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The Rapid Spread of Recombinants during a Natural In Vitro Infection with Two Human Immunodeficiency Virus Type 1 Strains

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We quantified a population of recombinants in a natural in vitro infection, using wild-type viruses without any pressure. It was found that recombinants emerged early after infection and constituted more than 20% of the whole proviral population 15 days after infection. Furthermore, recombinants were isolated as infectious viruses by simple limiting dilution. These results imply that, in addition to the high mutation rate of human immunodeficiency virus type 1 (HIV-1), recombination among HIV-1 strains plays a significant part in the development of the high diversity of HIV-1.

Many human immunodeficiency virus type 1 (HIV-1) isolates are known to be recombinants of viruses of different subtypes (2, 4, 8, 17, 19, 25), and recombination in a single patient has also been demonstrated (5, 6, 9, 11, 29). In contrast to these in vivo studies, in vitro studies have focused on the mechanism of recombination (7, 13, 23, 27) but not on the populational analysis of recombinants. Although the rapid emergence of recombinants under drug pressure has been reported (10, 15, 21), recombination in a natural in vitro infection has not yet been investigated. Thus, using PCR analyses, we quantified a population of recombinants in a natural in vitro infection, using wild-type viruses without any pressure, such as the use of drugs and antibodies. Two HIV-1 molecular clones, NL432 (1) and HAN2 (26), which belong to clade B and have 86% homology in the *env* gene, were used to simultaneously infect human T-cell line M8166 (3). Virus stocks of NL432 and HAN2 were prepared by transfection of M8166 cells, and the 50% tissue culture infective dose ($TCID_{50}$) was determined as described previously (16). M8166 cells (4×10^5) were infected with 4×10^4 $TCID_{50}$s of NL432 or HAN2 or were infected simultaneously with a mixture containing 2×10^4 $TCID_{50}$s each of NL432 and HAN2 (NL432/HAN2). After 24 h, cells were washed with phosphate-buffered saline and cultured in RPMI medium containing 10% fetal calf serum. Half of the medium was changed every 2 days, and 1×10^5 to 2×10^5 cells were newly added to cultures after day 3 or 5 because cultures showed severe syncytium formation. Recombinants between the two strains were then detected by the following PCR method. Two primer pairs that amplify the region from a sequence encoding a signal peptide to V5 of the *env* gene, about 1.4 kbp, were designed (Fig. 1). One is specific to NL432 and the other is specific to HAN2. The primers specific to NL432 were LAP1 (5′-CAGCACTTGTGGAGATGGGGGT GGAAA-3′, nucleotides [nt] 6242 to 6268 in NL432) and LAP2 (5′-GAAGATCTCGGACCCATTGTTGT-3′, nt 7618 to 7596 in NL432). The primers specific to HAN2 were HAP1 (5′-GAAAGTGAAGGAGACCAGGAAGAATTA-3′, nt 5898 to 5924 in HAN2) and HAP2 (5′-GATCTCGGTGGTAC TACTGTTATC-3′, nt 7290 to 7267 in HAN2). Proviral DNAs were amplified by PCR from DNA samples which were prepared by lysis of cells by proteinase K as described previously (22). Each DNA sample (0.2 μg) was amplified in a final volume of 50 μl by 30 cycles at 94°C for 1 min, 60°C for 2 min, and 72°C for 3 min.

Proviruses of NL432 and HAN2 were specifically detected in M8166 cells infected with NL432 and HAN2, respectively (Fig. 2A). From the culture infected with both NL432 and HAN2, PCR bands were detected with all the homologous and heterologous primer pairs (Fig. 2B). The PCR bands amplified by LAP1-HAP2 and HAP1-LAP2 showed an emergence of recombinants in this culture. No artificial product during PCR (20) was amplified in this system, because no PCR band was detected from the mixed DNA sample of NL432 and HAN2 (Fig. 2B, NL432, HAN2-mixed). Recombinants were detected from 3 days postinfection (dpi), when the first sample was collected (Table 1). In addition, when peripheral blood mononuclear cells from healthy humans were similarly infected with the viruses, recombinants were also detected from the culture at 7 and 13 dpi (data not shown). Thus, recombinants emerged early after infection with both NL432 and HAN2 in in vitro cultures.

To quantify the population of recombinants in the whole viral population, the amounts of proviruses of NL432, HAN2, and two types of recombinants were determined by the quantitative competitive PCR (QC-PCR) method (Table 1). In the QC-PCR method for proviral quantification, competitive DNA templates with a deletion were constructed for each virus. The competitors for NL432 and HAN2 were constructed by deleting an *Eco*T22I fragment (nt 6530 to 6738 in NL432) and a *Bgl*II fragment (nt 6287 to 6512 in HAN2), respectively. The competitors for the N-H recombinant (which has the 5′ portion of NL432 and the 3′ portion of HAN2 in the amplified region) and the H-N recombinant (which has the 5′ portion of HAN2 and the 3′ portion of NL432 in the amplified region) were constructed by ligating two parental plasmids using an *Stu*I site of NL432 (nt 6822) and a *Hin*cII site of HAN2 (nt 6460), a *Hin*cII site of HAN2 (nt 6460), and an *Nhe*I site of NL432 (nt 7250). QC-PCR was carried out with these competitors as described previously (14) by using [α-^{32}P]dTTP (400 Ci/mmol; Amersham Corp.). The amount of target DNA was determined from the equivalent point of the competitor/target ratio.

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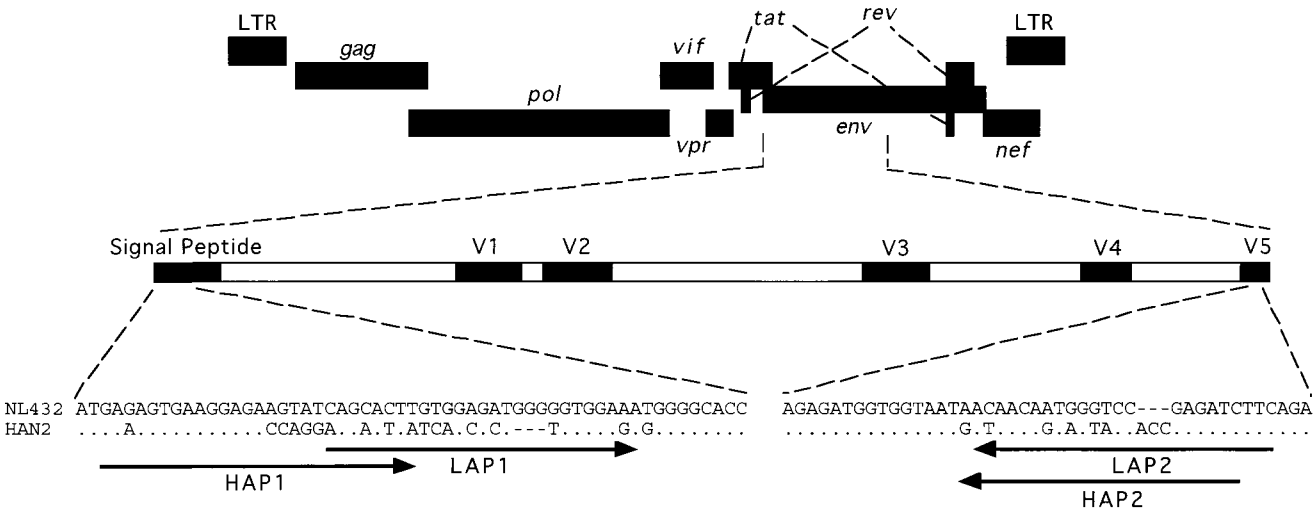

FIG. 1. Schematic representation of the region in which recombination was analyzed by PCR. Sequences to which each primer binds are at the bottom. LTR, long terminal repeat.

There was a large population of NL432 at 3 dpi, because NL432 replicated faster than HAN2 in M8166 cells (data not shown). Though N-H recombinants, which were detected by LAP1 and HAP2, were detected 6 to 10 times more frequently than H-N recombinants, which were detected by HAP1 and LAP2, the total number of recombinants accounted for 0.94% of the population at 3 dpi, 11.7% at 7 dpi, and 22.7% at 15 dpi. This frequency of recombinants in the population, 22.7% in a segment of only 1.4 kbp, is remarkably high. In addition, the spread of recombinants was very rapid. The population of recombinants increased 12.5 times from 3 to 7 dpi and about 1.9 times from 7 to 15 dpi. It was unclear whether this rapid spread of recombinants resulted from continuous production of new recombinants or from an efficient replication of the dominant recombinants. However, a sequencing analysis of several recombinants at 15 dpi suggested that there were no dominant viruses with the same recombination sites (data not shown).

In addition to the detection and the quantification of recombinants by PCR, recombinants were also investigated in the population of infectious viruses. Viruses were biologically cloned from the supernatant of the culture at 13 dpi by the limiting-dilution method. A total of 30 viruses were cloned and were identified as NL432, HAN2, or recombinants by PCR analysis (Table 2). Although seven of the samples had multiple viruses, 23 clones could be identified as a single virus. Of these 23 clones, 7 were recombinant viruses. The frequency of recombinants in the infectious clones almost coincided with the results of the QC-PCR analysis, though the number of viral clones was too small to correctly determine the frequency of recombinants. However, this result demonstrated that there was a large population of recombinants not only in proviruses but also in infectious viruses.

Previous studies have investigated recombination in detail using several retroviruses (12, 13, 27), including HIV-1 (7, 23). These studies showed that retroviruses are highly recombinogenic (12) and that recombination occurs during DNA synthesis by reverse transcriptase (7, 23, 27). However, these results were based on artificial systems that used mutant viruses and selection methods that involved drugs or antibodies. The de-

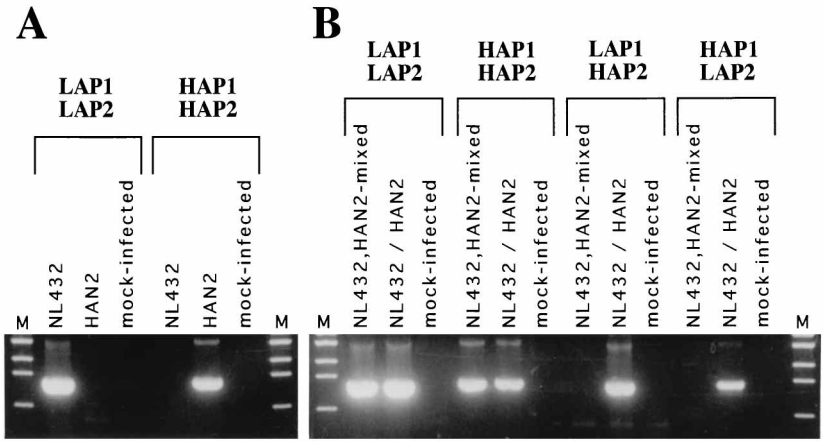

FIG. 2. Detection of recombinants by PCR with primer pairs specific to each virus. DNA samples were prepared from cultures infected with NL432, HAN2, and both NL432 and HAN2 (NL432/HAN2). DNA samples in which equal amounts of NL432 and HAN2 were mixed (NL432, HAN2-mixed) and normal M8166 cells (mock-infected) were analyzed as controls. Lanes M contain a size marker, λ DNA digested with *Eco*T14I.

TABLE 1. Copy numbers of proviruses in M8166 cells infected with both NL432 and HAN2[a]

Virus	Primer pair	Copy no. (% of population) at:		
		3 days	7 days	15 days
NL432	LAP1-LAP2	7.6×10^5 (93.4)	5.8×10^6 (68.3)	3.6×10^6 (50.6)
HAN2	HAP1-HAP2	4.6×10^4 (5.65)	1.7×10^6 (20.0)	1.9×10^6 (26.7)
N-H recombinant	LAP1-HAP2	6.7×10^3 (0.82)	9.1×10^5 (10.7)	1.4×10^6 (19.7)
H-N recombinant	HAP1-LAP2	9.7×10^2 (0.12)	8.8×10^4 (1.04)	2.1×10^5 (2.95)

[a] QC-PCR was performed for each proviral quantification.

tection and quantification of recombinants in a natural in vitro infection, which would not involve the above-mentioned methods, have not been done. For lack of in vitro estimation, it was difficult to estimate the contribution of recombination in the in vivo diversity of HIV-1. We analyzed a population of recombinants in a natural in vitro infection, which was done with wild-type viruses without any selective pressure. The high rate of recombination in vitro demonstrated in this study suggests that a region in the *env* gene may be frequently exchanged among HIV-1 strains during an in vivo infection. Various mutants that newly occurred may spread not only by replicating themselves but also by hybridizing with the other viruses. An experimental condition, like those in this in vitro infection, which was performed with a high multiplicity of infection, may be central to the likelihood of recombination. Recombination may occur in vivo in tissues such as the lymph nodes and spleen, whose viral burden is higher than those of other tissues. The high rate of mutation (18, 24) and the recombination which follows may generate quasispecies, a mixture of closely related viruses, in an infected individual. In a patient infected with multiple HIV-1 strains, recombination can produce more diverse viruses than can mutation. Since coinfections are not rare (28, 29), recombination among distinct viruses (6, 11, 29) is considered important for the emergence of diverse HIV-1 variants. Coinfection with multiple HIV-1 strains which belong to different subtypes may result in the emergence of recombinants among different subtypes (17, 19, 25). Since this study was limited to recombination between viruses which belong to the same clade, further studies are needed to analyze recombinants among different subtypes. Genotype exchange might result in the exchange of phenotypes, such as drug resistance, antigenicity, cytopathicity, and various replicative capacities. The rapid emergence of variants resistant to drugs (10, 15, 21) by recombination in vitro is apparent from the rapid emergence of recombinants shown in this study. Similarly, variants resistant to neutralization by antibodies may emerge rapidly by recombination. The rapid spread of recombinants in vitro may have important implications for the design of therapeutic and vaccine strategies.

TABLE 2. Viruses cloned by limiting dilution[a]

Virus(es)	Detection by primer pair				No. of clones (% of population)
	LAP1-LAP2	HAP1-HAP2	LAP1-HAP2	HAP1-LAP2	
NL432	+	−	−	−	14 (60.9)
HAN2	−	+	−	−	2 (8.7)
N-H recombinant	−	−	+	−	3 (13.0)
H-N recombinant	−	−	−	+	4 (17.4)
Multiple viruses	+	−	+	−	2
	+	+	+	+	5

[a] Infection was performed with 0.1 $TCID_{50}$ per well in 96-well plates. M8166 cells were infected with NL432 and HAN2, and the supernatant at 13 dpi was used for limiting dilution.

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