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Few Mutations in the 5′ Leader Region Mediate Fitness Recovery of Debilitated Human Immunodeficiency Type 1 Viruses

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Repeated bottleneck passages of RNA viruses result in fitness losses due to the accumulation of deleterious mutations. In contrast, repeated transfers of large virus populations result in exponential fitness increases. Human immunodeficiency virus type 1 (HIV-1) manifested a drastic fitness loss after a limited number of plaque-to-plaque transfers in MT-4 cells. An analysis of the mutations associated with fitness loss in four debilitated clones revealed mutation frequencies in *gag* that were threefold higher than those in *env*. We now show an increase in the fitness of the debilitated HIV-1 clones by repeated passages of large populations. An analysis of the entire genomic nucleotide sequences of these populations showed that few mutations, from two to seven per clone, mediated fitness recovery. Eight of the 20 mutations affected coding regions, mainly by the introduction of nonsynonymous mutations (75%). However, most of the mutations accumulated during fitness recovery (12 of 20) were located in the 5′ untranslated leader region of the genome, and more specifically, in the primer binding site (PBS) loop. Two of the viruses incorporated the same mutation in the primer activation signal in the PBS loop, which is critical for the $tRNA_3^{Lys}$-mediated initiation of reverse transcription. Moreover, 25% of the mutations observed were reversions. This fact, together with the presence of a large proportion of nonsynonymous replacements, may disclose the operation, during large population passages, of strong positive selection for optimal HIV-1 replication, which seems to be primarily affected by binding of the tRNA to the PBS and the initiation of reverse transcription.

Retroviruses, in particular human immunodeficiency virus type 1 (HIV-1), mutate and recombine at high rates (10^{-4} misincorporations per nucleotide copied) (34–36). Rapid genetic variation, together with the short replication time of HIV-1, generates complex and dynamic mutant swarms termed viral quasispecies (28, 40, 46). As a consequence, in natural HIV-1 populations, a large amount of genetic variability is observed between different isolates as well as within isolates (8, 18, 20, 25, 29, 31, 43, 44). The adaptive potential of viral quasispecies is manifested by quantitatively important fitness variations as viruses evolve in constant or changing environments (9, 10). This has been evidenced for HIV-1 by the identification of mutants that are capable of evading prior immune responses, variants with altered virulence and host cell tropism (24, 42), or mutants with decreased sensitivities to antiretroviral inhibitors (23, 31, 37). A better understanding of the dynamics of HIV-1 fitness variation and the effect of population size on virus adaptability may help in the design of effective vaccines or antiretroviral treatments as well as providing an understanding of the evolution of infection.

The measurement of fitness values under different experimental conditions in several RNA viruses has led to the observation that fitness variations are extremely dependent on the population size of the evolving RNA virus population. In particular, repeated bottleneck passages (the multiplication of viral genomes from only one or a small number of genomes) result in average fitness losses in several different RNA viruses (7, 11, 12). The decrease in fitness mediated by repeated bottlenecks has been interpreted as the result of an accentuation of Muller's ratchet effect (30) due to the incorporation of deleterious mutations and the lack of compensatory mechanisms (26) such as recombination (10–12, 27, 35). The operation of Muller's ratchet effect in viruses has been demonstrated with bacteriophage ϕ6 (7), vesicular stomatitis virus (11), foot-and-mouth disease virus (13), and HIV-1 (47, 48). In contrast, massive infections of cells with large numbers of infectious viruses lead to rapid fitness gains (10, 14, 32). As the population size of an RNA virus increases, the repertoire of multiple mutants that may be subjected to positive selection also increases. This process leads to the accumulation of more fit genomes and a loss or decrease in the frequency of less adapted genomes. For retroviruses, decreases in fitness as a result of serial bottleneck passages were first documented for HIV-1 after plaque-to-plaque passages on MT-4 cells (48). For this virus, fitness losses were unexpectedly drastic and rapid compared with those experienced by other RNA viruses, such as the bacteriophage ϕ6 (7), vesicular stomatitis virus (11), and foot-and-mouth disease virus (13), which were subjected to similar passage regimens. Only 4 of 10 HIV-1 clones produced viable progeny after 15 plaque-to-plaque transfers, and three of the four survivors displayed important decreases in fitness which were associated with an unusual distribution of mutations (47). The mutation frequencies in *gag* were threefold higher than those in *env*, and the complete absence of replace-

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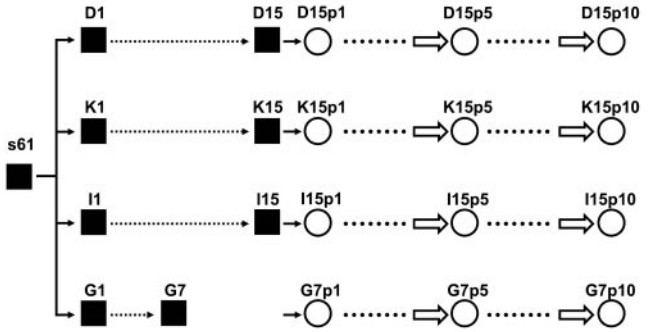

FIG. 1. Scheme of passages of HIV-1 clones subjected to plaque-to-plaque transfers (filled squares) and large population passages (open circles) in MT-4 cells. The experimental procedures and the origins of the natural isolate s61 and all of the different clones were previously reported (41, 48) and are given in Materials and Methods. HIV-1 clones are indicated by a letter followed by a number that gives the total number of plaque-to-plaque transfers and p1 to p10, indicating the number of large population transfers.

ments in the V3 loop of gp120 was remarkable. The amino acid replacements observed were also atypical, and several of the amino acid changes have not been previously recorded among natural HIV-1 isolates (47).

Here we report an increase in fitness and describe the associated mutations that occur when debilitated HIV-1 clones are subjected to large population passages. The results document a surprising preferential location of mutations in the 5′ untranslated leader sequence. The analysis also revealed that few mutations (an average of five mutations per clone) were associated with fitness recovery and that most of the mutations in the coding regions were amino acid replacements. The large numbers of reversions (25%) and amino acid substitutions to regain the most common amino acids found for the corresponding positions among natural HIV-1 isolates were particularly striking. These observations reflect a high selective pressure during fitness recovery.

MATERIALS AND METHODS

Cells, viruses, and biological cloning. The HIV-1 parental population, isolate s61 (41), was obtained from a 4-year-old child by standard coculture procedures. Ten biological clones were derived from this viral population by use of an MT-4 plaque assay (16, 41). D15, G7, I15, and K15 were obtained by subjecting clones D1, G1, I1, and K1 to serial plaque-to-plaque transfers. Plaque formation was not observed with clone G1 after passage 7 (47, 48).

Population passages of virus. Adaptive passages of large virus populations were performed as follows (Fig. 1). MT-4 cells (5×10^6) were infected with 5×10^5 PFU of each clone (multiplicity of infection of 0.1 PFU/cell) in a final volume of 5 ml. The virus was recovered in the supernatant when cytopathology was complete (at 5 to 7 days postinfection). For each passage, fresh MT-4 cells were infected with a 1:10 dilution from the supernatant of the previous passage, taking into account that viral titers do not change significantly during serial passages of HIV-1 (37). The cells were cultured in closed bottles, and all of the procedures were aimed at minimizing the possibility of cross-contamination, which was monitored by the use of uninfected control cultures which were maintained in parallel. No evidence of contamination was observed at any time.

Fitness assay. Fitness determination was performed by the use of growth competition experiments as previously described (48). Briefly, the assay consists of the coinfection of cultures with known amounts of the virus to be tested and a reference clone (J1). J1 was selected as the reference clone because the DNA from each of the biological clones analyzed in this study displayed a heteroduplex form with a distinct mobility when J1 was used as a probe (48). Coinfections were performed at different initial ratios (1:9, 1:1, and 9:1), and the viruses were allowed to compete for five passages. For each passage, a 1/10 dilution of the supernatant from the previous passage was used to infect 5×10^4 MT-4 cells. The proportions of the two viruses in each passage were quantified by a heteroduplex tracking assay (HTA) (8) as previously described (48). The proportion of the competing variant with respect to the reference strain (R_n) was divided by its proportion in the initial mixture (R_o), and this value (R_n/R_o) was plotted versus the competition passage to derive the fitness vector. Fitness values per competitive transfer were obtained from the exponential slope of each vector line, as described previously (19). To homogenize the fitness values, we modified previously obtained values (48) from the linear to the exponential slope.

RNA extraction, PCR amplification, and nucleotide sequencing. RNAs were extracted with a guanidinium isothiocyanate lysis buffer and glass milk as described previously (5). HIV-1 RNAs were amplified by reverse transcription-PCR and nested PCR. The first amplification was performed by use of the Access RT-PCR system (Promega) and six different pairs of primers that covered the entire genome of HIV-1. Internal amplifications were performed with Eco*Taq* DNA polymerase (Ecogen) and internal primers appropriate for each amplification. The primer sequences used for all amplifications are available upon request. Both external and internal amplifications involved 35 cycles, with temperatures chosen according to the compositions of the oligonucleotide primers. Negative amplification controls were run in parallel to monitor the absence of contamination.

Complete genomic nucleotide sequences were determined for the initial and final viral populations of the plaque-to-plaque passages and were reported in a previous work (47). The complete genomic nucleotide sequences of the final viral populations after the large population passages were determined for this study. Sequences were determined for the two cDNA strands by use of an ABI 373 automatic sequencer. Multiple sequence alignments were performed with the CLUSTAL W program (45). The numbering of nucleotide changes detected is reported relative to the numbering of isolate HXB2 included in the Los Alamos database alignment (21).

HTA. HTAs were performed as previously reported (48) by use of a cDNA copy of a 650-bp fragment corresponding to the V1-V2 region of the *env* gene. For amplification of this DNA, a nested PCR was performed with the primers 91ECU (5′ CTTAGGCATCTCCTATGGC 3′; positions 5956 to 5974 [numbering relative to HXB2]) and 92ECD (5′ GGAGCAGCAGGAAGCAC 3′; complementary to positions 7810 to 7827) and the nested primers 99ECU (5′ AGA GCAGAAGACAGTGGC 3′; positions 6205 to 6223) and 52EV1D (5′ TAAT GTATGGGAATTGGCTCAA 3′; complementary to positions 6874 to 6896). Amplifications were carried out for 35 cycles of 94°C for 1 min, 55°C for 55 s, and 72°C for 1 min, with a final extension cycle of 10 min at 72°C. In order to determine the proportion of each molecular species during competition passages, we labeled the amplified cDNA of clone J1 with [α-^{32}P]dCTP (3,000 Ci/mmol) by PCR amplification. About 10,000 cpm of this radioactive PCR probe was mixed with 50 to 100 ng of the unlabeled second-round PCR product from the competing virus in annealing buffer (0.1 M NaCl, 10 mM Tris-HCl [pH 7.8], and 2 mM EDTA). The DNA mixtures were denatured at 94°C for 2 min and then quickly cooled. Heteroduplexes were resolved in denaturing 8% polyacrylamide–15% urea gels in TBE (0.1 mM Tris-borate, 89 mM boric acid, and 2 mM EDTA, pH 8). Autoradiograms were obtained by exposing gels on a Fuji 2000 instrument for 2 h. Quantification of the ratio of the J1 cDNA (homoduplex) to the cDNA of the competing virus (heteroduplex) was performed by densitometry with the help of the PCBAS program.

RESULTS

Large population passages promote fitness recovery of debilitated HIV-1 clones. In order to study the effect of large population passages on HIV-1 fitness, we selected four biological clones that underwent severe fitness losses as a result of serial plaque-to-plaque transfers (48). These biological clones, termed D15, G7, I15, and K15, were serially passaged by infecting MT-4 cells as detailed in Materials and Methods. After 5 and 10 serial passages, fitness values were determined for each population by competition assays with the reference clone J1 as previously described (48). Clone G underwent a fitness increase in just one passage, from the preextinction population to the first large population passage, from <0.001 (limit of detection) to 1.73 (48). After five large population passages,

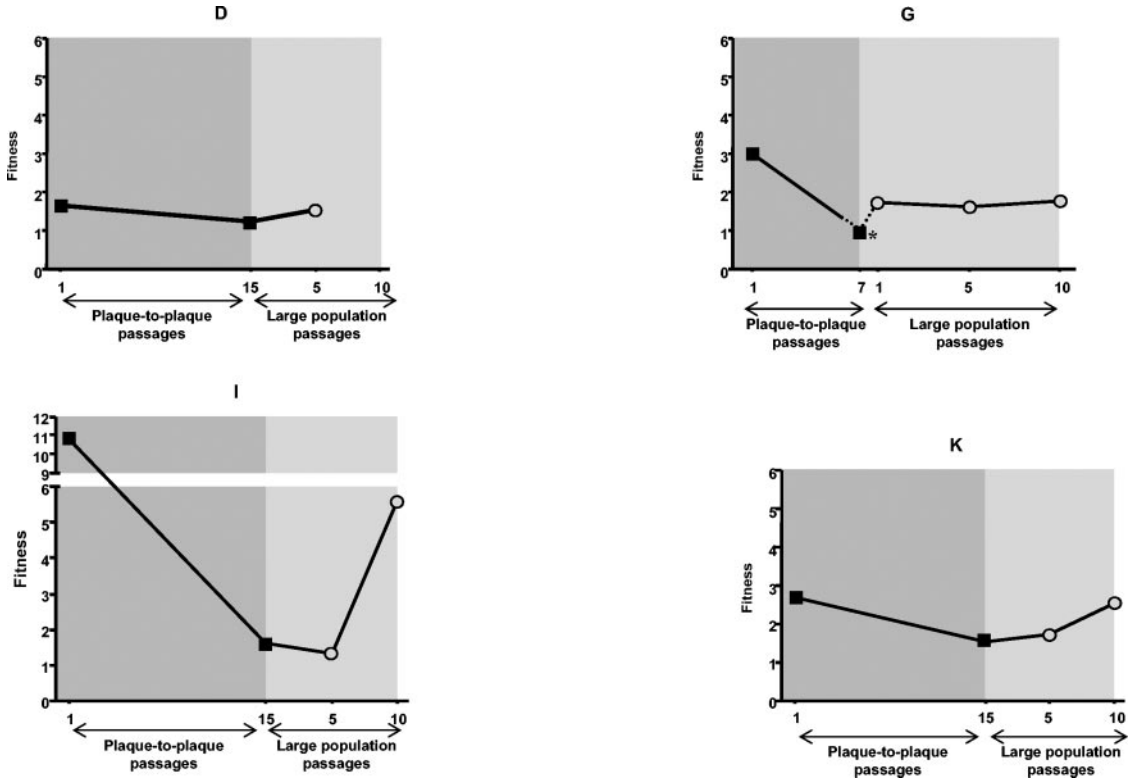

FIG. 2. Fitness evolution of each clone during plaque-to-plaque and large population passages. Fitness values were calculated from the exponential slopes of the corresponding fitness vectors (48) as described previously (19). Plaque-to-plaque transfers are indicated by filled squares, and large population passages are indicated by open circles. The fitness of clone D15p10 could not be calculated, as explained in Table 1. The asterisk indicates a debilitated clone (G7) that could not produce plaques after plaque-to-plaque transfer 7. The fitness of this clone could not be determined by competition passages, as described in Materials and Methods.

clones D15 and K15 had moderate fitness increases (28 and 10.4%, respectively), and two of the clones (I15 and G7) displayed moderate decreases in fitness (18 and 6.95%, respectively) (Fig. 2 and Table 1). After 10 large population passages, all clones had increases in fitness, although for clone D quantification was not possible (Table 1). Clone G had no significant change from the increase detected in passage 5, clone K had an increase of 62%, clone I had an increase of 250%, and clone G had an increase of 178,000% (Table 1). Thus, all HIV-1 clones gained fitness upon large population passages.

Genetic changes associated with fitness recovery. To determine the type and number of mutations accumulated during fitness recovery, we obtained the entire genomic nucleotide sequences of D15p10, G7p10, I15p10, and K15p10 and compared them with the sequences of the corresponding parental debilitated viruses after plaque-to-plaque passages (47). The number of mutations in the consensus sequence was six for clone D15p10, seven for clone G7p10, two for clone I15p10, and five for clone K15p10 (average of five mutations per clone) (see Fig. 4 and Table 2). Transitions were threefold more frequent than transversions, and A→G, C→T, and G→A changes accounted for 35, 20, and 15%, respectively, of the total mutations (Table 2). Mutation frequencies ranged from 2.1×10^{-4} to 7.5×10^{-4} substitutions per nucleotide (mean, 5.4×10^{-4} substitutions per nucleotide). Thirteen substitutions were in untranslated regulatory regions, and eight were in coding regions. Among the latter mutations, six were nonsynonymous (75% of the total) and two were synonymous. Most of these amino acid changes replaced residues which are present with low frequencies in the database with amino acids that are present in >90% of HIV-1 isolates (21), and the degree of

TABLE 1. Fitness values of clones after 15 plaque-to-plaque transfers and 1, 5, and 10 large population passages

Clone[a]	Fitness value				Maximal fitness increase[c]
	Final plaque-to-plaque population[b]	Large population passage			
		1	5	10	
D	**1.23**	ND[f]	**1.57**	ND[d,f]	0.34 (+28)
K	**1.54**	ND[f]	1.70	**2.5**	0.96 (+62)
I	**1.61**	ND[f]	1.32	**5.64**	4.03 (+250)
G	**<0.001**[e]	1.73	1.61	**1.78**	1.78 (+178,000)

[a] The origins of the HIV-1 clones are detailed in Materials and Methods and were described previously (48).

[b] All clones were plaque passaged 15 times, except for clone G, which went to extinction in passage 7.

[c] Maximal fitness increases were determined between the plaque-to-plaque and large population passages. The values used for the comparison are marked in bold. Values in parentheses are percentages for all clones.

[d] We were unable to determine the fitness of clone D15p10. This clone displayed a mixture of variants, and the HTA after the competition passages could not be analyzed.

[e] Limit of detection (see Materials and Methods).

[f] ND, not done.

TABLE 2. Numbers and types of mutations in the genomes of HIV-1 clones subjected to large population transfers

Clones compared[a]	Mutation frequency[b]	No. of mutations							
		A → G	C → A	C → T	G → A	G → T	T → C	T → G	Total
D15, D15p10	6.8×10^{-4}	3	1	0	1	1	0	0	6
G7, G7p10	7.5×10^{-4}	2	1	0	1	0	1	2	7
I15, I15p10	2.1×10^{-4}	1	0	1	0	0	0	0	2
K15, K15p10	5.4×10^{-4}	1	0	3	1	0	0	0	5
Total	5.4×10^{-4}	7	2	4	3	1	1	2	20

[a] The entire genomic nucleotide sequences of the indicated clones were determined as described in Materials and Methods.
[b] Mutation frequencies are the numbers of mutations found in the genomes of D15p10, G7p10, I15p10, and K15p10 (compared with the corresponding initial clones D15, G7, I15, and K15) divided by the total number of nucleotides sequenced; therefore, values are expressed in substitutions per nucleotide.

acceptability is high in most of the amino acid replacements (15) (Table 3).

The presence of reversions, which accounted for 25% of all mutations appearing during fitness recovery and were widespread in all populations except D15p10, was particularly striking (Table 3 and Fig. 3). The presence of accompanying mutations unique to each viral genome excluded the possibility that the observed reversions were the result of contamination (see Materials and Methods). Two of the reversions mapped in the 5′ long terminal repeat, and three mapped in the coding regions, with two in the Nef protein coding region (one in the region overlapping with U3) and another in nucleocapsid protein coding region (Table 3). This large proportion of reversions may represent an event of deterministic evolution.

We also determined the entire genomic nucleotide sequence of clone G7p1, in which fitness recovery occurred in one passage, and we found only one G-to-A mutation in the 5′ untranslated leader sequence that was also a reversion. It is noteworthy that despite the variation potential of HIV-1, a very limited number of mutations were associated with remarkable fitness increases in debilitated clones (Fig. 4 and Table 2).

Accumulation of mutations in the primer binding site hairpin. An analysis of the mutations that appeared during fitness recovery revealed that they were not randomly distributed along the genome, as 12 substitutions (60% of the total) were localized in the 5′ untranslated region, one was in the 3′ U3 region, and the remaining ones were situated within the coding regions. The 12 mutations in the 5′ untranslated leader region mapped within the primer binding site (PBS) loop (Fig. 3 and 4). The majority of these mutations correspond to conserved nucleotides in the sequence database (20). The 335-nucleotide 5′ untranslated leader region is structurally conserved in HIV-1 (21) and contains multiple sequence and structural motifs that play important roles in viral replication (4). Several mutations were found in more than one population. The G570A mutation was found in populations D15p10 and K15p10, and the A584G and T584G mutations were found in populations D15p10 and G7p10, although the A587G mutation which occurred in all four clones was more intriguing. The observation of the same mutation in independent populations is an example that illustrates evolutionary convergence and may support the deterministic behavior of RNA viruses when selection predominates (38, 39). Two of the mutations found in this region (G570A in K15p10 and T584G in G7p10) were reversions to the sequence present in the virus before fitness loss. The T584G reversion was also found to be the only mutation incorporated into clone G7p1 during fitness recovery. The mutation at position 584 is located in an eight-nucleotide motif in the U5 region (positions 576 to 584 [numbering according to HXB2]) (21) (Fig. 3) which is critical for the $tRNA_3^{Lys}$-mediated initiation of reverse transcription in vitro (3). This motif is referred to as the primer activation signal (PAS) and is important for the activation of the PBS-bound tRNA primer (2).

TABLE 3. Amino acid replacements and synonymous mutations during large population passages of HIV-1

Amino acid replacement[a]	Acceptability[b]	Frequency (%) in database[c]	HIV-1 protein[f]
L16F[d]	4	99.7	p7 nucleocapsid (mapped in first zing finger motif)
E203K	4	1.7	RT
K308E	4	98.1	RT
V417	6	95.3	RT
Y45H	3	55	Vpr
R604C	2	99.4	gp160
Stop65E[d,e]		90.3	Nef
D108	6	51.8	Nef (located in the U3 overlapping region)

[a] Amino acids are numbered for each individual protein according to the numbering for isolate HXB2 found in the HIV-1 database (21).
[b] The degree of acceptability of the amino acid substitution is given according to a previously described scale (15); the acceptability scale is from 0 to 6, with 6 representing replacements by the same amino acid.
[c] The database consulted was previously described (21).
[d] This amino acid replacement is a reversion to the amino acid that was present before the fitness loss.
[e] This mutation reverts an amber termination codon in Nef.
[f] RT, reverse transcriptase.

DISCUSSION

In this study, we have reported that very few mutations in the HIV-1 genome mediated the fitness recovery of debilitated clones, with a striking preference for mutations in the 5′ untranslated leader region. The small number of mutations associated with a fitness gain in HIV-1 is in contrast with the large number of mutations observed among natural populations (6, 18, 23, 25, 29, 31, 44). The fitness increase was not directly correlated with the number of mutations (Table 1). Clone I was particularly remarkable, as only two mutations were associated with a very large fitness increase (Table 1 and Fig. 2).

The large majority of mutations associated with fitness losses through repeated bottlenecks occurred in the *gag* gene (47), with mutation frequencies in *gag* being threefold higher than those in *env*. Surprisingly, most of the mutations associated

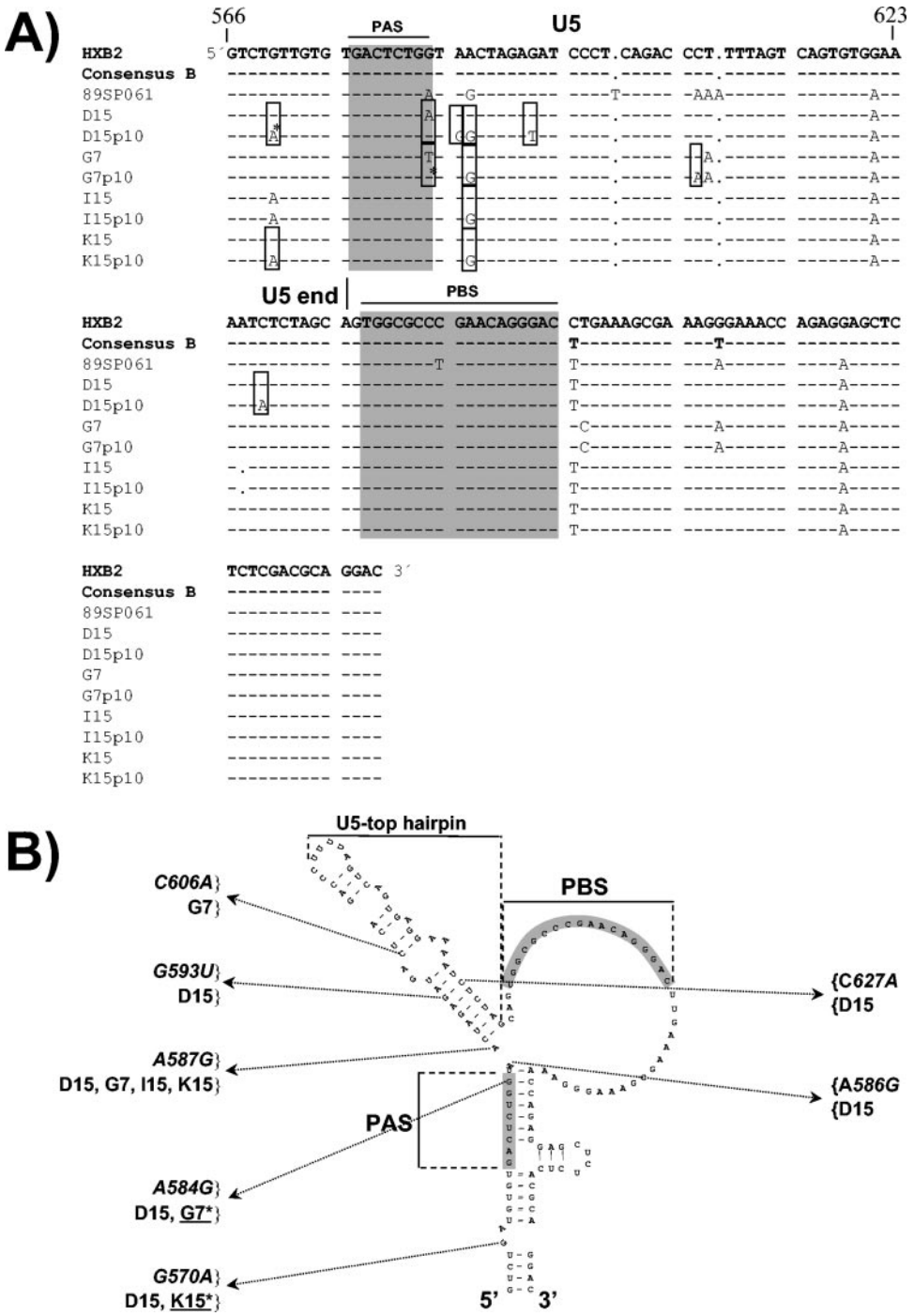

FIG. 3. Localization of mutations accumulated in the U5 PBS loop during large population passages. (A) Sequence alignment of viruses before and after fitness recovery in the U5 PBS region in comparison with the HXB2 and consensus B sequences, which are marked in bold. Boxes indicated nucleotide changes associated with large population passages. Asterisks indicate reversions to the sequence present before the fitness loss (47, 48). The PAS and PBS sequences are shaded. (B) Secondary structure model of the U5 PBS leader region of HIV-1 (LAI sequence). The locations of the PBS and the PAS are indicated. The positions of the mutations accumulated during fitness recovery in all final p10 viral populations are marked. Watson-Crick base pairing is marked with dashes. Clones that are underlined and marked with asterisks indicate reversions to the sequence that was present before the fitness loss (47, 48). Modified from reference 3.

with large population passages and fitness recovery were not located in any of the open reading frames, but were in the 5′ untranslated region. An analysis of the coding region mutations occurring during large population passages revealed that only one of the amino acid replacements had a low frequency in the HIV-1 sequence database (21), and four of the six amino acid replacements had a frequency above 90% (21) (Table 3).

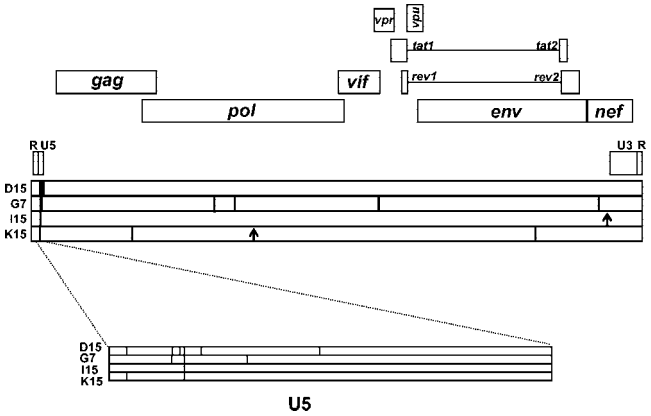

FIG. 4. Distribution in the complete genome of all mutations appearing during large population passages. Horizontal bars below the gene organization scheme represent the genomes of the four clones studied. Vertical lines illustrate nonsynonymous mutations, whereas vertical arrows indicate synonymous mutations. The 5′ U5 region was expanded in order to more precisely map the mutations accumulated in the clones.

The observation in our analysis of a preferential location of mutations in the 5′ leader region, with 12 of the 20 mutations associated with fitness recovery, was particularly interesting. The 5′ untranslated leader region of the viral genome is a highly structured RNA domain which is conserved among isolates of the same subtype (21). This region includes the PBS hairpin, displaying the PBS motif, which does not constitute a hot spot for genetic variation (21). Within this region, several interactions are required for efficient binding of the tRNA to the PBS in order to initiate reverse transcription, which is a critical step for viral replication (2, 3, 17). First, a cellular $tRNA_3^{Lys}$, which is used as a primer, binds the PBS motif in the 5′ end of the viral RNA (4). Second, a new critical motif, the PAS, was recently identified for $tRNA_3^{Lys}$ as a mediator of the initiation of reverse transcription (3). This motif interacts with the anti-PAS motif of the tRNA and triggers the initiation of retrotranscription. Mutations in the PAS motif have been associated with a reduced efficiency of tRNA-primed reverse transcription (1, 2). Interestingly, two of the low-fitness viruses in our study (D15 and G7) had mutations in the PAS motif. In clone G7, the mutation reverted after a fitness gain (T→G), and in D15 it changed to the common nucleotide (A→G), rendering in both cases the PAS wild-type sequence complementary to the anti-PAS sequence of the tRNA. RNA structure analysis performed with MFOLD (http://bioweb.pasteur.fr/seqanal/interfaces/mfold-simple.html) indicated a decrease in ΔG between the RNA structure for large populations and that for plaque-to-plaque passaged virus, showing that the T/A584G (in clones D15 and G7) and A587G (present in all recovered clones) mutations conferred more stability to the 5′ U5 structure (data not shown). Moreover, the accumulation of mutations in different positions of the PBS loop and around the PAS motif (Fig. 3) supports the proposed hypothesis (2) that additional interactions within this region are also required to assemble an initiation-competent and processive reverse transcription complex. The preferential location of mutations associated with fitness recovery in the PBS loop suggests that a more efficient initiation of retrotranscription is a critical factor for the optimization of in vitro HIV-1 viral replication.

Both fitness loss upon plaque-to-plaque transfers and fitness recovery upon large population passages were more rapid and larger for HIV-1 than for other RNA viruses analyzed (13, 14, 47, 48). During plaque-to-plaque transfers (44), 6 of 10 HIV-1 clones became extinct, in contrast to the results of other Muller's ratchet studies with viruses for which extinction was absent or rare (6, 10). For large population passages, the HIV-1 viral populations showed very significant changes in fitness values, ranging from 1.28- to 1,780-fold, which were much higher than those with other systems (14, 33) and those obtained with fewer serial passages.

In the first five large population passages, clones G and I showed slight decreases in fitness (Table 1). Although this decrease was not significant compared to the global fitness gains observed, this observation may be related to the increase in the viral population size and also the viral diversity occurring during the transition from homogeneous clonal populations to large population passages.

It is remarkable that five mutations associated with the recovery of fitness were reversions, with three located in the coding region (Table 3) and two located in the PBS loop. Reversions in the PBS loop were recently associated with an increase in replication capacity when low-replication viruses that employed the nonself $tRNA^{Pro}$ or $tRNA_{1,2}^{Lys}$ recovered the ability to use the $tRNA_3^{Lys}$ molecule (1). The occurrence of reversions in the PAS motif indicates a high degree of functional constraint, consistent with the high structural conservation observed in this region (2, 3). Also, the abundance of reversions suggests that in HIV-1, after drastic losses, reversion is the preferred mechanism for the in vitro recovery of viral fitness, at least in this genomic region. In contrast to what occurs in foot-and-mouth disease virus, in which reversions are virtually absent during fitness recovery (<10%) (14, 22), compensatory mutations were favored in HIV-1 and also in vesicular stomatitis virus (33).

The natural history of HIV-1 infection is characterized by fluctuations in the viral population size, with transitions from extremely large populations in many infected patients to periods with drastic reductions in population sizes such as occurs during transmission. The changes in viral populations when patients undergo antiretroviral therapy, from undetectable levels for successful potent antiviral therapy to exponential growth during treatment failures, or during structured treatment interruptions are also significant. This study gives a better understanding of in vitro HIV-1 population dynamics and of the dramatic consequences of bottleneck and large population passages on HIV-1 fitness, which may be relevant to in vivo infections.

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