JOURNAL OF VIROLOGY, Jan. 1995, p. 23–31 Vol. 69, No. 1
0022-538X/95/$04.00+0

pol Gene Quasispecies of Human Immunodeficiency Virus: Mutations Associated with Drug Resistance in Virus from Patients Undergoing No Drug Therapy

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Received 5 July 1994/Accepted 23 September 1994

The nucleotide sequences of two *pol* gene regions (codons 41 to 108 and 181 to 219 of reverse transcriptase) of 60 human immunodeficiency virus type 1 genomes obtained directly from primary lymphocytes from infected individuals are reported. In addition, the mutant spectra of several quasispecies have been sampled by repetitive sequencing of molecular clones representing the same *pol* genomic regions. Average mutation frequencies ranged from 1.6×10^{-2} to 3.4×10^{-2} substitutions per nucleotide for independent samples (relative to their consensus nucleotide sequence) and from 3.6×10^{-3} to 1.1×10^{-2} substitutions per nucleotide for individual quasispecies distributions. Several mutations leading to amino acid substitutions related to loss of sensitivity to reverse transcriptase inhibitors have been identified in samples from patients not subjected to antiretroviral therapy. Mutation frequencies in the codons previously identified as involved in resistance to reverse transcriptase inhibitors were very similar to the average mutation frequencies in the *pol* region analyzed. Thus, the finding of mutations related to drug resistance (even in the absence of positive selection by the corresponding drugs) is the expected consequence of the statistical distribution of mutations along the *pol* gene. The presence of such critical amino acid replacements in human immunodeficiency virus type 1 populations underscores the importance of viral quasispecies as reservoirs of phenotypic virus variants and has a number of implications for AIDS control.

RNA viruses replicate as complex and dynamic distributions of mutant genomes termed viral quasispecies (20, 21, 25). Since infected individuals often include 10^9 to 10^{12} infectious particles (20, 26) and mutation frequencies in clonal virus populations average 10^{-3} to 10^{-5} substitutions per nucleotide (19), viral quasispecies constitute huge reservoirs of genetic variants.

Retroviruses share with other RNA viruses the potential for rapid genetic and antigenic diversification (5, 7, 8, 11, 12–14, 23, 29, 49, 57, 61, 62). In particular, the human immunodeficiency virus type 1 (HIV-1) can reach dramatic levels of heterogeneity not only within infected individuals (1, 27, 28, 39, 60) but also within an organ of an infected individual (16). The amplitude and great complexity of HIV-1 quasispecies are probably influenced by the prolonged replication of the virus in diseased individuals, the tropism of the virus for a variety of cells involved in immune surveillance, and the expression of provirus triggered by T-cell activation, irrespective of the replicative fitness of the integrated viral genome (62).

It is becoming increasingly clear that the quasispecies structure of RNA viruses represents an important obstacle in the control of viral diseases either by vaccination or by drug therapy (18, 24). Of particular concern has been the repeated selection of HIV-1 variants resistant to antiretroviral agents from patients treated with any of the major reverse transcriptase inhibitors used in AIDS therapy (2, 4, 26, 31, 33–37, 44–46, 52, 53, 54, 56, 58, 63).

The ease with which drug-resistant reverse transcriptase variants may arise in evolving HIV-1 populations will greatly depend on the mutation frequencies in the *pol* gene, on the viral load, and on the number of mutations needed to confer the resistance phenotype (17–19). Mohri et al. (40) detected HIV-1 isolates resistant to 3′-azido-3′-deoxythymidine (AZT) in plasma or peripheral blood mononuclear cells (PBMC) of patients not treated with the inhibitor. Nájera et al. (42) have recently reported the presence of mutations associated with resistance to 2′,3′-dideoxycytidine (DDC), to 2′,3′-didehydro-2′,3′-dideoxythymidine (D4T), or to nevirapine in HIV-1 isolates from patients subjected to AZT therapy. However, the viruses analyzed were cocultured in donor PBMC and replicated in MT-2 cells before PCR amplification and nucleotide sequencing. Smith et al. (55) detected three mutations related to AZT resistance (those affecting codons 67, 70, and 215) in cell-free plasma culture isolates but not in uncultured isolates from treated patients. Since adaptation of HIV-1 to cell culture may perturb the natural HIV-1 quasispecies (39), we sought to explore the natural *pol* quasispecies in lymphocytes isolated from HIV-1-infected individuals, avoiding coculture of the virus with PBMC or adaptation to established cell lines. Here we report the presence of mutations related to drug resistance in HIV-1 populations from patients not subjected to drug therapy, as well as in individual components of the mutant

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TABLE 1. Origins of the lymphocyte samples from HIV-1-infected individuals analyzed in this study

Group and sample identification[a]	Epidemiological data[b] (infection; age, sex; sampling date)	Clinical status[c]	Antiretroviral treatment[d]
A			
137	IVDA/Hom.; 27, F; 11/92	IVC1	None
276	Hom./Het.; 28, F; 11/92	IVC1	None
26	Vertical; 3m, F; 27/4/93	P2ABCD2D3D1	None
17	Vertical; 9m, F; 19/4/93	P2A	None
34	IVDA; 30, F; 5/5/93	NK	None
35	Vertical; 8m, F; 5/5/93	P2AC	None
49	Vertical; 1, M; 17/5/93	P2AD3	None
106	IVDA; 33, F; 9/6/93	NK	None
108	Vertical; 8m, F; 23/9/93	P2A	None
GR34	IVDA; 27, F; 12/92	II	None
THF13/−2	Het.; 30, F; 26/12/91	II	None
SAF15	Het.; 37, F; 12/93	II	None
V75-1	Hom.; 27, M; 5/86	516[e]	None
V75-3	Hom.; 42, M; 8/90	217[e]	None
V75-5	Hom.; 43, M; 12/89	126[e]	None
V75-7	Hom.; 35, M; 3/86	64[e]	None
P5	Vertical; 8, F; 29/11/93	P2AD3	None
P6	Vertical; 3, F; 29/11/93	P2AD3	None
P9	Vertical; 3, F; 20/12/93	P2AD2 3B	None
P11	Vertical; 3, M; 4/1/94	P2D2	None
P12	Vertical; 3, F; 17/1/94	P2D2	None
P17	Vertical; 5, F; 31/1/94	P2AD3	None
P18	Vertical; 6, F; 31/1/94	P2D2AD3	None
P19	Vertical; 6, F; 31/1/94	P2A	None
58	Vertical; 5m, F; 9/6/93	P2D2D1	None
B			
304/PO	Transf.; 6, M; 15/1/91	P2D1	AZT-88
P2	Vertical; 11, M; 15/11/93	P2D2	AZT-16
P3	Transf.; 12, F; 15/11/93	P2ACD3	AZT-48
P4	Vertical; 7, M; 22/11/93	P2ABD2	AZT-60
P8	Vertical; 1, M; 13/12/93	P2AD2	AZT-20
P13	Vertical; 6, F; 17/1/94	P2D2	AZT-32
P14	Transf.; 13, F; 24/1/94	P2AD1D3	AZT-96
P15	Vertical; 1, F; 24/1/94	P2DAD1D3	AZT-60
P16	Vertical; 9, F; 24/1/94	ADCD1D3	AZT-96
P20	Het.; 14, F; 7/2/94	P2AD3	AZT-28
P21	Vertical; 3, M; 14/2/94	ABCD3D2	AZT-40
P23	Vertical; 4, F; 14/2/94	P2E	AZT-40
D12/+20	IVDA; 28, F; 12/12/91	IVC1	AZT-48
D12/+58	IVDA; 30, F; 11/11/93	IVC1	AZT-86
D17/+20	Het.; 39, M; 7/1/92	IVC1	AZT-116
D25/+24	IVDA; 29, M; 6/5/92	IVC1	AZT-84
D29/−2	IVDA; 33, F; 5/12/91	IVC1	AZT-24
D31/−2	IVDA; 24, F; 9/1/92	IVC1	AZT-32
D31/+8	IVDA; 24, F; 26/3/92	IVC1	AZT-42
D31/+24	IVDA; 25, F; 14/12/93	IVC1	AZT-124
D35/−2	Hom.; 46, M; 30/1/92	IVC1	AZT-24
V75-2	Hom.; 27, M; 10/86	315[e]	AZT-20
V75-9	Hom.; 32, M; 5/90	222[e]	AZT-12
V75-4	Hom.; 42, M; 6/91	96[e]	DDI-44
V75-6	Hom.; 43, M; 6/90	151[e]	DDC-24

Continued on following page

spectrum in genomic populations showing no evidence of such replacements in their consensus sequence. The results document that the preexisting high frequency of mutations in the *pol* quasispecies accounts for the presence of mutations related to resistance to inhibitors without the need to invoke an increased frequency of acquisition of drug resistance mutations in the presence of the drug. In addition to defining the *pol* quasispecies in quantitative terms, these results have a number of implications for AIDS control.

MATERIALS AND METHODS

Viruses. Two groups of HIV-1 samples were analyzed. Group A includes HIV-1 isolates from patients not subjected to specific antiretroviral drug therapy, and group B includes HIV-1 isolates from patients subjected to therapy with nucleoside analogs. The origins of the HIV-1 samples analyzed and clinical statuses of the corresponding patients are summarized in Table 1.

Preparation of nucleic acids from lymphocytes. Peripheral blood lymphocytes (1×10^6 to 10×10^6 cells) from infected individuals (Table 1) were collected by centrifugation and washed with phosphate-buffered saline. The pellet was resuspended in 0.5 ml of a solution of 10 mM Tris HCl (pH 7.5), 0.15 M NaCl,

TABLE 1—*Continued*

Group and sample identification[a]	Epidemiological data[b] (infection; age, sex; sampling date)	Clinical status[c]	Antiretroviral treatment[d]
D8/+44	IVDA; 27, M; 21/4/92	IVC1	AZT-8+DDI-44
D16/+52	IVDA; 33, M; 12/8/92	IVC1	AZT-40+DDI-52
D22/+28	Hom.; 35, M; 17/5/92	IVC1	AZT-64+DDI-28
D22/+36	Hom.; 35, M; 20/7/92	IVC1	AZT-64+DDI-36
D22/+48	Hom.; 35, M; 20/7/92	IVC1	O[f]
D28/+28	Hom.; 32, F; 27/7/92	IVC1	AZT-116+DDI-28
D29/+28	IVDA; 33, F; 22/7/92	IVC1	AZT-24+DDI-28
D35/+4	Hom.; 46, M; 12/3/92	IVC1	AZT-24+DDI-4
THF13/+24	Het.; 30, F; 7/7/92	II	(AZT+THF)-24
THF17/+4	IVDA; 24, F; 10/3/92	II	(AZT+THF)-4

[a] The code given allows identification of the corresponding nucleotide sequences in Fig. 1, 2, and 4. Samples identified by a D followed by the same number correspond to samples from the same patient taken at increasing times of treatment.

[b] Infection indicates infection route (IVDA, intravenous drug abuser; Hom., homosexual transmission; Het., heterosexual transmission; Transf., transfusion; Vertical, vertical transmission in pediatric patients). Age is given in years, except when the number is followed by m, which indicates months of age. Sex: M, male; F, female. Sampling is the date at which the patient's lymphocytes were obtained: day/month/year (when only two numbers are given, they represent the month and year of isolation). All samples were from patients living in the Madrid area, except for series V75 isolates, which were from San Diego, Calif., and samples 137, 276, and GR34, which were from Granada (Spain).

[c] Clinical status corresponds to the Centers for Disease Control surveillance case definition of AIDS (9). Descriptions starting with P refer to clinical status for pediatric patients as described elsewhere (10). NK, not known.

[d] For group A samples, None means no treatment with antiretroviral inhibitors. For group B samples, the inhibitor(s) used in the treatment is indicated with the abbreviated nomenclature (DDI, 2′,3′-dideoxyinosine); the number following the drug indicates weeks of treatment at the time of sampling of lymphocytes. Two patients (THF13/+24 and THF17/+4) were subjected to combination therapy with the drugs indicated in parentheses. A plus sign (+) between two drugs indicates two successive treatments with different inhibitors. The doses of AZT during treatment varied with body weight and clinical status but generally were 500 mg/day, except in cases with central nervous system involvement, in which they were 750 to 1,000 mg/day. For DDI, the standard dose was 400 mg/day.

[e] For this sample, the $CD4^+$ cell count is given.

[f] Treatment with AZT plus DDI was interrupted 4 weeks prior to sampling of lymphocytes.

and 10 mM EDTA, adjusted to 0.2 mg of proteinase K (self-digested; 37°C, 2 h) per ml and 0.4% sodium dodecyl sulfate, heated at 65°C for 1 h, and incubated at 37°C for 7 to 12 h with gentle stirring. Next, 0.5 ml of a solution of 10 mM Tris-HCl (pH 8.0), 0.65 M NaCl, and 10 mM EDTA was added, and the mixture was extracted three times with 1 volume of phenol-chloroform-isoamyl alcohol (25:24:1, vol/vol) and once with chloroform-isoamyl alcohol (24:1, vol/vol). Nucleic acids were recovered from the aqueous phase by ethanol precipitation. The pellets were washed and resuspended in 20 μl of a solution of 20 mM Tris HCl (pH 7.5) and 2 mM EDTA.

PCR amplification of viral sequences. Primers used for PCR amplification and for nucleotide sequencing correspond to well-conserved regions of the genomes of HIV-1 subtypes A, B, and D. The primers were designed on the basis of the BH10 sequence, which differs in some positions from the CAM-1 sequence (41), used here as a reference. Such positions are also variable in natural isolates and are indicated in the description of each primer. PCR amplification was carried out with the following external primers: 5′CTTATCTATTCCATCTAAAAAT AGT3′ (complementary to positions 4252 to 4228 of HIV-1 plus-strand cDNA; the numbering is that of HIV-1 isolate CAM-1 [41]) and 5′TAGGAACCAAA GAAAGATGGTTAAG3′ (complementary to positions 1940 to 1964 of minus-strand cDNA [41], except that residues 1957 and 1958 are C and T in CAM-1 RNA, instead of T and G, respectively). The reaction was initiated with an incubation at 94°C for 5 min and then 35 cycles of incubations at 94°C for 30 s, 59°C for 30 s, and 72°C for 1 min; these were followed by incubation at 72°C for 10 min. For nested PCR, the corresponding primers were 5′TCAGTCCAGCT GTCTTTTTCTGGC3′ (complementary to positions 3312 to 3289 of CAM-1 plus-strand cDNA [41], except that residues 3298 and 3312 are G and T in CAM-1 plus-strand cDNA, instead of A and A, respectively) and 5′GTATTAG TAGGACCTACACCT3′ (complementary to positions 2477 to 2497 of minus-strand cDNA [41]). The second amplification reaction was initiated with an incubation at 94°C for 5 min and then 35 cycles of 94°C for 30 s, 55°C for 30 s, and 72°C for 1 min; the last incubation was at 72°C for 10 min. The PCRs were carried out in a solution of 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$, a 0.1 mM concentration of each of the four deoxynucleotide triphosphates, and 0.01% gelatin.

Molecular cloning. PCR products were cloned into pGEM-5Zf(+) (Promega) by the protocol of Sambrook et al. (50) for blunt-end ligation. The efficiency of transformation was 5×10^6 to 5×10^7 transformants per μg of DNA. DNA from individual transformants was prepared by standard procedures (50).

Nucleotide sequencing. Consensus nucleotide sequences (defined by the dominant base in the population of amplified HIV-1 genomes at each position) were determined by the fmol method (Promega) on PCR-amplified DNA; sequence determinations were followed by treatment of the reaction mixture with terminal deoxynucleotidyl transferase to minimize ambiguities in sequencing gels derived from premature chain terminations (15). Two *pol* genomic regions were sequenced: nucleotides 2672 to 2875 (41) (which correspond to nucleotides 121 to 324 of the reverse transcriptase-coding region [amino acids 41 to 108]) and nucleotides 3092 to 3208 (which correspond to nucleotides 541 to 657 of the reverse transcriptase-coding region [amino acids 181 to 219]). The primers used to sequence nucleotides 2672 to 2875 were 5′GCACGATATCTA ATCCTGGTGTCTCA3′ (this primer is complementary to residues 2984 to 2962 of CAM-1 plus-strand cDNA [41], except that the four 5′-terminal residues do not match the CAM-1 viral sequence and that the authentic viral sequence includes three successive G residues instead of two at positions 2972 to 2974) and 5′GCCAGGAATGGATGGCCCAA3′ (complementary to residues 2590 to 2609 of the minus-strand cDNA [41]). The primers used to sequence nucleotides 3092 to 3208 were 5′TCAGTCCAGCTGTCTTTTTCTGGC3′ (complementary to positions 3312 to 3289 of CAM-1 plus-strand cDNA [41], except that residues 3298 and 3312 are G and T in CAM-1 plus-strand cDNA instead of A and A, respectively) and 5′GGATTAGATATCAGTACAATGTGCTT3′ (complementary to residues 2973 to 2998 of the minus-strand cDNA [41]).

To sequence individual genomic molecules, plasmid DNA from at least 20 transformants was sequenced with T7 DNA polymerase (Pharmacia Biotech) and with the same primers used for the determination of average sequences.

Nucleotide sequence accession numbers. The sequences reported here have been deposited in the GenBank database under accession numbers U14786 to U14903.

RESULTS

Mutations related to drug resistance in the *pol* gene of HIV-1 quasispecies from patients either not subjected to drug therapy or treated with unrelated drugs. Consensus nucleotide sequences were determined for the HIV-1 genomic regions encoding amino acids 41 to 108 and 181 to 219 of reverse transcriptase from 25 samples corresponding to patients not subjected to drug therapy (group A in Table 1) and 35 samples from patients treated with nucleoside analogs (in a few cases in combination with thymic humoral factor [THF]) (group B in Table 1). The results (Fig. 1 and 2) show mutations scattered along the entire region analyzed compared with the respective consensus sequence. Some mutations affected codons related to resistance to reverse transcriptase inhibitors. In particular, in seven of the samples from patients not subjected to antiviral therapy (34, 35, 106, GR34, THF13/−2, V75-5, and P9 [Table 1 and Fig. 1]), mutation A-209→G, corresponding to replacement K-70→R, which contributes to AZT resistance (37), was present. In sample THF17/+4, belonging to a patient subjected

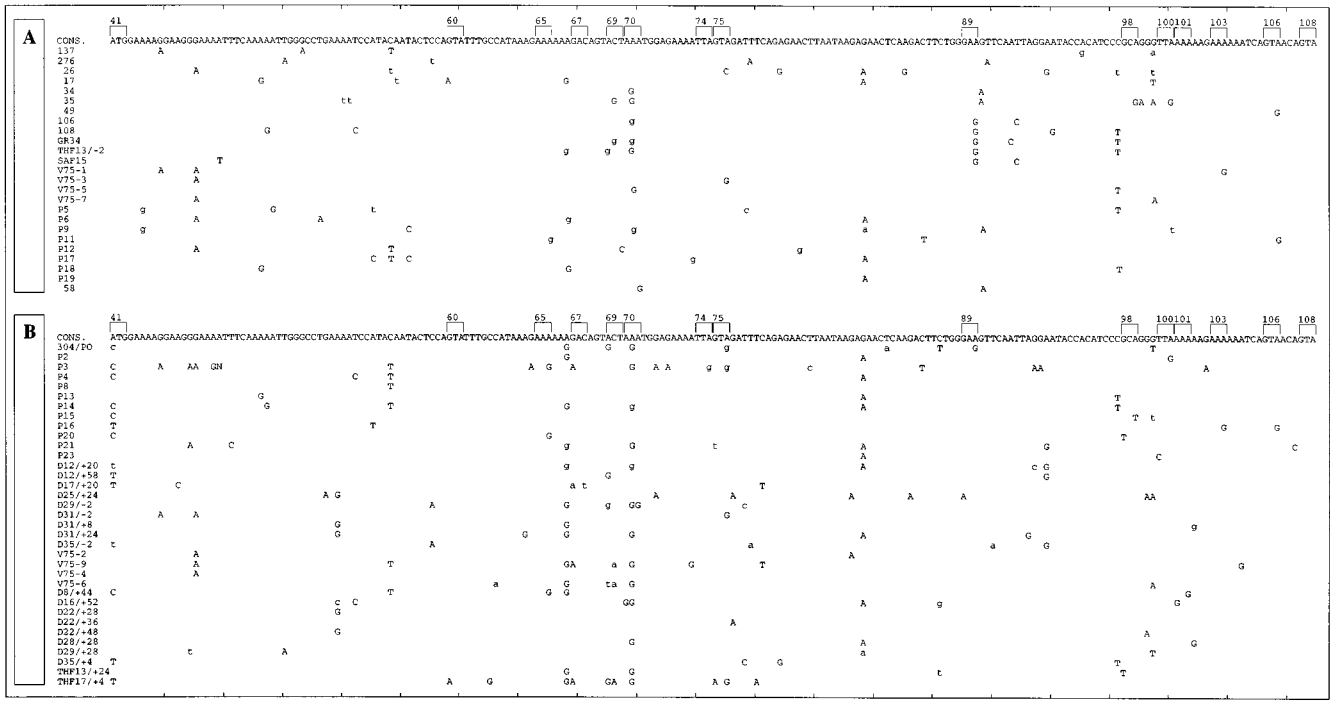

FIG. 1. Nucleotide sequence at residues 121 to 324 of the HIV-1 reverse transcriptase genomic region, encoding amino acids 41 to 108 of reverse transcriptase. The two sets of sequences (A and B) correspond to samples from group A (patients not subjected to antiretroviral therapy) and group B (patients subjected to antiretroviral therapy), as listed in Table 1. Only those nucleotides which differ from the consensus (cons.; defined for each group [A or B] of sequences analyzed) are indicated. The consensus for group A and B sequences differs from the HIV-1 CAM-1 sequence (41) in residues 135 (which is A in CAM-1) and 291 (which is T in CAM-1). Lowercase letters indicate an ambiguity in the sequencing gel, with predominance of the band corresponding to the nucleotide given; at least 90% of the sequences (including each position given as a lowercase letter) were determined for the two DNA strands with the primers and by the procedures described in Materials and Methods. Samples P3 and D25/+24 included a mutation leading to a termination codon (G-213→A, codon 71), presumably reflecting dominance of defective genomes in the proviral population. In particular, D25/+24 may be partially hypermutated (see also the legend to Fig. 2).

to combined AZT plus THF therapy (Table 1), amino acid replacement V-75→M (mutations G-223→A and A-225→G [Fig. 1]) was found; M-75 has been recently associated with a 30-fold loss of sensitivity to D4T (42). THF17/+4 included also amino acid replacement T-69→D (mutations A-205→G and C-206→A [Fig. 1]), which has been associated with resistance to DDC (26). Sample D16/+52 showed substitution K-101→E (mutation A-301→G [Fig. 1]), which is related to resistance to nonnucleoside analogs (6). As expected, among viruses from patients subjected to AZT therapy, multiple amino acid substitutions related to diminished sensitivity to this inhibitor (35) were observed. Additional mutations critical for loss of sensitivity to nucleoside or to nonnucleoside analogs have been identified both in the consensus sequence of some samples and within individual quasispecies (see below); the relevant amino acids are highlighted in Fig. 3.

Mutation frequencies explain the occurrence of mutations related to drug resistance in the absence of positive selection by the drugs. Mutation frequencies were calculated for each set of sequences relative to the corresponding consensus, as well as for the subset of codons previously identified as related to resistance to antiretroviral agents (52, 53) (Fig. 3). The results (Table 2) show mutation frequencies ranging from 0.8 $\times 10^{-2}$ to 3.4 $\times 10^{-2}$ substitutions per nucleotide, with similar values for the entire sequence analyzed and for the codons specifying amino acids related to loss of reverse transcriptase sensitivity to inhibitors.

Analysis of the mutant spectra of individual quasispecies. To sample the mutation composition of some quasispecies, we cloned and sequenced individual genomes from four viral samples. Viruses 137, V75-5, and D17/+20 (Table 1) were chosen at random, whereas virus 49 was selected for analysis because its consensus sequence suggested potential heterogeneity at codon 184 (Fig. 2). The nucleotide sequences of 20 or 21 clones analyzed for each viral sample are summarized in Fig. 4, and the amino acid replacements found in codons associated with changes in drug sensitivity are depicted in Fig. 3. In quasispecies 137, one clone included mutation A-209→G, which implies amino acid replacement K-70→R. In quasispecies 49, and in confirmation of the heterogeneity suggested by the consensus sequence, 9 clones showed A and 11 clones showed G at nucleotide 552; thus, two subpopulations about equally represented included amino acid M or I at position 184. The latter amino acid has been associated with resistance to (−)-β-2′,3′-dideoxy-5-fluoro-3′-thiacytidine (FTC) and (−)-β-2′,3′-dideoxy-3′-thiacytidine (3TC) (4, 58) (Fig. 3). In quasispecies V75-5, 8 of 20 clones included C-206→A, which leads to replacement T-69→N. This substitution by itself has not been associated with loss of sensitivity to any drug; however, C-206→A, along with A-205→G, is needed to produce replacement T-69→D, associated with resistance to DDC (26) (Fig. 3). Thus, this quasispecies included arrays of variants with diminished genetic distance to effect the transition from sensitivity to resistance to DDC. Again, extensive heterogeneity was seen at position 209, with genomes harboring either amino acid K-70 or amino acid R-70 (compare Fig. 3 and Fig. 4). Finally, sample D17/+20 corresponded to a patient which had undergone AZT therapy for 116 weeks (Table 1), and not surprisingly, its mutation spectrum depicted complete dominance of L-41 and of Y-215 (31, 35, 37). In addition, one clone

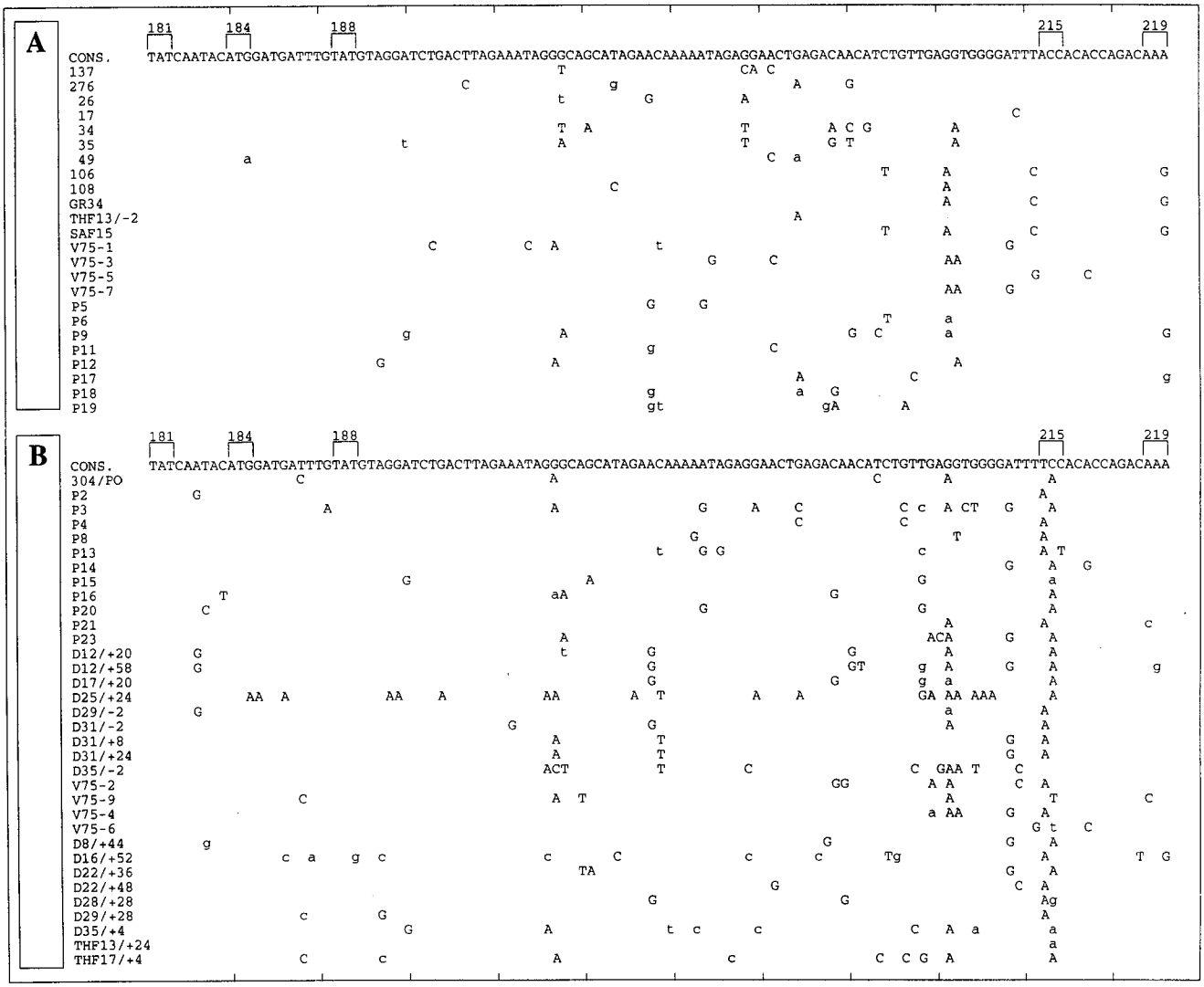

FIG. 2. Nucleotide sequence of residues 541 to 657 of the HIV-1 reverse transcriptase genomic region, encoding amino acids 181 to 219 of reverse transcriptase. Symbols are as in Fig. 1. The consensus (cons.) for group A sequences differs from the HIV-1 CAM-1 sequence (41) in residue 640 (which is C in CAM-1). The consensus for group B sequences differs from the HIV-1 CAM-1 sequence in residues 640 and 643 (which are C and A, respectively, in CAM-1); at least 90% of the sequences (including each position given as a lowercase letter) were determined for the two DNA strands with the primers and by the procedures described in Materials and Methods. Sample D25/+24 included two termination codons, each due to a double mutation (T-629→G, G-630→A, codon 210, and G-635→A, G-636→A, codon 212). This sample included 85% G→A transitions among the mutations scored, and it may correspond to a partially hypermutated genome according to published criteria (59).

showed mutation A-309→C, which induces amino acid replacement K-103→N, a substitution associated with resistance to pyridinones (6, 44).

***pol* mutation frequencies within quasispecies.** Average mutation frequencies within individual quasispecies ranged from 3.6×10^{-3} to 1.1×10^{-2} substitutions per nucleotide (Table 3). Since only 24 mutations affected codons specifying amino acids previously related to drug resistance—and 18 of them belonged to sample V75-5 (Fig. 4)—mutation frequencies for the last subset of codons were not reliable. However, in population V75-5, mutation frequencies at such codons were comparable to the average in all of the regions analyzed (Table 3).

Thus, the reverse transcriptase-coding region shows high mutation frequencies in natural HIV-1 quasispecies. Substitutions related to resistance to antiretroviral agents occur in viral sequences from patients not exposed to the relevant inhibitors. The comparison of average mutation frequencies with those at the critical codons suggests that the presence of mutations related to drug resistance—even in the absence of selection by the corresponding drugs—is the expected consequence of the high mutation frequencies in the *pol* gene.

DISCUSSION

High mutation frequencies in a functionally essential enzyme of HIV-1. *pol* gene mutation frequencies have been estimated from the number of mutations scored upon the sequencing of a total of 45,348 nucleotides (Fig. 1, 2, and 4). Values for the mutant spectra of individual quasispecies were, on average, fourfold lower than for the comparison of independent isolates (compare Tables 2 and 3). As an internal control for *Taq* polymerase errors, we have carried out repetitive sequencing of molecular clones derived from reverse

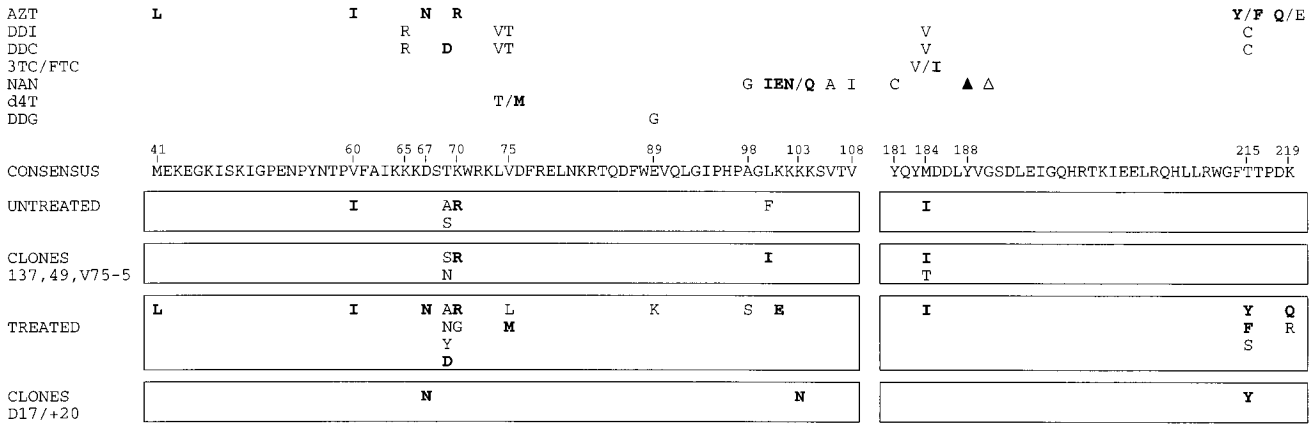

FIG. 3. Summary of amino acid replacements found at the codons specifying amino acids related to resistance to reverse transcriptase inhibitors. The single-letter amino acid code is used. The consensus amino acid sequence is that of all group A samples (Table 1) examined in the present study, and it is identical to the HIV-1 CAM-1 sequence (41). Above the consensus, amino acid replacements related to resistance to reverse transcriptase inhibitors (as compiled in references 47, 52, 53, and 63) are indicated (listed on the left of the corresponding line). Abbreviations for inhibitors are as follows: DDI, 2′,3′-dideoxyinosine; NAN, nonnucleoside inhibitors (a group which includes nevirapine, pyridinones, and TIBO derivates [given in reference 52]); DDG, 2′,3′-dideoxyguanosine. A bar separating two amino acids denotes their association with resistance to the same inhibitor; the filled triangle corresponding to position 188 for NAN indicates that resistance has been associated with amino acids C, H, and L (51); likewise, the empty triangle at position 190 indicates that resistance has been associated with amino acids A, E, and S. Below the consensus sequence, the amino acid replacements found in our analysis are grouped in boxes: UNTREATED, samples from group A (Table 1); CLONES 137, 49, and V75-5, mutant spectra of these three group A samples; TREATED, samples of group B (Table 1); CLONES D17/+20, mutant spectrum of this group B sample. Boldface letters indicate amino acids associated with drug resistance found in our analysis. Amino acid replacements related to loss of sensitivity to reverse transcriptase inhibitors have been recently reviewed (35, 52, 53).

transcription and PCR amplification of RNA from a foot-and-mouth disease virus (FMDV) clone highly adapted to its cell culture environment. FMDV RNA was extracted from a single plaque without any further viral replication. The plaque was grown in BHK-21 cells and was originated from a FMDV population which had been serially passaged 40 times in BHK-21 cells. The mutation frequency found was less than 1.2×10^{-4} substitutions per nucleotide; this should represent an upper limit for the introduction of errors by *Taq* polymerase during PCR amplification with HIV-1 in this study. In addition, independent amplifications of the same HIV-1 samples with either identical or different oligonucleotide primers have repeatedly yielded indistinguishable sequences (44a). Thus, even if it cannot be completely excluded that some mutations could arise during early cycles of PCR amplification, it is extremely unlikely that they have biased the estimates of mutation frequencies. Furthermore, each of the substitutions related to drug resistance was scored either by sequencing the two DNA strands or by sequencing the products of two (or more) different PCR amplifications. The two sequenced regions encode amino acids 41 to 108 and 181 to 219 of HIV-1 reverse transcriptase (Fig. 3). The latter domain is critical for nucleotide polymerization, and amino acids D-185 and D-186 are thought to be involved in catalysis (32, 38). Interestingly, among HIV-1 isolates from patients not subjected to antiretroviral therapy (group A in Table 1), but not among those subjected to therapy (group B in Table 1), mutation frequencies between codon 181 and 195 were substantially lower than for the region spanning codons 196 to 219; for group A patients, the values for these two segments were 6.4×10^{-3} and 4.3×10^{-2} substitutions per nucleotide, while for group B, the corresponding values were 1.7×10^{-2} and 5.1×10^{-2} substitutions per nucleotide, respectively (calculated from the data of Fig. 2). Perhaps replication of HIV-1 in the presence of reverse transcriptase inhibitors tends to fix constellations of compensatory mutations to preserve functionality of the critical domain spanning amino acids 181 to 195. It is highly unlikely that patients not subjected to antiretroviral therapy had been infected by individuals who had developed resistance to inhibitors and that the mutants we found were the result of further amplification in the recipient individuals of such resistant variants. No evidence from the clinical histories of the patients points to such a possibility. The only inhibitors available for routine treatment in Spain are AZT, 2′,3′-dideoxyinosine, and to a more limited extent, DDC (43). Administration of the other drugs listed in Fig. 3 was started later than the times of isolation of lymphocytes which included HIV-1 sequences with replacements related to the development of resistance, and their use has been restricted to a few experimental clinical trials. For example, D4T was not used until October 1993, whereas lymphocytes from sample THF17/+4, harboring M-75, were isolated in 1992 (Table 1). Quasispecies 49 (a pediatric sample isolated in May 1993 [Table 1]) included an abundant subpopulation with replacement M-184→I, asso-

TABLE 2. Mutation frequencies in the *pol* gene of HIV-1 samples from several patients

Sample group	Mutation frequency[a] (10^{-2})			
	Residues 121 to 324[b]		Residues 541 to 657[b]	
	Avg	Codons related to drug resistance	Avg	Codons related to drug resistance
Untreated (A in Table 1)	1.6	2.0	2.5	1.1
Treated (B in Table 1)	2.0	2.9	3.4	0.8

[a] Mutation frequency is defined as the proportion of mutation positions relative to the consensus nucleotide sequence for each sample group; the frequencies have been calculated by dividing the number of mutations (relative to the consensus) by the total number of nucleotides sequenced in the corresponding group (data are in Fig. 1 for residues 121 to 324 and in Fig. 2 for residues 541 to 657). Mutations shown in lowercase letters in Fig. 1 and 2 (ambiguities in sequencing gels) and mutations at positions 643 and 644 which show extreme heterogeneity (consensus defined by less than 60% of the sequences [Fig. 2]) have not been included in the calculations.

[b] Numbering is for the reverse transcriptase coding region; residues 121 to 324 and 541 to 657 encode amino acids 41 to 108 and 181 to 219, respectively.

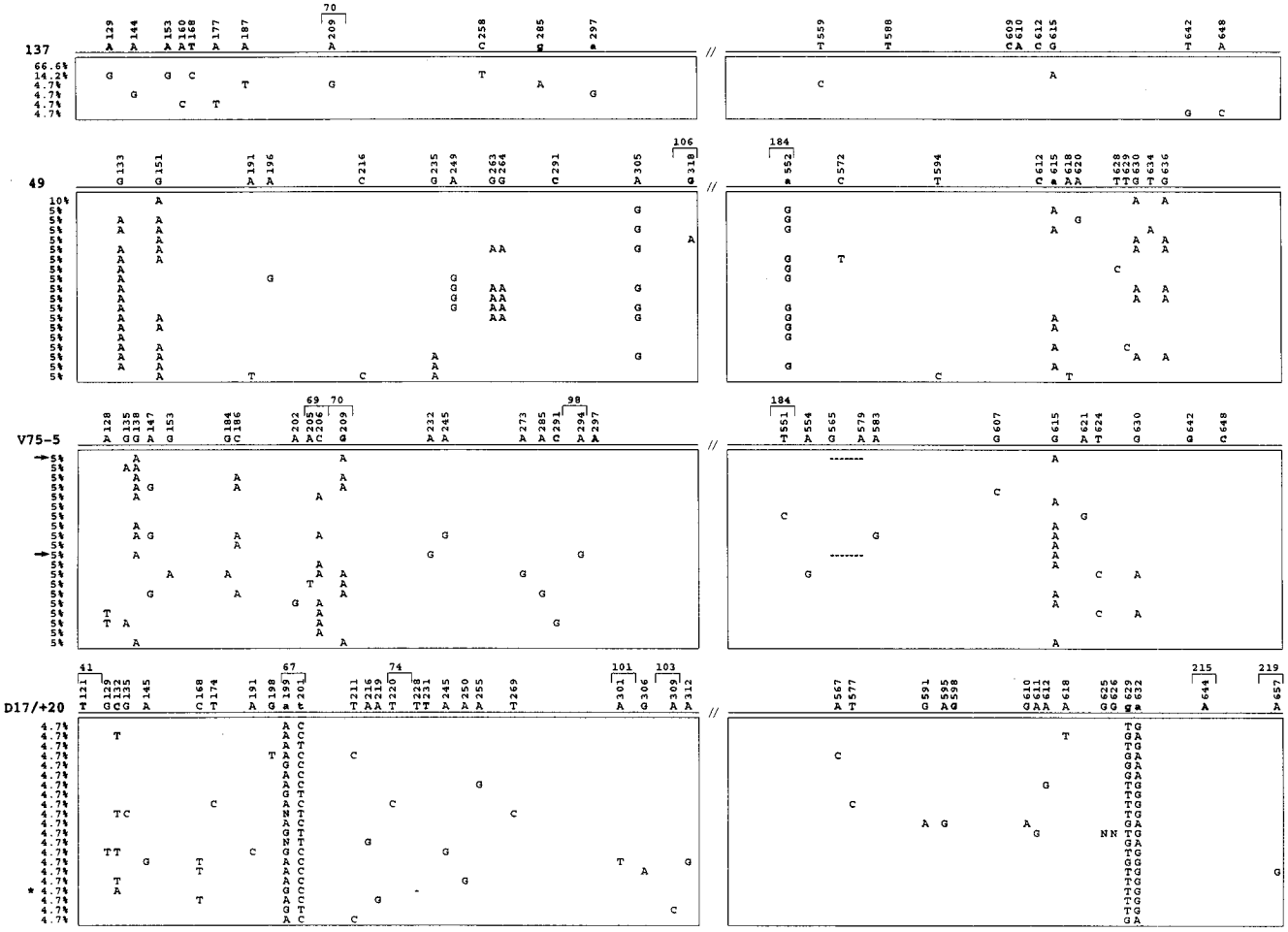

FIG. 4. Mutant spectra of quasispecies 137, 49, V75-5, and D17/+20. The origins of these HIV-1 samples are described in Table 1. In each box, unique nucleotide sequences in the entire sequenced region (left boxes, residues 121 to 324; right boxes, residues 541 to 657) are written on a separate line; the relative abundance of each particular sequence is given as the percentage of the total number of clones analyzed (20 for samples 49 and V75-5 and 21 for samples 137 and D17/+20). The line at the top of each box represents consensus nucleotides (determined experimentally by sequencing the uncloned PCR product) found variant with respect to any clone of the corresponding mutant spectrum; boldface letters indicate nucleotides which differ from the consensus for the corresponding group of samples (group A for quasispecies 137, 49, and V75-5, and group B for quasispecies D17/+20), as given in Fig. 1 and 2. Lowercase letters correspond to positions showing ambiguities (multiple bands) in the sequencing gel patterns. The two arrows in the V75-5 box point to clones with a deletion spanning nucleotides 565 to 579 (amino acids 189 to 193); this deletion did not originate a change in the open reading frame. The asterisk in the D17/+20 box points to a clone with a deletion of residue T-228; this deletion leads to a change in the reading frame and to an amber codon 16 triplets downstream; thus, this clone must correspond to a defective HIV-1 genome. Mutant frequencies (Table 3) have been calculated by excluding extremely heterogeneous positions (positions 133, 151, and 552 in quasispecies 49, and positions 199, 201, 629, and 632 in D17/+20).

ciated with resistance to FTC or 3TC (Fig. 3 and 4); 3TC was in very restricted use beginning only in July 1993, and FTC is not yet available. Furthermore, since mutation frequencies at the codons related to resistance to reverse transcriptase inhibitors are comparable to the average (Tables 2 and 3), it is expected that, for statistical reasons, mutations will occasionally affect the relevant codons. As many as 13 amino acids previously associated with diminished sensitivity to retroviral inhibitors were identified in the sampling of natural HIV-1 quasispecies (Fig. 3). About equal numbers of amino acid replacements relevant to drug resistance and replacements not associated with resistance (within the same subset of codons) were recorded (Fig. 3). This supports the proposal that the critical substitutions found are the result of the occurrence of random mutations in the absence of selection by the inhibitors.

Implications for AIDS control. The selection of drug-resistant RNA viruses in vivo or in vitro has been previously reported for several viruses (reviewed in reference 17). Among other examples, influenza viruses resistant to amantadine and rimantadine (3), rhinoviruses with various degrees of resistance to isoxazole derivatives (WIN compounds) (30), or Sindbis virus resistant to ribavirine (51) reflect the abilities of viral proteins to vary to counteract the effect of inhibitors while remaining functional. The tolerance of reverse transcriptase to accept amino acid substitutions is clearly illustrated by our analysis of average sequences in clinical specimens (Fig. 1 and 2) as well as by the sampling of molecular clones from mutant spectra (Fig. 3). In particular, mutation A-209→G (amino acid replacement K-70→R) was found in seven samples from untreated patients (Fig. 1) and in the mutant spectra of quasispecies 137 and V75-5 (Fig. 4). This is the same substitution previously identified in isolates from untreated patients from an unrelated geographical area (40), and it may correspond to a genomic position particularly tolerant to change. Indeed, the relevant nucleotide is located in a predicted unpaired bulge loop in *pol* RNA (53), and the

TABLE 3. Mutation frequencies within individual HIV-1 *pol* quasispecies

Sample[a]	Mutation frequency[b]			
	Residues 121 to 324		Residues 541 to 657	
	Avg	Codons related to drug resistance	Avg	Codons related to drug resistance
137	4.4×10^{-3}	1.0×10^{-3}	3.6×10^{-3}	$<3.2 \times 10^{-3}$
49	7.8×10^{-3}	1.1×10^{-3}	8.7×10^{-3}	$<3.2 \times 10^{-3}$
V75-5	1.1×10^{-2}	1.8×10^{-2}	8.5×10^{-3}	3.3×10^{-3}
D17/+20	6.3×10^{-3}	3.3×10^{-3}	3.6×10^{-3}	3.3×10^{-3}

[a] The origins of the samples are given in Table 1. The nucleotide sequences on which calculation of mutation frequencies are based are given in Fig. 4.

[b] Mutant frequencies, defined as in footnote *a* to Table 2, have been calculated by dividing the number of mutations (relative to the corresponding consensus for each quasispecies) by the total number of nucleotides sequenced (given by each box in Fig. 4). Mutations at residue 552 of quasispecies 49 and at residues 199, 201, 629, and 632 of quasispecies D17/+20, which show extreme heterogeneity (consensus defined by less than 60% of the sequences [Fig. 4]), have not been included in the calculations.

corresponding amino acid is placed at an exterior loop between β-sheets numbers 3 and 4 in the fingers domain of reverse transcriptase (32). In support of the remarkable tolerance of reverse transcriptase to variation is that the alignment of 31 functional reverse transcriptases from widely different origins revealed that only 3 of about 300 residues were invariant in all the enzymes (22). A similar functional flexibility is apparent for retroviral proteases (62). Unless the genetic changes needed for HIV-1 to become resistant to an antiviral agent (or to the combined action of several agents) entailed profound fitness losses in the virus, resistant HIV-1 isolates requiring one or a few amino acid substitutions will appear with high probability during HIV-1 replication in infected individuals, given the estimated mutation frequencies reported here and the population numbers involved during disease progression (62). It must be emphasized that the absence of a phenotypically relevant mutation among the molecular clones sampled does not ensure that such a mutation may not be present at a lower proportion in the same population or that it may arise de novo on subsequent replication events. Mutation and recombination may both contribute to rescuing high-fitness HIV-1 variants harboring phenotypically relevant genetic alterations. Even partially hypermutated molecules may serve to expand the phenotypic reservoir, since such molecules are not necessarily associated with defective genomes, as recently documented for hypermutated forms of viable respiratory syncytial virus (48). The potential of evolving quasispecies to replenish permanently the variant pool cautions against indiscriminate use of antiretroviral agents (19, 24) unless clinical benefits can be clearly documented during the period of treatment before resistant variants become dominant.

ACKNOWLEDGMENTS

We are indebted to C. Escarmis for valuable suggestions, to D. D. Richman for the supply of sample series V75, to A. Bernal for series THF, and to I. González for samples 137, 276, and GR34.

Work at the Centro de Biología Molecular "Severo Ochoa" was supported by grant FIS n. 93/0003-01 and the Fundación Ramón Areces. Work at Instituto Carlos III was supported by a Concerted Action between Brystol-Myers Squib and Instituto Carlos III and by grant FIS 94/0317. I.N. was supported by a predoctoral fellowship from Instituto de Salud Carlos III-Bristol Myers Squibb (Spain), A.H. was supported by a predoctoral fellowship from Comunidad Autónoma de Madrid (Spain), and M.E.Q.-M. was supported by a predoctoral fellowship from CONICIT (Venezuela).

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