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High Rates of Human Immunodeficiency Virus Type 1 Recombination: Near-Random Segregation of Markers One Kilobase Apart in One Round of Viral Replication

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One of the genetic consequences of packaging two copies of full-length viral RNA into a single retroviral virion is frequent recombination during reverse transcription. Many of the currently circulating strains of human immunodeficiency virus type 1 (HIV-1) are recombinants. Recombination can also accelerate the generation of multidrug-resistant HIV-1 and therefore presents challenges to effective antiviral therapy. In this study, we determined that HIV-1 recombination rates with markers 1.0, 1.3, and 1.9 kb apart were 42.4, 50.4, and 47.4% in one round of viral replication. Because the predicted recombination rate of two unlinked markers is 50%, we conclude that markers 1 kb apart segregated in a manner similar to that for two unlinked markers in one round of retroviral replication. These recombination rates are exceedingly high even among retroviruses. Recombination rates of markers separated by 1 kb are 4 and 4.7% in one round of spleen necrosis virus and murine leukemia virus replication, respectively. Therefore, HIV-1 recombination can be 10-fold higher than that of other retroviruses. Recombination can be observed only in the proviruses derived from heterozygous virions that contain two genotypically different RNAs. The high rates of HIV-1 recombination observed in our studies also indicate that heterozygous virions are formed efficiently during HIV-1 replication and most HIV-1 virions are capable of undergoing recombination. Our results demonstrate that recombination is an effective mechanism to break the genetic linkage between neighboring sequences, thereby reassorting the HIV-1 genome and increasing the diversity in the viral population.

Retroviruses package two copies of RNA into one virion, with each copy containing all the genetic information needed for viral replication (16, 29). Although two copies of RNA are packaged, generally only one provirus is generated from each infectious event; therefore, retroviruses are considered pseudodiploid and not diploid (21). Retroviruses have been shown to undergo frequent recombination. One of the prerequisites for recombination is the formation of heterozygous virions, which contain two copies of RNA with different genetic information (21, 53). Recombination occurs during reverse transcription when portions of genetic information from both packaged RNA copies are used to generate a hybrid DNA copy (7, 52). It is difficult to identify genetic recombination events in the progeny from homozygous virions; these virions package two identical copies of RNA, and therefore, the recombinants will have the same genotype as the parental viruses.

Previously, we determined that the recombination rates of spleen necrosis virus (SNV) and murine leukemia virus (MLV) in one round of viral replication are 4 and 4.7%, respectively, with two markers 1 kb apart (1, 21). In MLV, the recombination rates increase to 5.0 and 7.4% with markers 1.3 and 1.9 kb apart, respectively (1, 2). However, when markers are separated by 7.1 kb, the recombination rate is 8.2%, which is not significantly different from the 7.4% rate observed for markers separated by 1.9 kb (1).

Recombination can reassort mutations in the viral genome to increase the diversity of the viral population, which can improve the probability of the survival of the viral population in a changing environment (7, 52). The selective advantage of having frequent recombination events is clearly illustrated in human immunodeficiency virus type 1 (HIV-1), which causes AIDS. It was estimated that significant portions of the currently circulating strains of HIV-1 are recombinants (9, 26, 45, 46). Recombinant strains of HIV-1 have become dominant in the AIDS epidemic in certain geographical regions; for example, the A/E recombinant causes many of the HIV-1 infections in Thailand (18, 35). HIV-1 recombination has also been directly observed in infected patients (15, 34, 47, 56).

Much research effort has been devoted to understanding HIV-1 recombination. Recombination has been observed during reverse transcription in vitro using purified nucleic acids and proteins (3, 12–14, 38, 39). Frequent HIV-1 recombination events have also been observed in cell culture systems (6, 22, 30, 42, 50). More recently, the frequency of HIV-1 recombination was estimated by studying recombination between two similar strains of HIV-1 and mapping the crossovers by using a heteroduplex-tracking assay (23, 57). These studies revealed that within limited cycles of replication, many HIV-1 genomes

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contained more than one recombination event; an average of two to three crossovers per genome was estimated. These studies are highly informative; however, direct comparisons among the HIV-1, SNV, and MLV recombination rates are difficult because of the differences in experimental protocols and strategies used to calculate the recombination frequencies.

In this report, we measured HIV-1 recombination rates in one round of viral replication. We found that HIV-1 recombines at an exceedingly high frequency even when compared with other retroviruses, such as MLV and SNV. These comparisons were made possible by using the same target sequences measured in the MLV recombination studies.

MATERIALS AND METHODS

Nomenclature and plasmid construction. The names of all plasmids used in this study begin with p, but the names of the viruses derived from these plasmids do not; for example, pTR-HyIN refers to a plasmid and TR-HyIN refers to the virus or provirus derived from this plasmid. Plasmids were constructed using standard molecular cloning techniques (48). Plasmid pTR-HyIN was constructed from pJS30 (1) and pKD-HIV(PIN), which was a generous gift from Vinay K. Pathak and a derivative of pHR′-CMVLacZ (37). Plasmids pKD-HIV(PIN) and pJS30 were digested with *Bam*HI and *Bsr*GI, respectively, treated with the Klenow fragment of *Escherichia coli* DNA polymerase I (Klenow) to fill in the 3′ ends of DNA fragments, and then digested with *Bcl*I. The 1.8-kb DNA fragment derived from pJS30 containing hygromycin phosphotransferase B gene (*hygro*) (19) and internal ribosomal entry site (IRES) from encephalomyocarditis virus was ligated to the backbone from pKD-HIV(PIN) to generate pTR-HyIN, which contains *hygro* and the neomycin phosphotransferase gene (*neo*) (24). To generate pTR-HyS2NIN, pTR-HyIN was digested with *Sac*II, treated with T4 DNA polymerase to remove the protruding 3′ termini, and self ligated. This treatment generated an inactivating frameshift mutation in *hygro*, destroyed the *Sac*II site, and generated an *Ngo*MIV site. To generate pTR-HyN2MIN, pTR-HyIN was partially digested with *Nde*I, and then a linker (5′-TATGACGCGTCA-3′) was inserted; these treatments generated a 10-bp addition to the sequence, which resulted in an inactivating frameshift mutation in *hygro* and an additional *Mlu*I site. To generate pTR-HyINE2B, a 0.9-kb *Msc*I-to-*Bam*HI region in pTR-HyIN was replaced by its counterpart from pJA32-1kb (1); the resulting plasmid contained the same structure as pTR-HyIN except for a 4-bp inactivating insertion in *neo* that destroyed an *Ehe*I site and added a *Bss*HII site. Plasmid pTR-HyIN was partially digested with *Nco*I, treated with Klenow enzyme, and ligated to generate pTR-HyN2NIN and pTR-HyINN2N, which contained a 4-bp insertion that inactivated *hygro* and *neo*, respectively.

Cells, transfections, and infections. Cultured cells were propagated in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum, penicillin (50 U/ml) and streptomycin (50 U/ml). Cells were maintained in a 37°C incubator with 5% CO_2.

DNA transfections were performed by the calcium phosphate method (48) using an MBS mammalian transfection kit (Stratagene). Cells were plated at a density of 4×10^6 per 100-mm-diameter dish and transfected 18 h later with a mixture of DNA. After incubating for 4 h in a 37°C incubator with 3% CO_2, the DNA mixture was removed, fresh medium was added to the cells, and the cells were transferred to a 37°C incubator with 5% CO_2. Viral supernatants were harvested 24 h later; cellular debris was removed by filtering the supernatants through a 0.45-μm-pore-size filter. Viral supernatants were either used immediately or stored at −80°C prior to infection. Helper construct pCMVΔR8.2 (36) together with pSV-A-MLV-*env* (31) or pHCMV-G (54) was transfected into cells to generate viruses. Plasmid pCMVΔR8.2 expresses all HIV-1 viral proteins needed for infection except Env. Plasmid pSV-A-MLV-*env* or pHCMV-G expresses amphotropic MLV Env or vesicular stomatitis virus G protein (VSV-G), respectively. DNA mixtures used for transfection were either at a 5:1:4 (pCMVΔR8.2/pSV-A-MLV-*env*/vector) or 2:1 (pCMVΔR8.2/pHCMV-G) ratio.

For the infection procedure, target cells were plated at a density of 10^5 per 60-mm-diameter dish 24 h prior to infection. Serial dilutions were generated from each viral stock and used for infection in the presence of Polybrene at a final concentration of 50 μg/ml. Viruses were removed 4 h later, and drug selections were carried out 24 h postinfection. Selection with hygromycin, G418, or hygromycin plus G418 was performed at 170, 609, or 170 plus 473 μg/ml, respectively.

Southern hybridization analyses. Genomic DNAs from infected cells were purified using the QIAamp DNA blood mini kit (Qiagen) or AquaPure genomic DNA isolation kit (Bio-Rad). Southern hybridization was performed following standard procedures (48). A 1.0-kb *Hin*dIII-to-*Ngo*MIV DNA fragment derived from pWH390 (1) containing the 3′ portion of the IRES and the 5′ 0.6-kb portion of *neo* was used to generate a ^{32}P-labeled probe by random priming (17) (random primed DNA labeling kit; Roche). Southern hybridization results were obtained by autoradiography or PhosphorImager analyses.

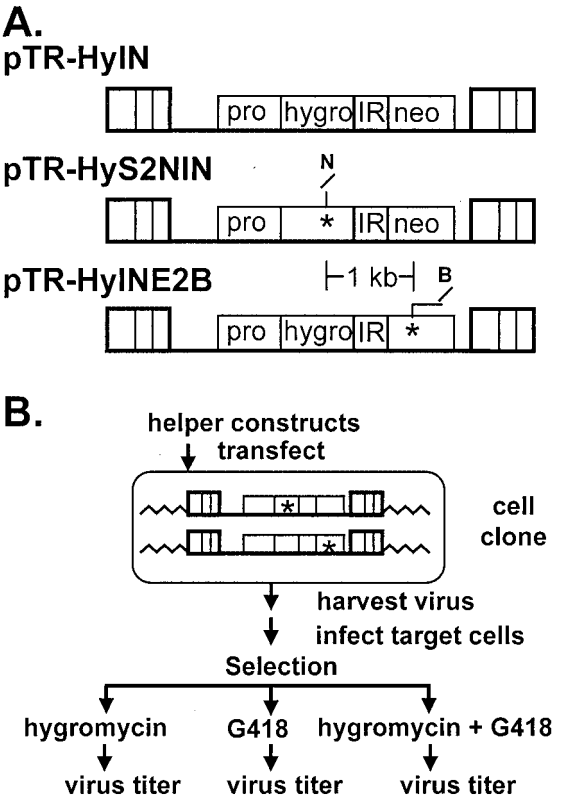

FIG. 1. Viral vectors and protocol used to measure HIV-1 recombination rates with markers 1 kb apart. (A) General structures of the vectors. pro, CMV promoter; hygro, hygromycin phosphotransferase B gene; IR, internal ribosomal entry site; neo, neomycin phosphotransferase gene; asterisk, inactivating frameshift mutation; N, *Ngo*MIV; B, *Bss*HII; S and E with slash, destroyed *Sac*II and *Ehe*I sites, respectively. (B) Protocol used to measure recombination rates.

RESULTS

Strategy used to measure HIV-1 recombination rates. The following constructs and strategy were used to measure the HIV-1 recombination rates. We constructed an HIV-1-based vector (pTR-HyIN) that expresses *hygro* and *neo*, which confer resistance to hygromycin and G418, respectively. An internal cytomegalovirus promoter directs the transcription of *hygro* and *neo*, and the translation of *neo* is enhanced by an encephalomyocarditis virus IRES (Fig. 1). Plasmid pTR-HyIN also contains all the *cis*-acting elements essential for viral genome replication; however, it does not express HIV-1-encoded viral proteins.

Two vectors were derived from pTR-HyIN, each containing an inactivating mutation in one of the drug resistance genes. The vector pTR-HyS2NIN contains an inactivating frameshift mutation in *hygro* that destroys a *Sac*II site and generates an *Ngo*MIV site, whereas pTR-HyINE2B contains an inactivating frameshift mutation in *neo* that destroys an *Ehe*I site and

generates a *Bss*HII site (Fig. 1). The distance between the *Sac*II site in *hygro* and the *Ehe*I site in *neo* is 1 kb. Because these two vectors each contain only one functional drug resistance gene, a provirus derived from pTR-HyS2NIN or pTR-HyINE2B can confer resistance only to one drug selection. However, if recombination occurs within the 1-kb distance separating the mutations, it could generate a provirus containing two functional drug resistance genes conferring resistance to double drug selection. By measuring the frequency at which recombinants with two functional genes are generated, we can calculate the recombination rate between markers 1 kb apart.

The protocol used to measure the HIV-1 recombination rate is illustrated in Fig. 1B. TR-HyS2NIN- or TR-HyINE2B-containing virions were generated separately by transfecting 293T cells with vector plasmid, pCMVΔR8.2, and pSV-a-MLV-*env*. The helper construct pCMVΔR8.2 expresses most HIV-1-encoded proteins except Env, whereas pSV-A-MLV-*env* expresses amphotropic MLV Env, which can functionally replace HIV-1 Env to generate infectious pseudotyped HIV-1 virions. These viruses were used to infect 293 cells simultaneously, and the resulting hygromycin-plus-G418 doubly resistant cell clones were selected and characterized by Southern analyses. Only cell clones containing one copy of each provirus with intact structures were selected and used for further experiments.

To measure the recombination rate, these characterized cell clones were transfected with the helper constructs pCMVΔR8.2 and pHCMV-G, which expresses VSV-G. VSV-G can be used to generate infectious pseudotyped HIV-1 virions. Twenty-four hours after transfection, viruses were harvested and serial dilutions were generated. These viral stocks were used to infect 293 target cells in triplicate; these cells were then selected with hygromycin, G418, or hygromycin plus G418. The numbers of drug-resistant cell colonies were determined, and titers of virus were calculated. Hygromycin-plus-G418-resistant cell clones were isolated, and the molecular nature of the proviruses was characterized. These data were used to calculate the frequency at which recombinants with two functional drug resistance genes were generated. This frequency was then used to calculate the recombination rate.

Comparisons of TR-HyIN-derived viral titers resulting from different drug selections. The strategy described above relied on viral titers generated from different drug selections to calculate how frequently recombinants with two functional genes were generated. Therefore, it was important to first define the relative viral titers from different drug selections. Using a protocol similar to that described above, we generated cell clones containing a single copy of TR-HyIN provirus. Viruses were generated from these cells and used to infect 293 target cells; hygromycin, G418, or hygromycin-plus-G418 viral titers were determined. A summary of viral titers generated from five different cell clones is shown in Table 1. In each of the five cell clones, viral titers generated by the three selection regimens were similar. Therefore, viral titers generated from single and double drug selections reflect the number of infection events and can be used to calculate recombination rates.

Measuring HIV-1 recombination rates with two markers 1 kb apart. Using the aforementioned protocol, we generated cell clones containing a copy of each TR-HyS2NIN and pTR-HyINE2B provirus with intact structures. These characterized cell clones were transfected with the helper constructs pCMVΔR8.2 and pHCMV-G to generate viruses; the resulting titers of virus from five cell clones are summarized in Table 2. In general, titers of virus for hygromycin selection and G418 selection within each cell clone were similar, indicating that the two parental proviruses were expressed at similar levels; in addition, the titers of virus for hygromycin-plus-G418 selection were approximately 20% of the viral titers for single-drug resistance. Each of the parental viruses can confer only single-drug resistance; a doubly resistant cell can be generated by the presence of both parental viruses or by the presence of a recombinant virus with two functional drug resistance genes. To characterize the molecular nature of the proviruses in the doubly resistant cells, we isolated 11 hygromycin-plus-G418-resistant target cell clones and performed Southern analyses (Fig. 2). The two parental viruses are identical in sequence except for the two inactivating mutations, which convert a *Sac*II site in *hygro* to an *Ngo*MIV site and an *Ehe*I site in *neo* to a *Bss*HII site. Therefore, the nature of the proviruses can be distinguished by Southern analyses. A representative Southern analysis is shown in Fig. 2B. DNA samples were digested with *Ngo*MIV plus *Ehe*I and hybridized with a probe generated from a DNA fragment containing the 3′ half of IRES and most of *neo*. A provirus derived from one of the parental viruses should generate either a 1-kb band plus a 0.5-kb band (TR-HyS2NIN) or a 2.3-kb band (TR-HyINE2B), whereas a recombinant provirus with two functional genes should generate a 1.8-kb band plus a 0.5-kb band. Southern analyses of DNA samples from two types of cells are shown: virus-producing cells and doubly resistant target cells infected with virus generated from the producer cells. Lanes labeled 1.0B2, 1.0C3, and 1.0C4 contained DNA isolated from virus producer cell clones doubly infected with the two parental viruses. In each of these lanes, three bands were detected, corresponding to the expected 2.3-, 1.0-, and 0.5-kb fragments. In contrast, a 1.8-kb band and a 0.5-kb band were observed in DNA samples isolated from resistant target cell clones (lanes labeled B2B1,

TABLE 1. Titers of virus generated by cell clones containing TR-HyIN proviruses

Clone	Titer (10^2 CFU/ml) after selection by:		
	Hygromycin	G418	Hygromycin + G418
HN A1	1.4	1.8	1.2
HN C1	2.2	2.8	2.4
HN C3	4.1	5.0	3.6
HN E2	3.7	5.2	2.8
HN E4	0.3	0.7	0.4

TABLE 2. Titers of virus generated by cell clones containing TR-HyS2NIN and TR-HyINE2B proviruses

Clone	Titer (10^2 CFU/ml) after selection by:			Recombination rate (%)[a]
	Hygromycin	G418	Hygromycin + G418	
1.0B2	0.9	1.1	0.2	44.4
1.0C2	1.4	1.8	0.3	42.8
1.0C3	1.4	2.5	0.3	42.8
1.0C4	11.4	11.2	2.6	46.4
1.0D1	7.7	6.7	1.2	35.8

[a] The average (±SE) recombination rate was 42.4% ± 1.8%.

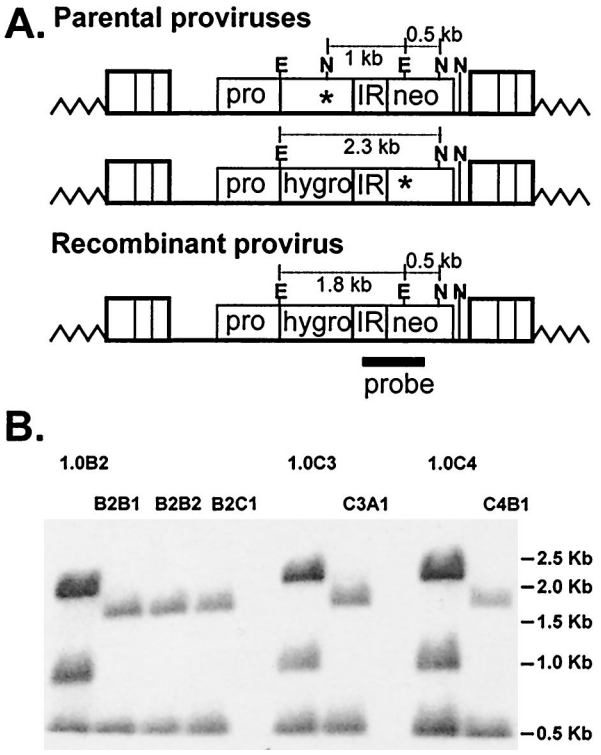

FIG. 2. Characterization of the proviral structures by Southern analyses. (A) Partial restriction enzyme maps of parental and recombinant proviruses. A 1.0-kb DNA fragment (probe) was used for the random-priming reaction to generate a probe for Southern hybridization analysis. E, *Ehe*I. (B) A representative Southern analysis of virus-producing cells and the doubly resistant cell clones. 1.0B2, 1.0C3, and 1.0C4 were doubly infected cell clones used to produce virus to measure recombination rates. B2B1, B2B2, and B2C1 were hygromycin-plus-G418-resistant target cell clones infected by viruses harvested from 1.0B2; C3A1 and C4B1 were doubly resistant target cell clones infected by viruses harvested from 1.0C3 and 1.0C4, respectively. Molecular size markers are indicated on the right of the Southern blot. All abbreviations are the same as in Fig. 1.

B2B2, B2C1, C3A1, and C4B1). All of the 11 target cell clones contained recombinant proviruses (Fig. 2 and data not shown). These results indicate that most of the hygromycin-plus-G418-resistant virus was generated from infection of recombinant proviruses rather than double infection of the two parent viruses. Therefore, the titers of doubly resistant virus reflect the amount of recombinants containing two functional drug resistance genes.

Two types of recombinants can be generated in this system: recombinants with two functional drug resistance genes and recombinants with two inactivated genes. Because the double drug selection protocol specifically detected recombinants with the two functional drug resistance genes, only half of the recombination events were measured. To calculate the recombination rate, the viral titers for hygromycin-plus-G418 selection were divided by the lower of the two single-drug selection titers and then multiplied by 2. The average recombination rate from the five clones is 42.4% ± 1.8% (Table 2, standard error [SE]).

Theoretical recombination rate of unlinked markers in this system. We performed the following calculation to estimate the recombination rate of two unlinked markers. Assuming that in the virus-producing cells, RNA expression of the two proviruses is equal and the formation of homozygous and heterozygous virions is random, then 50% of the virions should be heterozygous, 25% of the virions should be homozygous containing two copies of TR-HyS2NIN RNA, and 25% of the virions should be homozygous containing two copies of TR-HyINE2B RNA. Homozygous virions generate progeny proviruses with the same phenotypes as their parents. In contrast, heterozygous virions can generate four different types of progeny proviruses: the TR-HyS2NIN phenotype, the TR-HyINE2B phenotype, the recombinant phenotype with two functional drug resistance genes, and the recombinant phenotype with two inactivated genes. If the mutations in *hygro* and *neo* are unlinked, then random segregation is predicted, which implies that the four different phenotypes of progeny proviruses should be generated at the same frequencies. Therefore, of the 50% heterozygous virions, 12.5% of each phenotype of progeny is generated. Totaling all of the progeny generated by both homozygous and heterozygous virions, 50% of the proviruses should confer resistance to a single drug selection (25% from the homozygous virions, 12.5% from the parental phenotype generated from heterozygous virions, and 12.5% from the double drug resistance phenotype), whereas 12.5% of the proviruses should confer resistance to double drug selection. Therefore, the recombination rate should be 50% [(12.5%/50%) × 2].

HIV-1 recombination rate does not increase significantly when markers are 1.3 or 1.9 kb apart. The rate that we measured with two markers 1 kb apart was 42.4%, which approaches the rate at which two markers reassort randomly. To investigate whether the HIV-1 recombination rate can increase further, we measured the recombination rates when two markers are 1.3 or 1.9 kb apart. These two distances were chosen because using the same target sequences we have previously measured the MLV recombination rates when markers were 1.3 or 1.9 kb apart (1, 2); additionally, the 1.9-kb recombination rate is significantly higher than the 1.0-kb recombination rate (1).

Three vectors were derived from pTR-HyIN; the structures of their proviruses are shown in Fig. 3A and 4A. The vector pTR-HyN2MIN contains an inactivating frameshift mutation in *hygro* that introduced an *Mlu*I site in an *Nde*I site; when paired with the previously described pTR-HyINE2B, the recombination rate with markers 1.3 kb apart can be determined (Fig. 3A). The vector pTR-HyN2NIN contains an inactivating mutation in *hygro* that changed an *Nco*I site to an *Nsi*I site, and pTR-HyINN2N contains an inactivating mutation in *neo* that changed an *Nco*I site to an *Nsi*I site (Fig. 4A); the distance between the *Nco*I site of *hygro* and the *Nco*I site of *neo* is 1.9 kb.

Using a protocol identical to that used to measure the recombination rate between markers 1.0 kb apart, we generated and characterized cell clones containing one copy each of TRHyN2MIN and TR-HyINE2B. Helper constructs were used to transfect these cell clones, viruses were harvested, target cells were infected, and titers of virus were determined. Titers of virus generated from five different cell clones are summarized in Table 3. Because all the inactivating mutations are accompanied by restriction enzyme site alterations, the structures of the proviruses with a parental or recombinant genotype can be distinguished by Southern analyses. To characterize the molecular nature of proviruses in the hygromycin-plus-

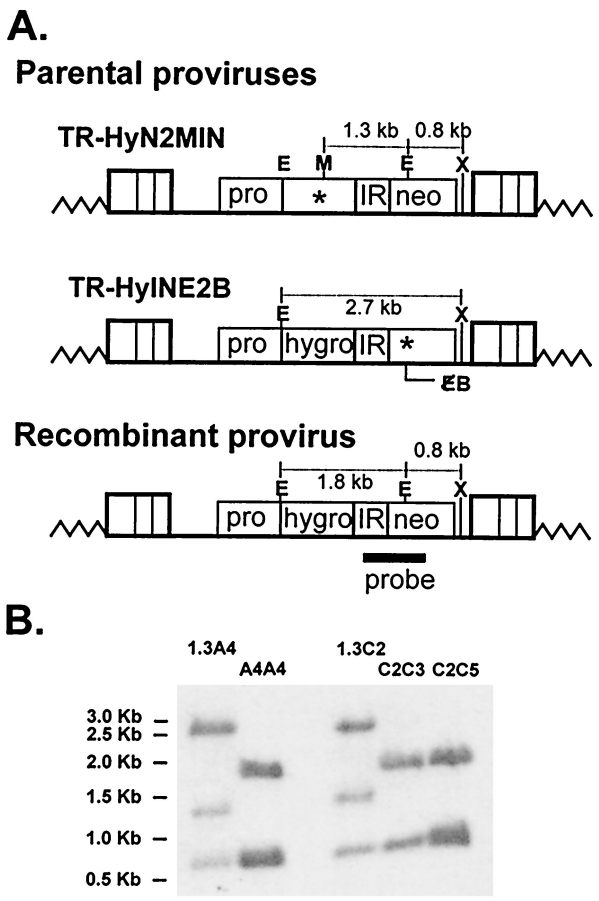

FIG. 3. Southern analyses of the doubly resistant cell clones from the 1.3-kb marker distance study. (A) Partial restriction enzyme maps of proviruses. M, *Mlu*I; X, *Xho*I. (B) A representative Southern analysis of virus-producing cells and the doubly resistant target cell clones. 1.3A4 and 1.3C2 were doubly infected cell clones used to produce virus to measure recombination rates. A4A4, C2C3, and C2C5 were hygromycin-plus-G418-resistant target cell clones infected by viruses harvested from 1.3A4 and 1.3C2. Molecular size markers are indicated on the left of the Southern blot. Other abbreviations are the same as for Fig. 1 and 2.

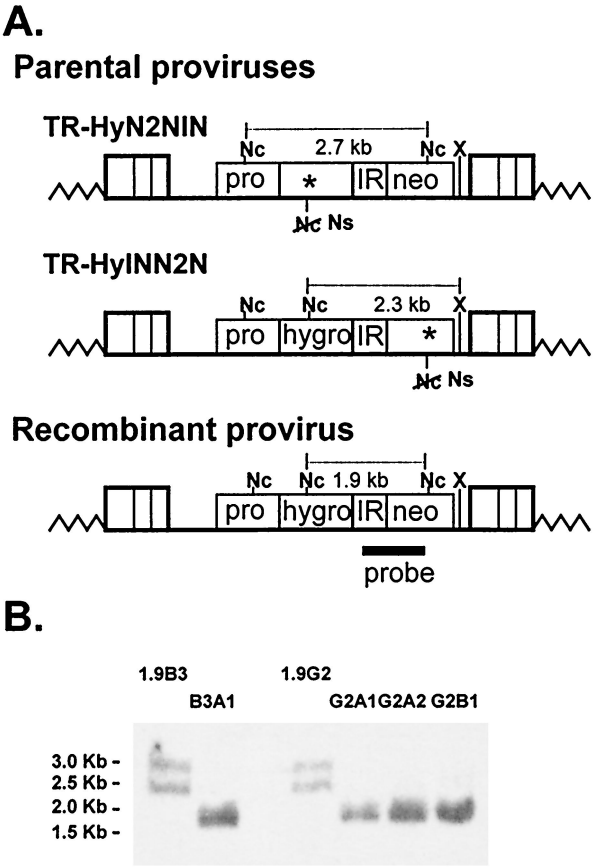

FIG. 4. Southern analyses of the doubly resistant cell clones from the 1.9-kb marker distance study. (A) Partial restriction enzyme maps of proviruses. Ns, *Nsi*I; Nc, *Nco*I; Nc with slash, destroyed *Nco*I. (B) A representative Southern analysis of virus-producing cells and the doubly resistant target cell clones. 1.9B3 and 1.9G2 were doubly infected cell clones used to produce virus to measure recombination rates. B3A1, G2A1, G2A2, and G2B1 were hygromycin-plus-G418-resistant cell clones infected by viruses harvested from 1.9B3 and 1.9G2. Molecular size markers are indicated on the left of the Southern blot. All abbreviations are the same as in Fig. 1, 2, and 3.

G418-resistant cells, we isolated and analyzed DNA from doubly resistant target cell clones. As illustrated in Fig. 3A, after digestion with *Mlu*I, *Ehe*I, and *Xho*I and hybridization with probes generated from the aforementioned DNA fragment, proviruses with parental genotypes are expected to have either a 1.3-kb band plus a 0.8-kb band or a 2.7-kb band, whereas recombinant proviruses with two functional genes are expected to generate a 1.8-kb band and a 0.8-kb band. A representative Southern blot is shown in Fig. 3B; the two virus-producing cell clones (1.3A4 and 1.3C2) contained the two parental proviruses, whereas the three doubly resistant target cell clones (A4A4, C2C3, and C2C5) contained recombinant proviruses with two functional genes. All of the eight doubly resistant target cell clones that were analyzed contained recombinant proviruses with two functional drug resistance genes (Fig. 3 and data not shown). These data indicate that the titers of hygromycin-plus-G418-resistant virus reflect the numbers of recombinant proviruses containing two functional drug resistance genes. Therefore, the average recombination rate of two markers 1.3 kb apart is 50.4% ± 3.7% (Table 3, SE).

Using the same protocol, we established and characterized cell clones containing a copy each of TR-HyN2NIN and TR-HyINN2N proviruses. Viral titers were measured from seven of these cell clones; these data are summarized in Table 4. Similar to data generated from cell clones containing provi-

TABLE 3. Titers of virus generated by cell clones containing TR-HyN2MIN and TR-HyINE2B proviruses

Clone	Titer (10^2 CFU/ml) after selection by:			Recombination rate (%)[a]
	Hygromycin	G418	Hygromycin + G418	
1.3A1	9.5	8.3	2.2	53.0
1.3A4	13.7	13.6	3.0	44.1
1.3C2	9.1	10.3	2.2	48.4
1.3E3	4.4	8.5	1.4	63.6
1.3F4	9.7	8.8	1.9	43.1

[a] The average (±SE) recombination rate was 50.4% ± 3.7%.

TABLE 4. Titers of virus generated by cell clones containing TR-HyN2NIN and TR-HyINN2N proviruses

Clone	Titer (10^2 CFU/ml) after selection by:			Recombination rate (%)[a]
	Hygromycin	G418	Hygromycin + G418	
1.9B1	2.5	3.1	0.51	40.8
1.9B3	3.9	4.1	1.1	56.4
1.9B4	7.5	8.2	2.0	53.3
1.9C2	9.5	6.7	1.6	47.8
1.9D1	10.7	11.5	2.4	44.9
1.9G2	11.3	9.3	1.9	40.9
1.9I2	11.7	15.2	2.8	47.9

[a] The average (±SE) recombination rate was 47.4% ± 2.2%.

ruses with markers 1.0 and 1.3 kb apart, within each cell clone, the two single-selection titers are generally comparable whereas the double drug selection titers are approximately 20 to 25% of the single-selection titers. Doubly resistant target cell clones were isolated, and the proviral structures were analyzed. As shown in Fig. 4A, when digested with *Nco*I and *Xho*I and hybridized with probes, the two parental proviruses are expected to generate a 2.7-kb band and a 2.3-kb band, whereas the recombinants with two functional genes are expected to generate a 1.9-kb band. A representative Southern blot is shown in Fig. 4B; the two virus-producing cell clones (1.9B3 and 1.9G2) contained the two parental viruses, and the four doubly resistant target cell clones contained the recombinant proviruses (B3A1, G2A1, G2A2, and G2B1). We examined nine double-drug-resistant target cell clones, and all of them contained recombinant proviruses with two functional drug resistance genes (Fig. 4 and data not shown). Therefore, the average recombination rate with markers 1.9 kb apart is 47.4% ± 2.2% (SE, Table 4). Therefore, the recombination rate for HIV-1 does not increase significantly when the marker distance is increased from 1.0 kb to 1.3 or 1.9 kb apart. Furthermore, all three rates remain similar to the 50% predicted rate on unlinked markers.

The protocols used in these experiments mainly measured events that occurred during one round of HIV-1 replication. Viruses were harvested from cells 24 h after transfection; in order for more than one round of viral replication to occur, viral proteins have to be expressed from the transfected helper plasmids, and virions have to be generated, infect new target cells, complete the viral replication cycle, express the proviral genome, and form new viruses within 24 h. We estimate that only a very small percentage of the events measured in the system will be derived from more than one round of replication, and these infrequent events should not affect the recombination rate that we measured. To test this, we generated two cell pools, one containing TR-HyN2NIN proviruses and one containing TR-HyINN2N proviruses. Equal numbers of cells from these two pools were mixed together, and transfected with helper constructs, and then viruses were harvested and used to infect target cells by the same protocol described above. Because the two cell pools each contained only one parental virus, only homozygous virions should be produced; thus, recombinant proviruses with two functional drug resistance genes should not be generated in one round of viral replication. However, cells containing both parental proviruses could be generated after the first round of viral infection, and recombinant proviruses could be generated at later replication cycles. We observed that the titer of hygromycin-plus-G418 double-resistant virus was less than 2% of the single-selection viral titers, which is in sharp contrast with the previous experiments using coinfected cell clones. This experiment demonstrated that the majority of the events measured in this system occurred in one round of viral replication.

DISCUSSION

High rates of HIV-1 recombination and their implications. In this report, we described a series of experiments measuring HIV-1 recombination rates. We found that the recombination rate in one round of viral replication is 42.4% with markers 1 kb apart, which predicts that two markers separated by 1 kb can reassort at a frequency similar to that of unlinked markers. This observation indicates that recombination is an incredibly powerful tool to break the linkage between neighboring sequences in the viral genome, thereby generating diversity in the viral population and increasing the evolutionary capacity of HIV-1. This heightened ability to generate diversity also presents more challenges in the development of effective anti-HIV-1 treatments and vaccines. Although many anti-HIV-1 drugs have been developed in the past decades, resistance-conferring mutations in the HIV-1 genome have been observed for every Food and Drug Administration-approved drug. For example, the mutation L10I in protease confers resistance to indinavir and lopinavir (8, 28), and the mutation T215Y in reverse transcriptase confers resistance to zidovudine (27, 32). These two mutations are separated by approximately 1 kb and should segregate randomly if recombination occurs between a virus with L10I and a virus with T215Y. Therefore, the high rates of recombination can accelerate the generation of multidrug-resistant HIV-1 strains. In addition, high recombination rates are also important to the generation of novel strains of HIV-1. HIV-1 isolates are classified into groups M, N, and O based on sequence identity; the vast majority of the isolates belong to group M, which is further divided into different subtypes (44). Recombination can occur between closely related strains (15, 34, 47, 56), between isolates from different subtypes (4, 5, 10, 33, 43, 49, 55), or even between different HIV-1 groups (41, 51). Currently, many of the circulating strains of HIV-1 are intersubtype recombinants (9, 26, 45, 46). The high rates of recombination indicate that the mixing of the viral genomes from different strains can occur much faster than previously anticipated, and novel HIV-1 variants can also be generated at an accelerated pace.

Efficient formation of heterozygous HIV-1 virions. Previously, we demonstrated that the formation of the heterozygous virions is critical to SNV and MLV recombination (21; J. A. Anderson and W.-S. Hu, unpublished data). Here, we also show that high rates of recombination occurred when viruses were harvested from cell clones containing both parental viruses (thus allowing the formation of heterozygous viruses) but not from coinfection of two stocks of homozygous viruses. Therefore, similar to simple retroviruses, the high frequency of HIV-1 recombination also requires heterozygous virion formation.

We calculated the hypothetical recombination rate of un-

linked markers with the assumptions that RNA expressed from the two parental viruses would be equal and the formation of homozygous and heterozygous virions would be random. The calculated hypothetical rate (50%) is similar to our measured rate (42.4 to 50.4%). Our titer data indicate that within each cell clone, the two parental viruses were expressed at similar levels (Tables 2 to 4). These results validate the assumption regarding RNA expression. The observed high recombination rates in these experiments allow us to conclude that heterozygous virions must be formed efficiently during HIV-1 replication.

Comparison of SNV, MLV, and HIV-1 recombination. The HIV-1 recombination rates described in this report are approximately six- to ninefold higher than the MLV recombination rates (1-kb distance: 4.7% versus 42.4%; 1.9-kb: 7.4% versus 47.4%). Because the same target sequences were used in the measurement of HIV-1 and MLV recombination rates, the large difference in recombination rates most likely reflects the difference between MLV and HIV-1 replication. Previously, we observed that in SNV and MLV, intramolecular template switching occurred far more frequently than intermolecular template switching (1, 11, 20, 21, 25). We hypothesized that although most viruses are capable of undergoing intramolecular template switching, only a subpopulation of the virions are capable of undergoing intermolecular template switching (recombination) (20). We provided two possible explanations for the limited recombination subpopulation: nonrandom copackaging of viral RNA (inefficient heterodimer formation) or altered structures of reverse transcription complex, which hampered the access of both RNA copies to be used as the template during reverse transcription (20). Currently, we do not know the mechanisms that limit the intermolecular template-switching events in MLV and SNV. However, HIV-1 does not appear to have the same constraints, because we were able to measure a 42.4 to 50.4% recombination rate in HIV-1, similar to the 50% hypothetical recombination rate of unlinked markers. Therefore we can also conclude that a major portion of HIV-1 viruses are capable of undergoing intermolecular template switching (recombination) events.

As we were completing the manuscript, a report was published concluding that HIV-1 recombines more frequently than MLV (40). There are several differences in the systems used and the conclusions of these two studies. In our study, viruses were harvested from producer cell clones that were characterized to contain a copy of each parental provirus, whereas in the other study viruses were generated by transiently cotransfecting two vectors and helpers. Cell clone-produced viruses have several advantages. First, viral RNAs were generated from proviruses, and we measured any bias in the transport and sorting of RNA expressed from proviruses integrated at different locations on the host chromosomes. Second, the homogeneity of the characterized cell clones allows assessment of the expression levels of the two proviruses, which can be used to estimate the efficiency of heterologous virions formation. Such estimation cannot be easily obtained using viruses generated from a transfected pool, because the heterogeneity of such pools creates uncertainty about the numbers of doubly transfected cells and the expression levels of the vectors in each cell. Third, DNA recombination during transfection is not a complicating factor in our system. In addition, our system allows the simultaneous scoring of the two parents and the recombinants. In both systems, recombination is scored by the simultaneous presence of the two parental phenotypes, which can be complicated by double infection of the two parental viruses. We have characterized target cell clones to ensure that our measurements reflected the recombination events.

There are differences in our conclusions as well. We have measured HIV-1 recombination rates at three marker distances; we have observed that markers 1 kb apart can segregate in a manner similar to unlinked markers in one round of retroviral replication, which has strong implications for HIV-1 evolution and the development of antiviral treatments and vaccines. In addition, we concluded that heterozygous virions are formed efficiently in HIV-1, and most of the HIV-1 virions are capable of carrying out recombination events.

The results from our study add to a growing body of evidence that HIV-1 recombination is an important factor in generating diversity in the viral population. Many questions about HIV-1 recombination remain to be answered, such as the mechanisms of recombination and the factors that affect recombination. However, our present understanding indicates that the rapid redistribution of mutations in the viral genomes must be taken into account when designing new treatment regimens and developing effective vaccines.

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