the HIV-1 genome present in CRF07_BC were lacking, like for CRF08_BC. Similarly, short subtype B' segments in the 3' portions of the genomes (corresponding to *nef* regions) suggested a close structural relationship to either CRF07_BC or CRF08_BC (Fig. 2A to D).

Since the possible involvement of CRF07_BC and CRF08_BC in the structural makeup of these two strains was suggested, we further scrutinized their recombinant structures by bootscanning analyses testing the relationship to CRF07_BC

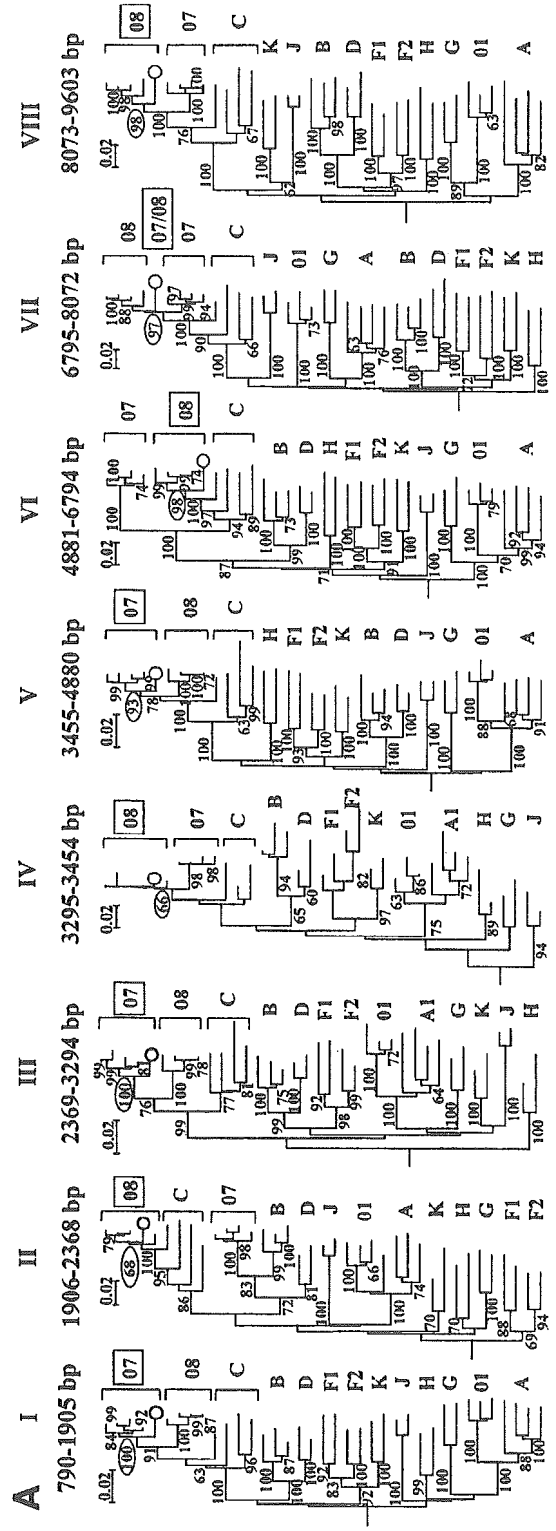


FIG. 3. Exploratory tree analysis. (A) 00CN-HH069; (B) 00CN-HH086. Using bootscanning and distance scanning techniques (Fig. 2G and H), the plausible recombination breakpoints were identified and HIV-1 genomes were then divided into eight segments (I through VIII). Each segment was then used in separate phylogenetic analyses based on the neighbor-joining method to confirm the subtype (or CRF) origin of the segment. The stability of the nodes was assessed by using maximum parsimony (6) with a bootstrap value of 100 replications (5). $SIV_{CPZ}GAB$ was used as an outgroup but is not shown for simplicity. Nucleotide positions are numbered by the HXB2 numbering engine (http://hiv-web.lanl.gov/content/hiv-db/NUM-HXB2/HXB2.MAIN.html). The analysis starts from *gag* open reading frame. The bootstrap values with which the cluster containing each query strain is supported are marked with ovals at the corresponding nodes. ○, 00CN-HH069; ●, 00CN-HH086. The genotype assignments in the separate phylogenetic analyses are indicated within the box at the right of the respective tree. 07, CRF07_BC; 08, CRF08_BC; 07/08, equidistant to both CRF07_BC and CRF08_BC.

TABLE 1. Summary of genotype assignment, based on exploratory free analysis, in different regions of HIV-1 genomes of two putative inter-CRF recombinants (00CN-HH069.1 and 00CN-HH086.1)

Region in HIV-1 genome[a]	00CN-HH069.1		00CN-HH086.1	
	Position[b]	Genotype[c]	Position	Genotype
I	790–1905	07 (100)	790–1905	07 (99)
II	1906–2368	08 (68)	1906–2628	08 (100)
III	2369–3294	07 (100)	2629–3777	07 (100)
IV	3295–3454	08 (66)	3778–3878	08 (67)
V	3455–4880	07 (93)	3879–5314	07 (97)
VI	4881–6794	08 (98)	5315–6620	08 (100)
VII	6795–8072	07/08 (97)	6621–7897	07/08 (100)
VIII	8073–9603	08 (98)	7898–9603	08 (100)

[a] On the basis of recombination breakpoints estimated by bootscanning plots (Fig. 2G and H), HIV-1 genomes of strains 00CN-HH069.1 and 00CN-HH086.1 were divided into 8 segments (I through VIII). Each segment was subjected to neighbor-joining tree analysis (Fig. 3).

[b] Nucleotide positions are as numbered by the HXB2 numbering Engine (http://hiv-web.lanl.gov/content/hiv-db/NUM-HXB2/HXB2.MAIN.html).

[c] 07, CRF07_BC; 08, CRF08_BC; 07/08, equidistant to CRF07_BC and CRF08_BC. Numbers in parentheses indicate the percentage of the bootstrap value with which the adjacent cluster containing the indicated strain is supported in phylogenetic tree analysis.

(97CN001) and CRF08_BC (97CNGX6F), in comparison with the representative reference strains of HIV-1 subtypes, including HIV-1 subtypes A and D and CRF01_AE (Fig. 2E to H). As shown in Fig. 2G and H, these two strains appear to be comprised of several patches of CRF07_BC and CRF08_BC in the 5′ halves of the genomes, while the 3′ halves of the HIV-1 genomes consisted mostly of CRF08_BC.

To confirm their recombinant structures, exploratory tree analyses were performed along the HIV-1 genomes of these two strains. Based on the data on the putative recombination breakpoints estimated by the profiles of bootscanning plots (Fig. 2G and H), the HIV-1 genomes of these two strains were divided into eight segments (I through VIII in Fig. 3). Each segment was then analyzed by constructing a neighbor-joining tree to confirm the genotype (Fig. 3 and Table 1). The stability of the nodes was assessed by bootstrap analysis (5) with 100 replications, using maximum parsimony (6). As shown in Fig. 3 and Table 1, each query strain showed congruence to either the CRF07_BC or CRF08_BC cluster, with high bootstrap confidence (93 to 100%) in most of the areas (segments I, II, III, V, VI, and VIII). These two strains formed a significant cluster with CRF08_BC, with a modest level of bootstrap confidence (66 to 67%) in segments IV, while those segments were very short (159 bp for 00CN-HH069.1 and 100 bp for 00CN-HH086.1) (Fig. 3 and Table 1). Since the middle parts of the *env* regions were approximately equidistant phylogenetically to those of CRF07_BC and CRF08_BC (segments VII in Fig. 3), the bootstrap values were lower than those in the other areas (Fig. 2G and H). However, even in these areas, 00CN-HH069.1 and 00CN-HH086.1 were more closely related phylogentically to CRF07_BC and CRF08_BC (with bootstrap confidence of 97 to 100%) than the other subtype C reference strains (segments VII in Fig. 3).

We further defined the recombination breakpoints by informative-site analysis as described previously (7; D. L. Robertson, P. M. Sharp, F. E. McCutchan, and B. H. Hahn, Letter, Nature **374**:124-126, 1995). Using this approach, we identified alternating patches of the regions that were assigned as either CRF07_BC or CRF08_BC with high statistical significance (Table 2). The only disagreement between the informative-site analysis and the bootscanning and exploratory tree analyses was for a short CRF08_BC segment in the middle of the *pol* region in 00CN-HH086 (the second CRF08_BC segment from the left in Fig. 2H) (Table 2). This short CRF08_BC segment

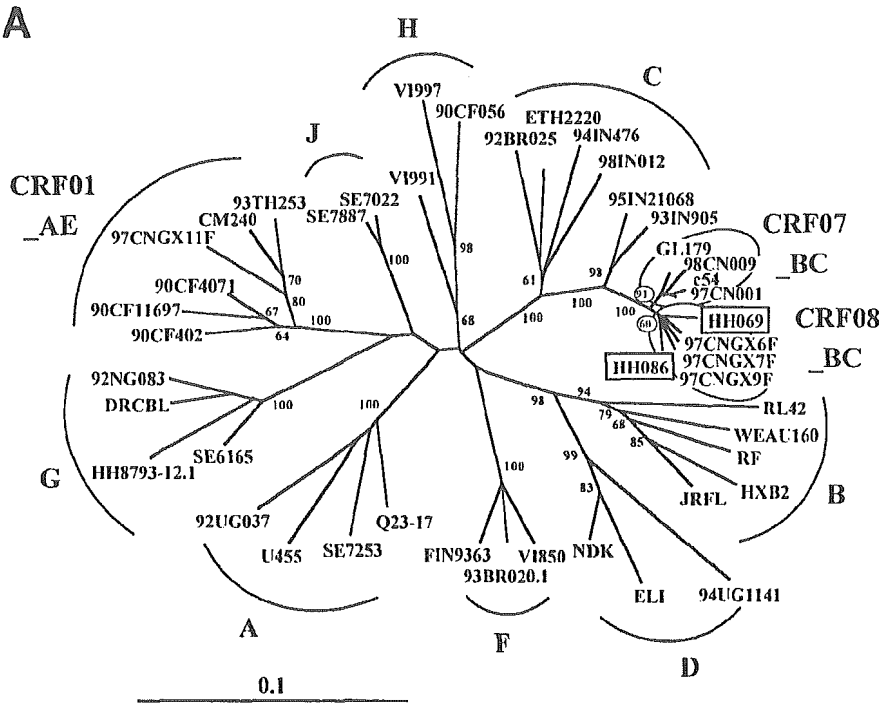
TABLE 2. Informative-site analyses of putative inter-CRF recombinants (HH069 and HH086) comprised of CRF07_BC and CRF08_BC[a]

Strain	Region[b]	Genotype	No. of informative sites in:			P[c]
			CRF07	CRF08	Outgroup	
00CN-HH069.1	790–2028	CRF07	15	5	5	
						<0.0001
	2091–2375	CRF08	0	17	0	
						<0.0001
	2390–3517	CRF07	18	6	2	
						0.05
	3614–3851	CRF08	0	3	0	
						0.01
	3857–5241	CRF07	7	1	3	
						<0.0001
	5337–9631	CRF08	14	72	31	
00CN-HH086.1	790–1909	CRF07	14	5	3	
						<0.0001
	2040–2696	CRF08	1	27	2	
						<0.0001
	2885–5193	CRF07	23	1	4	
						<0.0001
	5337–9631	CRF08	12	85	18	

[a] To determine the recombination breakpoints, each putative recombinant sequence was compared with two predicted parental sequences (CRF07_97CN001 and CRF08_97CN6F) and an outgroup (A_92UG037) as previously described (7, 19).

[b] The nucleotide positions were determined with the HXB2 numbering engine (http://hiv-web.lanl.gov/content/hiv-db/NUM-HXB2/HXB2.MAIN.html). The nucleotide positions of the identical pairs of recombination breakpoints are in boldface.

[c] P values for the resultant divisions of sites were calculated by using Fisher's exact test.



B

		NFκB III		NFκB II		NFκB I
	Subtype B (Con)	....		GGGACTTTCC	GCTG	GGGACTTTCC
Subtype C	ETH2220	GGGACTTTCC	GCC	----------	A--.	---G-G----
	96BW0502	GGGACTTTCC	GCT	----------	AA-.	---G-G----
	96ZM751.3	GGGACTTTCC	GCT	----------	A--.	---G-G----
	93IN905	GGGACTTTCC	GCT	----------	A--.	---G-G----
	93IN301904	GGGACTTTCC	GCT	----------	A--.	---G-G----
	92BR025	.....	GCT	----------	A--.	---G-G----
CRF07_BC	97CN001	GGGACTTTCC	GCG	----------	A--.	---GTG---t
	98CN009	GGGACTTTCC	GCG	----------	A--.	---GTG---t
	97CNXJ54	GGGACTTTCC	GCG	----------	A--.	---G-G---t
	CNGL179	GGGACTTTCC	GCG	----------	A--.	---G-G---t
CRF08_BC	97CNGX6F	GGGACTTTCC	GC-	----------	A-C.	---G-G----
	97CNGX7F	GGGACTTTCC	GC-	----------	A-C.	---G-G----
	97CNGX9F	GGGACTTTCC	GC-	----------	A-C.	---G-G----
	98CNGS006	GGGACTTTCC	GC-	----------	A--.	---G-G----
ICR07/08	00CN-HH086	aGGACTTTCC	GC-	----------	A--.	---G-G----
	00CN-HH069	GGGACTTTCC	GC-	----------	A--.	---G-G----

FIG. 4. Phylogenetic relationship and possible sequence signatures of LTRs of 00CN-HH069 and 00CN-HH086. (A) Neighbor-joining tree based on 3′-LTR sequences of 00CN-HH069 and 00CN-HH086 with HIV-1 group M and CRF reference sequences (7) with bootstrapping (100 replications). Bootstrap values (>60) are shown at the corresponding nodes. The bootstrap values with which the cluster containing each query strain is supported are marked in ovals on the corresponding nodes. Subtype and CRF assignments are shown outside the tree. HH069, 00CN-HH-69; HH086, 00CN-HH086. (B) Nucleotide sequence alignment of enhancer-core regions of the 3′ LTR in comparison with HXB2 and other subtype C strains. Dashes indicates the sequence identity with HXB2. Periods indicate gaps introduced to improve the alignment. The NF-κB-binding motif (consensus sequence, 5′-GGGRNNYYCC-3′) is boxed. Lowercase letters in the box with the gray margin are the substitutions that differ from the NF-κB consensus. Shaded areas are the putative sequence signatures specific to CRF07_BC (uniform nucleotide substitution from C to T in the proximal NF-κB site; G substitution at 3′ end of trinucleotide spacer sequence between NF-κB II and III) or CRF08_BC (GC dinucleotide spacer sequence between NF-κB II and III). ICR, putative second-generation inter-CRF recombinants.

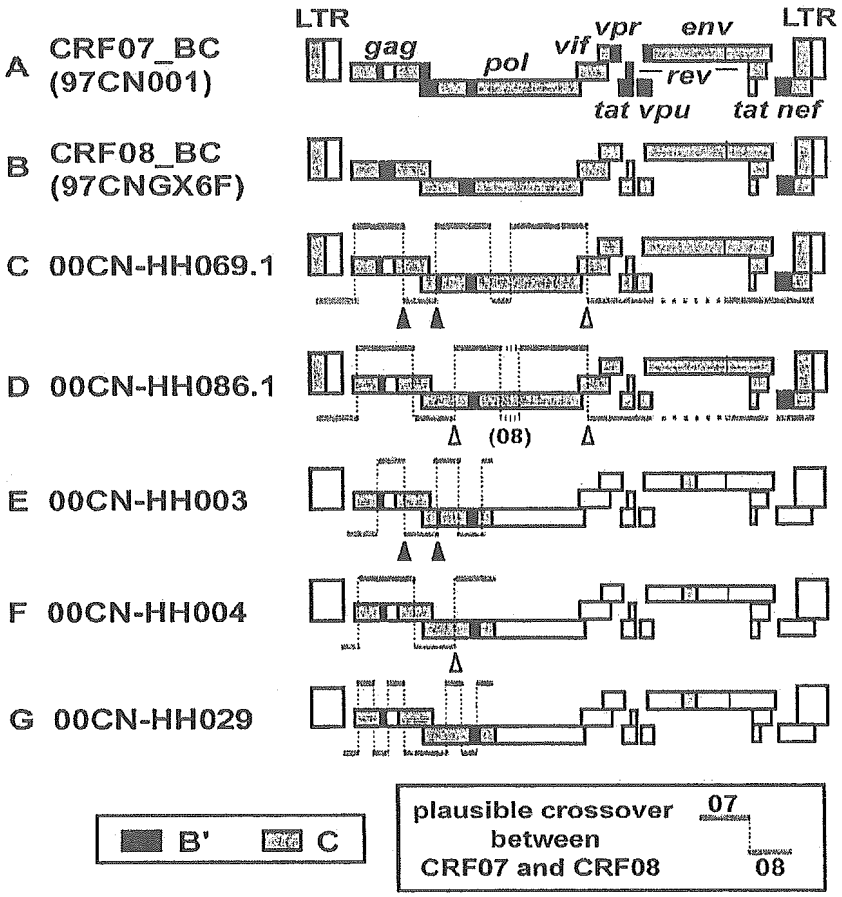


FIG. 5. Schematic representation of subtype structures and deduced profiles of crossovers between CRF07_BC and CRF08_BC in new second-generation inter-CRF recombinants identified in Honghe Prefecture of Yunnan Province. The regions of subtypes B′ and C are as indicated at the bottom. The predicted profiles of crossovers between CRF07_BC (07) and CRF08_BC (08) deduced from bootscanning plots (Fig. 2 and 7), exploratory tree analyses (Fig. 3; Table 1), and informative-site analyses (Tables 2 and 3) are superimposed on the subtype structures of plausible inter-CRF recombinants. (A) CRF07_BC (97CNXJ001); (B) CRF08_BC (97CNGX6F); (C) 00CN-HH069.1; (D) 00CN-HH086.1; (E) 00CN-HH003; (F) 00CN-HH004; (G) 00CN-HH029. The dotted lines in the segments of CRF08_BC in the 3′ half of the genome, corresponding to *env* open reading frames, are the areas that are approximately equidistant genetically to both CRF07_BC and CRF08_BC but are distantly related to other subtype C strains. (08) in panel D indicates the area that was detected and supported by both bootscanning plots (Fig. 2) and exploratory tree analysis (Fig. 3) but was not supported by informative-site analysis (Table 2). The subtype and CRF assignments of LTR regions were based on the data presented in Fig. 4. Positions of identical recombination breakpoints between CRF07_BC and CRF08_BC, which were predicted from informative-site analyses (boldface numbers in Tables 2 and 3), are marked with arrowheads with different shadings.

in 00CN-HH086 was detected and supported by both bootscanning plots (Fig. 2) and exploratory tree analysis (Fig. 3) but was not supported by informative-site analysis (Table 2), probably because the segment was too short (100 bp [see above]) to verify the recombination breakpoint with statistical significance. Taken together, all lines of evidence support the notion that the most of the genomes of these two strains were likely to be generated by recombination between CRF07_BC and CRF08_BC circulating in China.

Since the subtype assignment of the long terminal repeat (LTR) was not clear from the recombination identification programs described above, we constructed a separate phylogenetic tree for the LTR regions (Fig. 4A) and examined possible sequence signatures in enhancer-promoter regions of LTRs (Fig. 4B). The neighbor-joining tree analysis revealed that 3′ LTRs of both 00CN-HH069.1 and 00CN-HH086.1 belonged to subtype C and formed a cluster with CRF08_BC reference strains with modest level of bootstrap confidence (60%) (Fig. 4A). Moreover, the nucleotide sequence alignment of enhancer-promoter regions in LTRs suggested that the overall configurations of the LTRs of 00CN-HH069.1 and 00CN-HH086.1 were related more closely to that of CRF08_BC by the following two criteria. (i) The spacer sequences between two distal NF-κB sites (NF-κB II and NF-κB III) of these two strains were comprised of two nucleotides (5′-GC-3′) that appear to be specific to CRF08_BC strains known to date (four of four). In contrast, three or four spacer nucleotides are commonly observed in subtype C and CRF07_BC strains (mostly GCC or GCT for subtype C strains and GCG for CRF07_BC). (ii) All known CRF07_BC strains (four of four) harbor the unique nucleotide substitution at the 3′ end of the proximal NF-κB motif (NF-κB I), being GGGGCGTTCT or GGGGTGTTCT

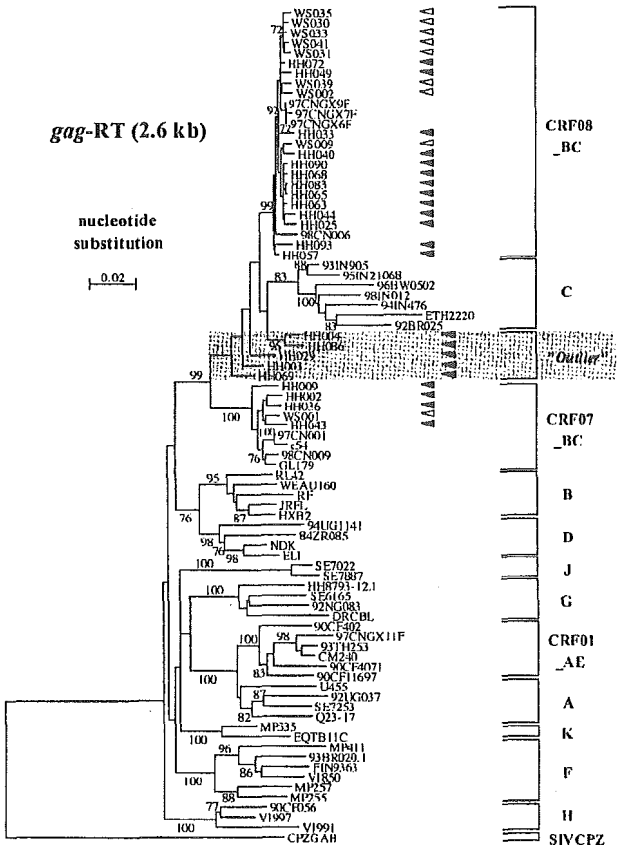


FIG. 6. Neighbor-joining tree analysis based on the nucleotide sequences of 2.6-kb *gag*-RT regions of HIV-1 samples with outlier genotypes from Yunnan Province. The HIV-1 samples with outlier genotypes (shaded) were placed between the clusters of CRF07_BC and CRF08_BC. 00CN-HH069.1 and 00CN-HH086.1 are boxed. The arrowheads indicate the HIV-1 samples analyzed in the present study: solid arrowheads, HIV-1 samples from Honghe Prefecture; open arrowheads, HIV-1 samples from Wenshan Prefecture.

instead of GGGGCGTTCC of the subtype C consensus. In contrast, 00CN-HH069.1 and 00CN-HH086.1 had the proximal NF-κB motif (5′-GGGGCGCCTT-3′) specifically found in most subtype C strains and all known CRF08_BC (four of four) strains (Fig. 4B).

As deduced from bootscanning plots (Fig. 2E and F) and exploratory tree (Fig. 3 and 4A; Table 1) and informative-site (Table 2) analyses, the overall subtype structures and the plausible profiles of crossovers between CRF07_BC and CRF08_BC in these two strains are schematically illustrated in Fig. 5, in comparison with the subtype structures of the prototype strains of CRF07_BC and CRF08_BC (Fig. 5A and B). As seen in Fig. 5C and D, 00CN-HH069.1 and 00CN-HH086.1 showed almost identical profiles of recombination, suggesting that they were of closely related origin, if not identical. One of the major structural differences between these two strains was that the putative recombination breakpoint between CRF08_BC and CRF07_BC in the 5′ part of the *pol* region was shifted by approximately 500 bp to the 5′ direction in 00CN-HH069.1 (Table 2), and thereby a small segment of subtype B′ derived from CRF07_BC was observed in 00CN-HH069.1 (Fig. 2C and 5C).

It is tempting to speculate that these two novel inter-CRF recombinants may belong to a new but as-yet-undefined class of CRF. We searched for a similar category of recombinants among a total of 71 specimens from Yunnan, including 57 specimens from eastern Yunnan Province (Honghe and Wenshan Prefectures) and 14 specimens from western Yunnan Province (Dehong Prefecture). We found three additional candidates with outlier genotypes (00CN-HH003, 00CN-HH004, and 00CN-HH029) by neighbor-joining tree analysis based on 2.6-kb *gag*-RT regions (Fig. 6): 00CN-HH003, from a 29-year-old female IDU with a 1 2/3-year history of injecting drug use; 00CN-HH004, from a 19-year-old female IDU with a 1-year history of injecting drug use; and 00CN-HH029, from an 18-year-old male IDU with a 2/3-year history of injecting drug use. The bootscanning plot (Fig. 7) and informative-site analyses (Table 3) based on the nucleotide sequences of 2.6-kb *gag*-RT regions revealed that these three additional HIV-1 samples with outlier genotypes (00CN-HH003, 00CN-HH004, and 00CN-HH029) were indeed comprised of the segments derived from CRF07_BC and CRF08_BC, similar to 00CN-HH069.1 and 00CN-HH086.1. Of note, 00CN-HH086.1 and 00CN-HH004 showed identical profiles of recombination in *gag*-RT regions (Fig. 7D and 7F and Fig. 5). Their precise recombination breakpoints defined by informative-site analysis were essentially the same (Table 3). They formed a monophyletic cluster with a bootstrap confidence of 96% in the neighbor-joining tree shown in Fig. 6. 00CN-HH086.1 and 00CN-HH004 are thus likely to be identical forms of recombinants, although the near-full-length nucleotide sequence of 00CN-HH004 is not available at this time. Similarly, some of the same recombination breakpoints were shared between 00CN-HH069.1 and 00CN-HH003 (Table 3; Fig. 7C and E and 5C and E), suggesting that they were evolved from a common progenitor strain comprised of CRF07_BC and CRF08_BC. The subtype structure and the plausible profiles of crossovers between CRF07_BC and CRF08_BC based on partial nucleotide sequences of these three specimens with outlier genotypes are summarized in the lower panel of Fig. 5.

From these lines of evidences we conclude that these five HIV-1 strains from Yunnan Province were new "second-generation" inter-CRF recombinants comprised of two previously established CRFs (CRF07_BC and CRF08_BC) circulating in China. These five HIV-1 samples were all from Honghe Prefecture (5 of 44; 11.4%), while none was detected in Wenshan (0 of 13) (far east) and Dehong (0 of 14) (west) Prefectures, suggesting that these new recombinants appears to spread focally among IDUs in Honghe Prefecture of Yunnan Province. Although further investigation is needed, we predict that some forms of the recombinants of this category may constitute a new but yet-undefined class of CRF, as represented by 00CN-HH086.1 and 00CN-HH004.

The inherent ability of the retroviral replication machinery to switch template leads to the generation of diverse forms of recombinants, and such recombination between heterologous RNA molecules may conceivably generate strains with novel qualities (4). It is believed that nonrecombinant forms of HIV-1 subtype C had been introduced into Yunnan Province

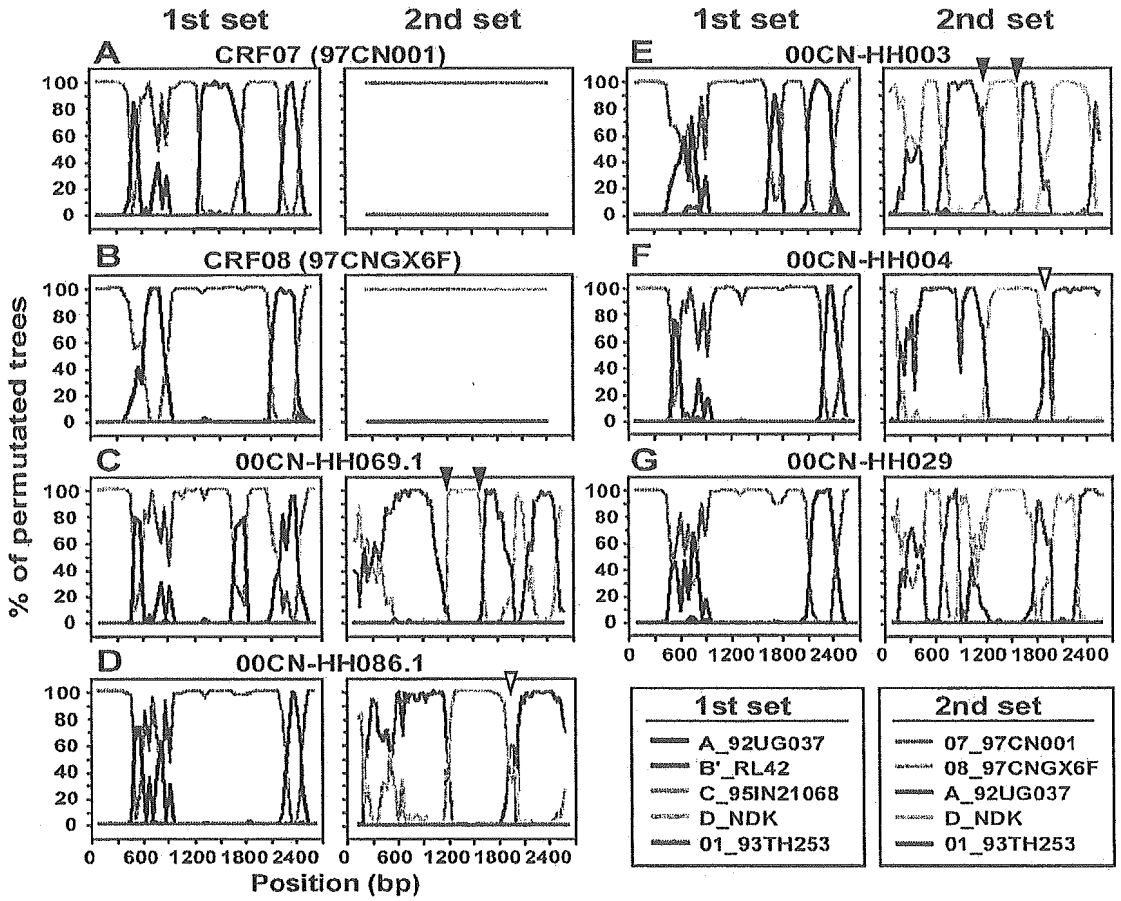


FIG. 7. Bootscanning analyses based on the nucleotide sequences of 2.6-kb *gag*-RT regions of HIV-1 samples with outlier genotypes. Analyses were performed as described for Fig. 2. The first sets of the bootscanning plots, depicting the relationship to the representative strains of HIV-1 subtypes (A, B', C, and D) and CRF01_AE, are shown at the left of each panel. The second sets of bootscanning plots, depicting the relationship to the reference strains for CRF07_BC (97CN001) and CRF08_BC (97CNGX6F) with the respective representatives of HIV-1 subtypes A and D and CRF01_AE, are shown at the right of each panel. (A) 97CN001 (CRF07_BC); (B) 97CNGX6F (CRF08_BC); (C) 00CN-HH069.1; (D) 00CN-HH086.1; (E) 00CN-HH003; (F) 00CN-HH004; (G) 00CN-HH029. The bootstrap values are plotted for a window of 200 bp moving in increments of 30 bp along the alignment. Reference strains used for two different sets of bootscanning plots are shown in the boxes at the bottom right: A_92UG037, B'_RL42, C_95IN21068, D_NDK, 01_93TH253, 07_97CN001, and 08_97CNGX6F. Positions of identical recombination breakpoints between CRF07_BC and CRF08_BC, which were predicted from informative-site analyses (boldface numbers in Tables 2 and 3), are marked with vertical arrowheads with different shadings.

by the early the 1990s (11). However, the nonrecombinant form of HIV-1 subtype C was not detected among samples from IDUs in Yunnan in 2000 and 2001, apparently having being replaced by newly emerged recombinants (24). It is thus tempting to speculate that this drastic shift of strains was not caused by chance but may reflect a yet-undefined selective advantage(s) of recombinants over the preexisting parental strains. Recombination events might provide an opportunity to yield more fitness (e.g., more efficient transmission) compared to that of the parental viruses. The regions in southwestern China (24) and northern Myanmar (14) where new recombinants appear to be arising continually might provide unique opportunities to investigate the natural history and biological consequences of HIV-1 recombination. The present study also suggests that the mixing of different lineages of HIV-1 strains in highly exposed populations and in the social networks known in southwestern China could quickly lead to the evolution of new forms of recombinants and even second-generation recombinants between the previously established first-generation CRFs.

The emergence of such new generations of recombinants could further complicate the development of effective vaccines to limit HIV-1 spread. HIV vaccine efficacy, once established, might be quickly challenged by novel recombinant strains acquiring new sets of escape mutations in viral epitopes by shuffling different parts of HIV-1 genomes. The present study may provide insight into the genesis of the HIV-1 epidemic in China and implications for future vaccine strategies.

Nucleotide sequence accession numbers. The near-full-length nucleotide sequences of 00CN-HH069.1 and 00CN-HH086.1 and the nucleotide sequences of the 2.6-kb *gag*-RT regions of 00CN-HH003, 00CN-HH004, and 00CN-HH029 are available under GenBank accession numbers AP005206, AP005207, AB090997, AB090998, and AB090999, respectively.

TABLE 3. Summary of informative-site analysis based on the nucleotide sequences of 2.6-kb *gag*-RT regions of HIV-1 samples with outlier genotypes from eastern Yunnan Province[a]

Strain	Region[b]	Genotype	No. of informative sites in:			P[c]
			CRF07	CRF08	Outgroup	
00CN-HH069.1	790–1904	CRF07	15	5	4	
						<0.0001
	2010–2357	CRF08	0	17	1	
						<0.0001
	2372–3362	CRF07	16	6	0	
00CN-HH003	790–1524	CRF08	4	13	1	
						0.0005
	1542–1997	CRF07	8	0	1	
						<0.0001
	2010–2357	CRF08	0	16	1	
						<0.0001
	2461–2637	CRF07	8	0	0	
						0.0008
	2675–3405	CRF08	3	9	3	
00CN-HH086.1	790–1912	CRF07	15	5	3	
						<0.0001
	2022–**2678**	CRF08	1	27	2	
						<0.0001
	2867–3365	CRF07	12	0	0	
00CN-HH004	790–1912	CRF07	16	2	5	
						<0.0001
	2073–**2678**	CRF08	2	26	0	
						<0.0001
	2867–3365	CRF07	13	0	0	
00CN-HH029	790–1041	CRF08	0	3	1	
						0.06
	1058–1305	CRF07	5	1	1	
						0.008
	1306–1443	CRF08	0	6	0	
						0.007
	1456–1590	CRF07	5	1	2	
						<0.0001
	1755–2435	CRF08	1	23	0	
						0.0002
	2517–2636	CRF07	3	0	1	
						0.003
	2678–2978	CRF08	0	7	0	
						<0.0001
	3107–3406	CRF07	9	0	0	

[a] To determine the recombination breakpoints, each putative recombinant sequence was compared with two predicted parental sequences (CRF07_97CN001 and CRF08_97CN6F) and an outgroup (A_92UG037) as described for Table 2.
[b] Nucleotide positions are as numbered by the HXB2 numbering engine. The nucleotide positions of the identical pairs of recombination breakpoints are in boldface.
[c] *P* values for the resultant divisions of sites were calculated by using Fisher's exact test.

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functioning, role functioning, and health transition showed statistically significant differences ($P < 0.05$) at baseline. The mean MOS-HIV scores for the 210 non-TI patients did not show significant trends over 96 weeks, and remained essentially stable.

For patients experiencing a TI, mean HRQOL scores decreased in nine out of 11 dimensions, with five dimensions declining significantly. When HAART treatment was re-initiated, the mean HRQOL scores increased in seven dimensions but decreased in four dimensions. In only one dimension, health transition, did the mean scores reach or exceed the pre-TI scores.

The reason for a TI may significantly impact on HRQOL. Patients starting a TI as a result of viral failure showed significant decreases in HRQOL scores during the TI but rebounded to near pre-TI levels after re-initiating therapy. For patients stopping treatment because of adverse effects HRQOL scores showed more variability. When these patients re-initiated treatment, HRQOL declined for most dimensions, indicating that there was a negative impact on their perception of health. HRQOL scores for patients choosing to stop treatment increased during the TI and decreased when therapy was re-initiated. Small sample sizes preclude overinterpretation of the data; however, the trends seen here indicate important differences in HRQOL between these subsets when the reason for the TI is taken into consideration. Our on-going study continues to analyse these differences.

TI are indicated for a variety of reasons. Whereas the virological, immunological, economic, and clinical merits of a TI remain debatable, our study has shown that a TI does not necessarily increase HRQOL, but we did see an improvement in HRQOL on restarting therapy.

Overall, we found that TI negatively affects HRQOL both during and after a TI. The findings of this study suggest that healthcare providers and patients should view a TI as a medical decision and not as a means to increase HRQOL.

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Different subtype distributions in two cities in Myanmar: evidence for independent clusters of HIV-1 transmission

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A molecular epidemiological investigation was

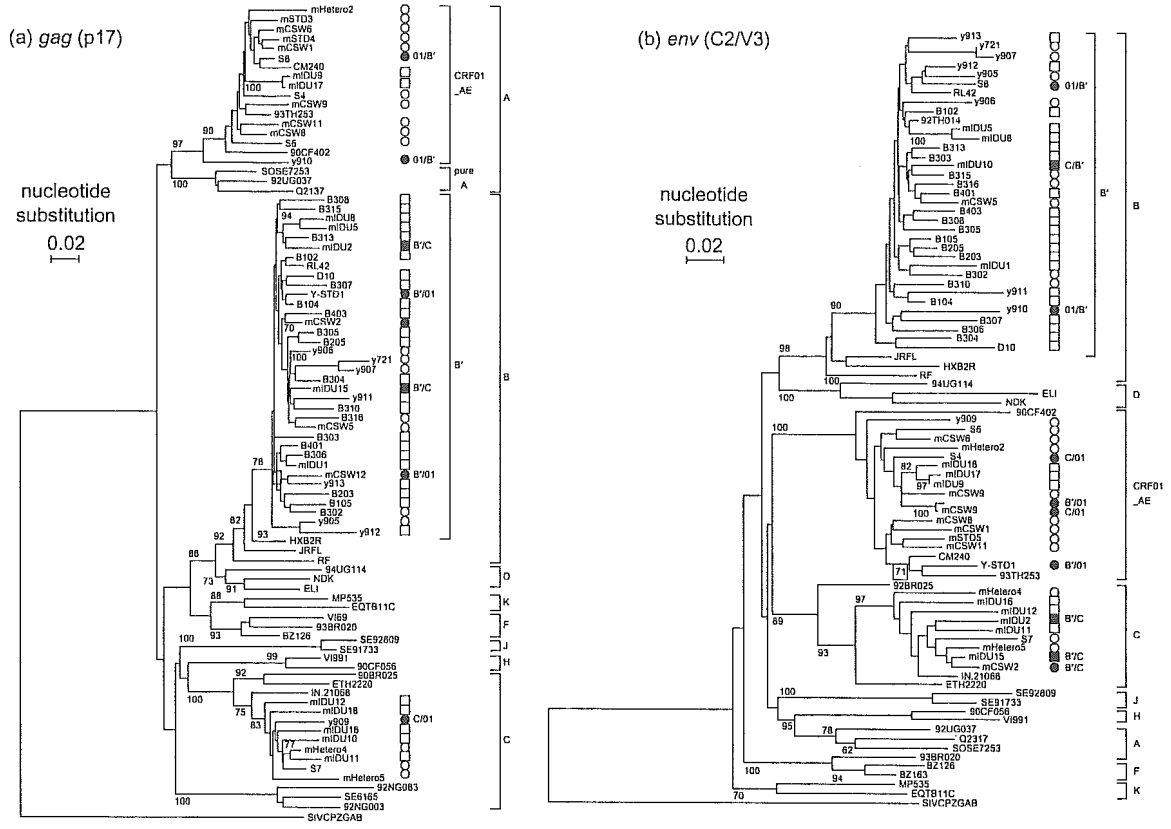

Fig. 1. Substantial portions of HIV-1 samples from two major cities in Myanmar showed subtype discordance in different regions in the HIV-1 genome. Neighbor-joining tree analysis based on the nucleotide sequences of the (a) *gag* (p17) and (b) *env* (C2/V3) regions with HIV-1 group M (subtype A–D, F–H, J, K) and CRF01_AE reference sequences. $SIV_{CPZ}GAB$ was used as an outgroup. Bootstrap values (> 80) are shown at the corresponding nodes. Subtypes and CRF are indicated at the right side of trees. The specimens from Myanmar are marked with squares (injecting drug users) and circles (individuals presumably infected sexually). Closed symbols indicate the specimens that show the discordance between the *gag* (p17) and *env* (C2/V3) subtypes. The subtype assignment of the *gag* (p17) and *env* (C2/V3) regions are shown after each closed symbol: for example, B'/01 indicates subtype B' in the *gag* (p17) region and CRF01_AE in the *env* (C2/V3) region.

conducted in two major cities in Myanmar (Yangon and Mandalay). The study revealed a unique predominance of HIV-1 subtype B' (Thailand variant of subtype B) among injecting drug users in Yangon, indicating the strong founder effect of this variant. In contrast, multiple lineages of HIV-1 strains were found in Mandalay, leading to the evolution of various forms of intersubtype recombinants. The results showed independent clusters of HIV-1 transmission in Myanmar.

The face of the HIV/AIDS epidemic in Myanmar is changing rapidly. As of June 2000, a total of 29 636 HIV cases with 4063 AIDS cases had been reported. Approximately 30% of officially reported HIV cases are attributed to injecting drug use and 68% to heterosexual transmission. The 1999 UNAIDS estimates indicated approximately 530 000 HIV cases in Myanmar, the second largest number in Southeast Asia, after only Thailand. The initial HIV-1 outbreak in Myanmar began among injecting drug users (IDU) in 1989, and the prevalence among IDU reached 54% by 1999. More recently, sentinel surveillance data [1] indicated an increase in prevalence among female commercial sex workers in two major cities from 4% in 1992 to 57% in Mandalay in September 1999.

In the present study, we investigated the molecular epidemiology of HIV among IDU and individuals at risk of sexual exposure in two major cities, Yangon (central) and Mandalay (north central), to explore the networks of HIV spread in Myanmar. A total of 58 specimens were collected from consenting IDU and individuals presumably infected sexually during 1995–2002. For the screening of the HIV-1 genotype, the nucleotide sequences of the *gag* (p17) and *env* (C2/V3) regions were determined from plasma RNA, and phylogenetic tree analyses were performed (Fig. 1) [2].