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Sequencing-Based Detection of Low-Frequency Human Immunodeficiency Virus Type 1 Drug-Resistant Mutants by an RNA/DNA Heteroduplex Generator-Tracking Assay

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Drug-resistant viruses may be present as minority variants during early treatment failures or following discontinuation of failed antiretroviral regimens. A limitation of the traditional direct PCR population sequencing method is its inability to detect human immunodeficiency virus type 1 (HIV-1) variants present at frequencies lower than 20%. A drug resistance genotyping assay based on the isolation and DNA sequencing of minority HIV protease variants is presented here. A multiple-codon-specific heteroduplex generator probe was constructed to improve the separation of HIV protease genes varying in sequence at 12 codons associated with resistance to protease inhibitors. Using an RNA molecule as probe allowed the simple sequencing of protease variants isolated as RNA/DNA heteroduplexes with different electrophoretic mobilities. The protease gene RNA heteroduplex generator-tracking assay (RNA-HTA) was tested on plasma quasispecies from 21 HIV-1-infected persons in whom one or more protease resistance mutations emerged during therapy or following initiation of salvage regimens. In 11 of 21 cases, RNA-HTA testing of virus from the first episode of virologic failure identified protease resistance mutations not seen by population-based PCR sequencing. In 8 of these 11 cases, all of the low-frequency drug resistance mutations detected exclusively by RNA-HTA during the first episode became detectable by population-based PCR sequencing at the later time point. Distinct sets of protease mutations could be linked on different genomes in patients with high-frequency protease gene lineages. The enhanced detection of minority drug resistance variants using a sequencing-based assay may improve the efficacy of genotype-assisted salvage therapies.

A frequent cause of treatment failure in human immunodeficiency virus type 1 (HIV-1)-infected persons is the emergence of viruses resistant to antiretroviral (ARV) drugs. A number of studies have shown that viral drug resistance genotyping can improve virologic outcome (6, 9, 10, 22, 74). Resistance to ARV drugs can be determined by identifying primary drug resistance mutations known to confer increased resistance to specific ARV drugs and secondary drug resistance mutations that further increase resistance and can improve the replicative fitness of viruses carrying primary drug resistance mutations (25). Recent studies have also indicated that the presence of minority drug-resistant variants may also be an independent predictor of virological failure (37, 40). This may be particularly relevant in persons in whom drug-resistant variants are only beginning to emerge or who have discontinued treatment and whose drug-resistant variants become displaced by preexisting fitter wild-type variants (14, 40).

Sequence-based genotyping can be performed either by direct PCR product sequencing (also called population-based or bulk sequencing) or by sequencing multiple subclones derived from a PCR product. Direct PCR sequencing is primarily used in the clinical setting, but one of its major limitations is its inability to consistently detect minority variants present at frequencies below 10 to 25% (47, 49, 64, 76). The presence of mixed bases in clinical samples is also largely responsible for discordant results when the same samples are analyzed in different laboratories or using different nonsequencing methods (20, 28, 36, 38, 66). The laborious nature of sequencing multiple plasmid subclones, where the major variant may be resequenced multiple times (50), largely restricts this approach to research settings (3, 11, 30, 36, 38, 42, 48, 54, 56, 57, 62).

To increase the sensitivity of current sequencing-based genotyping methods, we developed a method for the separation and sequencing of minority drug-resistant variants. We present here this method and its application, using clinical samples from persons in whom HIV-1 developed new drug resistance mutations while on a failing treatment regimen(s), and we compare the results to direct PCR population sequencing.

MATERIALS AND METHODS

Synthesis of the HIV-1 protease gene universal heteroduplex generator (UHG). The DNA template used for synthesis of the RNA probe was synthesized by assembling 18 oligonucleotides (each 30 to 48 nucleotides long) into a highly mutated version of the HIV-1 protease gene. Gene assembly was carried out as described elsewhere, with minor modifications (70). A 250 μM concentration of each oligonucleotide was mixed, and the mixture was subsequently diluted 100-fold in 50 μl of a PCR buffer containing 10 mM Tris-HCl (pH 9.0), 50 mM KCl, 2.5 mM $MgCl_2$, 0.1% Triton X-100, a 2.5 mM concentration of each deoxynucleoside triphosphate, 3.5 U of *Taq* polymerase, and 0.05 U of *Pfu* polymerase (Promega, Madison, Wis.). The PCR program consisted of 50 cycles of 94°C for 30 s, 50°C for 30 s, and 72°C for 30 s. The oligonucleotides used were the following (5′ to 3′): PF1, GAAGCAGGAGCCGATAGACAAGGAAC TGTATCCTTTAACT; PF2, TCCCTCAGATCACTCTTTGGCAACGACCG CTCGTCACAAT; PF3, AAAGATAGGGGGGCAACTAAAGGAAGCTCT

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ATTAGATACA; PF4, GGAGCAGATCGATACTGTATTAGAACAAATG AATTTGCC; PF5, AGGAAGATGGAAACCAAAAAAGATAGGCGGGA AATGGA; PF6, GGTTTTAATCAAAGTAAGACAGTATGATCAGATAC TCATA; PF7, GAAATCTGTGGACATAAAGCATTAGGTACAGTATTA GTAG; PF8, GACCTACACCTGATCAACAATAATTGGAAGTAATCTG TCTGACTC; PF9, AGATTGGTTGCACTTTAAATTTTCCCATTAGCCC TATTGAGACTGTACCAG; PR1, CTGGTACAGTCTCAATAGGGCTAA TGGGA; PR2, AAATTTAAAGTGCAACCAATCTGAGTCAGACAGATTA CTTCCAA; PR3, TTATTGTTGATCAGGTGTAGGTCCTACTAATACTGT ACCTAATGCTTTATG; PR4, TCCACAGATTTCTATGAGTATCTGATC ATACT; PR5, GTCTTACTTTGATTAAAACCTCCATTTCCCGCCTATC TTTTT; PR6, TGGTTTCCATCTTCCTGGCAAATTCATTTCTTCTAAT ACA; PR7, GTATCGATCTGCTCCTGTATCTAATAGAGCTTCCTTTAG; PR8, TTGCCCCCCTATCTTTATTGTGACGAGCGGTCGTTG; and PR9, CCAAAGAGTGATCTGAGGGAAGTTAAAGGATACAGTTCCTTGTCTA TCGGCTCCTGCTTC. After the initial gene assembly PCR, the reaction mixture was diluted 40-fold in 100 μl of the same PCR buffer, with deoxynucleoside triphosphates plus 10 pmol of each flanking primer: EDPR3, GAAGCAGGAG CCGATAGACAAGG (HXB2 positions 2211 to 2233); EDPR4, CTGGTACA GTTTCAATAGGACTAATGG (HXB2 positions 2551 to 2577). The second PCR program consisted of three cycles of 94°C for 45 s, 57°C for 45 s, and 72°C for 45 s, followed by 34 cycles of 94°C for 30 s, 57°C for 30 s, and 72°C for 30 s, and final extension at 72°C for 5 min. A 100-μl aliquot of the PCR product was run in a 1.5% agarose gel, and the 360-bp band was purified (QIAQuick gel extraction kit; QIAGEN Inc.). The mutated protease gene DNA fragment was subcloned into the pGEM-T plasmid vector (Promega), and 10 subclones were sequenced using M13 reverse primer. One subclone with the expected sequence was used in all subsequent experiments. The resulting plasmid (pAK1-pro1UHG), necessary for generating the protease RNA probe, is available upon request.

RNA probe synthesis. The mutated protease gene insert of pAK1-pro1UHG was amplified from 10 ng of plasmid as above but for only 20 PCR cycles with primers EDPR3 and T7-EDPR4 (T7 RNA polymerase promoter region [TAA TACGACTCACTATAGGG] added to the 5′ end of primer EDPR4). A 100-μl aliquot of the PCR product was run in a 2% agarose gel, and the 370-bp band was purified from the gel. One microliter of the purified PCR product was added to 100 μl of transcription mixture containing 20 μl of 5× transcription buffer, 10 μl of 0.1 M dithiothreitol, 20 μl of 2.5 mM (each) ribonucleoside triphosphate, 100 U of human placental RNase inhibitor, and 45 U of T7 RNA polymerase. The mixture was incubated for in vitro transcription at 37°C for 90 min, followed by 70°C for 15 min to inactivate the enzyme.

Virus isolates. The plasma samples of persons undergoing direct PCR population sequencing at Stanford University Hospital Diagnostic Virology Laboratory meeting the following criteria were chosen for further evaluation: (i) two genotypes were performed within the course of 1 year, each following virologic failure on a protease inhibitor-containing regimen; and (ii) the second genotype contained at least one major protease inhibitor resistance mutation that was not observed in the direct PCR-based sequence at the time of the first virologic failure. Further analyses were performed on the first virologic failure sample of all patients meeting this criteria and on the second virologic failure sample if that sample was also available. The study was approved by the Stanford University Hospital and University of California San Francisco human subjects committees. All testing was performed anonymously.

Viral RNA isolation and reverse transcription-nPCR. Viral RNA was extracted from plasma and reverse transcribed as previously described (21). PCR primers used for the first round of nested PCR protease gene amplification were EDPR1 (GAGCAGACCAGAGCCAACAGCCCCA [HXB2 positions 2139 to 2163]) and EDPR2 (TTGTTTAACTTTTGGGCCATCC [HXB2 positions 2597 to 2618]). Second-round primers were EDPR3 and EDPR4. Each of the nested PCR (nPCR) programs consisted of three cycles of 94°C for 45 s, 57°C for 45 s, and 72°C for 45 s, followed by 27 cycles of 94°C for 30 s, 57°C for 30 s, and 72°C for 30 s, and final extension at 72°C for 5 min. Using pNL4-3 dilutions, the sensitivity of this nPCR was determined to be between 1 and 10 copies (21).

RNA/DNA heteroduplex formation and gel electrophoresis. For hybridization reactions, 2 μl of the UHG-pro1 RNA probe was mixed with 3 μl of patients' nPCR products and 0.5 μl of 10× heteroduplex annealing buffer (100 mM NaCl, 10 mM Tris-HCl [pH 7.5], and 2 mM EDTA), incubated at 95°C for 2 min, and rapidly transferred to ice for hybridization at 4°C. The RNA/DNA heteroduplexes were then separated in a 7.5% gel (29:1 acrylamide-bis solution) with 1.25× Tris-acetate-EDTA electrophoresis buffer at 250 V in a temporal temperature gradient of 26 to 45°C over 6 h (ramp time of 3.5°C increase per h) using the Decode Universal mutation detection system electrophoretic gel apparatus (Bio-Rad Laboratories). Temporal temperature gradient electrophoresis exploits the principle of sequence-dependent differences in DNA melting and slightly improves the separation of protease sequence variants.

Isolation of RNA/DNA heteroduplexes and DNA sequencing. After electrophoresis, polyacrylamide gels were stained with ethidium bromide (0.5 μg/ml) and placed on a UV illuminator. Following photographic documentation using a charged-coupled device camera, five small gel pieces of approximately 10 μl were taken from each gel lane by using a 1,000-μl PCR pipette tip whose opening was slightly widened by cutting it with a razor blade. In RNA-HTA gels where multiple distinct RNA/DNA heteroduplex bands were visible, small gel pieces were taken from all bands. In patient samples where only one broad RNA/DNA heteroduplex band was visible, one gel piece below the center of the fluorescent signal, one from the center of the signal, and three immediately above it were taken. A distance of approximately 3 mm was maintained between sampled gel pieces. The gel pieces were ejected into a 50-μl PCR mix with second-round primers EDPR3 and EDPR4 for reamplification of the protease gene. An initial melting step of 5 min at 95°C was added to the second-round primer PCR amplification cycles. Since the RNA probe is not PCR amplified from the RNA/DNA heteroduplex, only the annealed DNA variant strand was reamplified. PCR products were then purified and subjected to dideoxy cycle sequencing using 10 pmol of EDPR3 primer and an ABI Prism 3700 capillary sequencer with ABI Prism BIG-DYE terminators.

Measurement of variant frequencies. The percentage of mutated base at a nucleotide position in any single sequencing electropherogram was estimated using the formula $[M^{PH}/(M^{PH} + W^{PH})] \times 100$ (where M^{PH} and W^{PH} are the sequencing electropherogram peak heights of mutant and wild-type bases at the same position). The program used for the analysis of multiple aligned sequence electropherograms derived from the same sample was SeqMan from DNASTAR version 5.0.

RESULTS

HTA with an RNA probe based on the UHG concept. To improve heteroduplex tracking analysis (HTA) so that electrophoretically separated sequence variants differing by only one or a few nucleotide substitutions could be distinguished and then purified for sequencing, we designed an HTA probe based on the UHG concept (60). To be able to sequence amplified protease genes and avoid sequencing the HTA probe present in a DNA heteroduplex, we changed the probe from DNA to a single-stranded RNA molecule. The use of a UHG-based RNA probe resulted in RNA/DNA heteroduplexes exhibiting greatly reduced electrophoretic mobilities with no overlap with the much-faster DNA/DNA heteroduplexes formed between the quasispecies variants themselves. The DNA/DNA heteroduplexes contained only a few nucleotide mismatches and comigrated with the DNA homoduplexes (data not shown). The use of a single-stranded RNA probe also allowed the extraction of RNA/DNA heteroduplexes from gels and their direct sequencing, as only the DNA strand of the RNA/DNA heteroduplexes was reamplified following PCR. The assay was named RNA-HTA for RNA UHG tracking assay.

Design of the UHG-pro1 sequence. UHGs are DNA molecules designed to increase the electrophoretic separation of sequence variants with which they form DNA heteroduplexes (7, 12, 60, 63, 79, 80, 82, 83). The protease UHG probe used here (pro1) was based on the HIV-1 subtype B protease gene consensus sequence and was constructed using a series of 18 overlapping oligomers (see Materials and Methods). The pro1 RNA-HTA probe was designed based on the multiple-site-specific protease UHG approach described by Resch et al. (60) with the following modifications. The pro1 probe was made of RNA, it included all 99 codons of the HIV protease, and its mutation-detecting features consisted of nucleotide insertions as well as substitutions. Single-nucleotide insertions were introduced into or immediately flanking six targeted primary

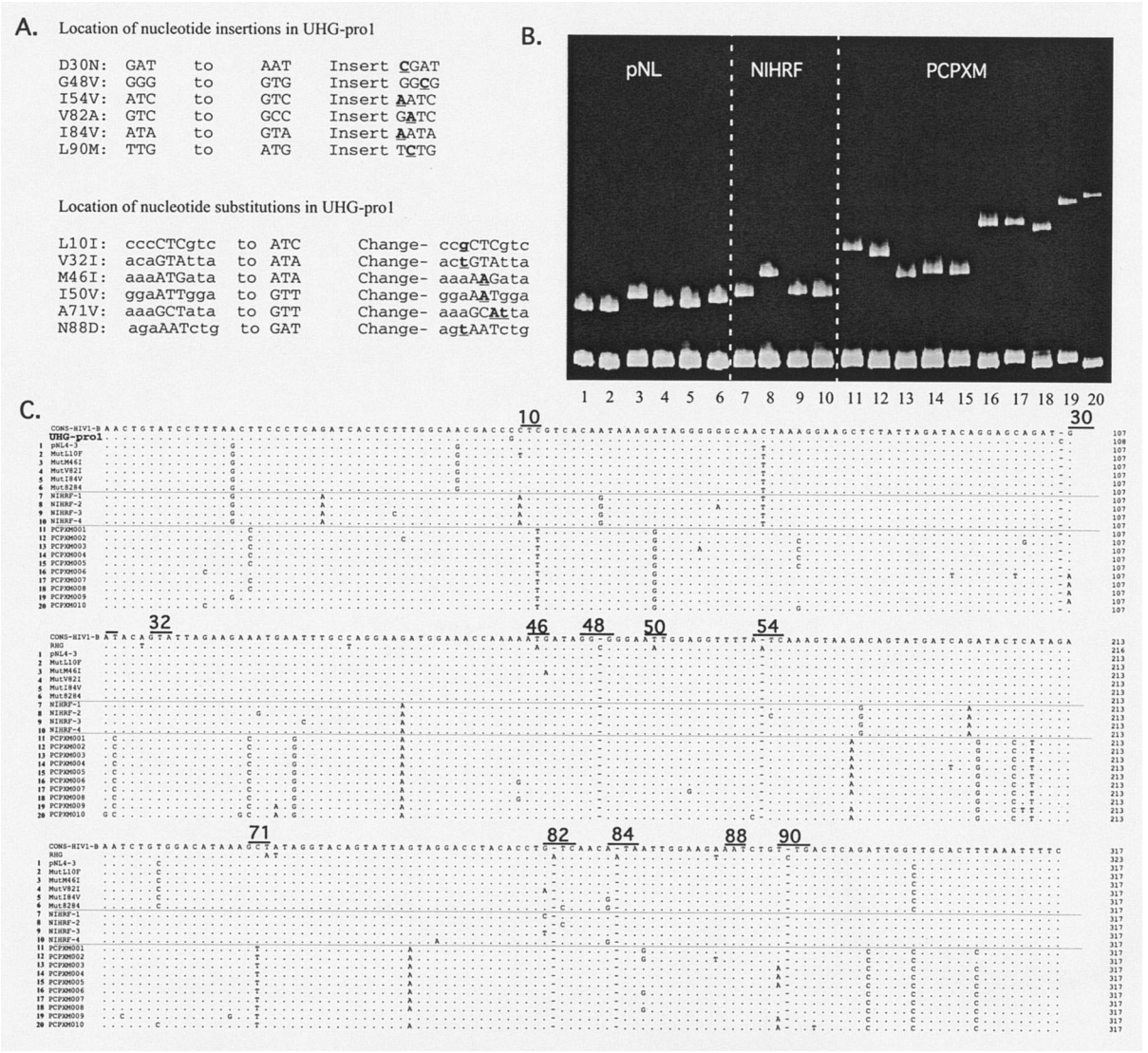

FIG. 1. RNA-HTA pro1 probe design and its use with protease variants. (A) Mutations engineered in the protease subtype B consensus sequence. Targeted codons are shown in capital letters. Six primary and six accessory drug resistance mutations were targeted by nucleotide insertion and substitutions, respectively (underlined). (B) RNA-HTA using protease variants from three different isolates (pNL, NIHRF, and PCPXM variants). The protease variants used in each lane correspond to the sequence numbers in panel C. The gel is shown from the loading wells to the DNA homoduplexes. (C) Alignment of the subtype B consensus, UHG-pro1 probe, and protease variants of pNL, NIHRF, and PCPXM used in panel B. Drug resistance codons targeted by the UHG-pro1 probe are underlined.

drug resistance codons (protease amino acids 30, 48, 54, 82, 84, and 90), while single- or double-nucleotide substitutions were introduced into or immediately flanking six targeted accessory drug resistance codons (protease amino acids 10, 32, 46, 50, 71, and 88) (Fig. 1A). The pro1 probe was therefore designed to improve electrophoretic separation of coamplified variants within PCR products that differed at 1 or more of the 12 codons where mutations are most often selected by protease inhibitor treatment (46).

When RNA/DNA heteroduplexes are generated with patients' protease variants, the six extra inserted nucleotides in the RNA strand are accommodated by looping them out of the heteroduplex, causing a highly kinked double-stranded structure whose electrophoretic mobility through polyacrylamide is greatly retarded (Fig. 1B) (39). The exact structure of the heteroduplex molecule at each of the nucleotide insertion sites is highly dependent on the regional nucleotide sequence. Different sequences in the six codons targeted by insertions therefore result in different heteroduplex structures, which in turn lead to different electrophoretic mobilities. For example, a wild-type codon at position 30 will form a structure with an unmatched cytosine looping out of the RNA/DNA heteroduplex. A D30N mutant will form a structure that will also include a flanking mismatched nucleotide base pair.

Nucleotide substitutions were also introduced into the consensus sequence of the RNA probe such that the presence of an accessory drug resistance mutation would increase by 1 bp an already-present region of heteroduplex melting. For exam-

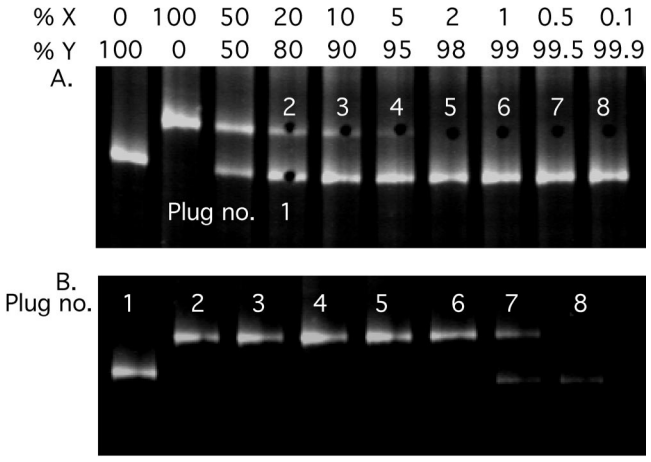

FIG. 2. Detection of low-frequency variants using RNA-HTA. (A) Mixtures of two protease variant PCRs (PCPXM002 and PCPXM003) were separated by RNA-HTA, and gel pieces (plugs) were taken from the gel. Plug holes are visible in the gel. (B) RNA-HTA of the PCR product generated from the gel plugs.

ple, a wild-type protease variant annealed to the pro1 RNA probe would have a single mismatched base pair at codon 10, while the L10I mutant will increase the region of mismatch to two neighboring base pairs. Such mismatched nucleotides result in heteroduplex "bubbles" (39) that also reduce electrophoretic mobility through polyacrylamide.

RNA-HTA using cloned sequence variants from the same patients. To confirm the ability of RNA-HTA to separate variants with point mutations, we reannealed the pro1 RNA probe to PCR products derived from sequenced plasmid subclones and fractionated the RNA/DNA heteroduplexes by polyacrylamide gel electrophoresis (Fig. 1B). Collectively distorting the structure of the RNA/DNA heteroduplexes and slowing their electrophoretic mobilities were, therefore, (i) unmatched (i.e., inserted) nucleotides introduced in the six targeted primary resistance codons, (ii) mismatched base pairs resulting from the substitutions introduced at the six targeted accessory resistance codons, and (iii) other mismatched base pairs due to differences between the viral strain being analyzed and the subtype B consensus. Lanes 1 to 6 show the mobility of variants of pNL4-3 (the pNL4-3 protease differs from the subtype B consensus by five nucleotides). Lanes 7 to 10 show the mobility of variants of RF (the RF protease consensus differs from the subtype B consensus by seven mutations with a range 8 to 12 differences). Lanes 11 to 20 show the mobility of variants from patient PCPXM (PCPXM consensus protease differs from the subtype B consensus by 15 mutations with a range 17 to 24 differences). As expected from prior studies of DNA/DNA heteroduplexes (18, 19, 21), mobility retardation generally increased with greater divergence between the reannealed target DNA and RNA probe strands. The higher level of divergence between PCPXM and the subtype consensus is more typical of currently circulating HIV-1 strains than the lower level of divergence seen with the pNL4-3 and RF strains, both collected early in the subtype B epidemic (26, 32, 51).

Different pNL4-3-derived mutants showed minor but reproducible mobility differences, except for V82I and I84V, whose mobilities were undistinguishable, and L10F, whose mobility was identical to that of pNL4-3. Among RF-derived mutants, the mobility of variants NIHRF-1, -3, and -4 were also only slightly different. These three RF variants' sequence differences were a single base pair difference in either primary resistance codon 82 or 84 and three to five substitutions in nontargeted (i.e., non- drug resistance-associated) codons. The highly distinct mobility of NIHRF-2 was associated with its unique mutation in targeted primary resistance codon 54 (Fig. 1B and C).

A set of patient-derived variants (PCPXM) was also compared to further analyze the effect of sequence differences at targeted versus nontargeted codons. The PCPXM clones were divided into five groups according to their differences in targeted primary drug resistance codons (amino acids 30, 54, and 90) (Fig. 1C). Variants within each group (PCPXM01 and PCPXMO2, D30, I54, and L90; PCPXMO3, PCPXMO4, and PCPXMO5, D30, I54, and L90M; PCPXMO6, PCPXMO7, and PCPXMO8, D30N, I54, and L90; PCPXMO9, D30N, I54, and L90M; PXM10, D30, I54L, and L90M) displayed electrophoretic mobilities that were more similar than those of variants from the other groups (Fig. 1B). Electrophoretic mobility differences were also observed within the five PCPXM groups. Group 1 variants (PCPXMO1 and PCPXMO2) differed by one substitution in codon 87 immediately upstream of a targeted accessory codon (at position 88) and at four nontargeted sites. Group 2 variants with different mobilities (PCPXMO3, PCPXMO4, and PCPXMO5) differed by single nucleotide differences in nontargeted sites. Group 3 variants PCPXMO6, PCPXMO7, and PCPXMO8 differed by four to seven nucleotides in nontargeted sites.

We conclude that differences in primary drug resistance-targeted codons result in a stronger effect on mobility than mutations in nontargeted regions of the protease gene. Nonetheless, some targeted mutations (e.g., L10F in the pNL4-3 background) did not result in noticeable mobility changes, and mutations in nontargeted regions also influenced the mobility of RNA/DNA heteroduplexes (e.g., PCPXM variants with identical targeted codon sequences).

Isolation and sequencing of HIV protease drug-resistant variants present at low frequencies. The sensitivity of RNA-HTA for the detection of minority drug-resistant protease variants was measured using mixtures of two clones with different primary drug resistance mutations. The PCR products were mixed such that one variant ranged in frequency from 50 to 0.1% of the PCR product. Following RNA-HTA, the presence of the minority variant was readily detected by ethidium bromide staining when it was present at a frequency of ≥5% (Fig. 2A). Using a pipette tip, small pieces of the gel were taken over the location of the minority RNA/DNA heteroduplex (Fig. 2A). The DNA strands of the RNA/DNA heteroduplexes were then reamplified by PCR (see Materials and Methods). The PCR products were then hybridized with the RNA probe and analyzed in another RNA-HTA (Fig. 2B). The resulting RNA-HTA mobility indicated which of the two variants had been amplified from the gel and showed that the minority variant was readily purified even when originally present at a frequency of ≥0.5%. At the 0.5% concentration, a 50:50 mixture of both the minority and majority variant was amplified by RNA-HTA, a likely consequence of a fraction of the faster RNA/DNA heteroduplex trailing (streaking) over the position

FIG. 3. Viral load and ARV drug history of 21 patients analyzed by RNA-HTA. The baseline and follow-up time points are shown with vertical lines. ARV drug history is presented in each box.

TABLE 1. Comparison of protease gene drug resistance genotypes obtained for baseline and follow-up samples by using direct PCR and RNA-HTA enhanced sequencing[a]

Patient	Baseline genotype from direct PCR (population) sequencing	Baseline RNA-HTA genotype	Follow-up genotype from direct PCR (population) sequencing	Interval (wks)
606	L10I, A71VAIT, L90M	L10I, A71VAIT, L90M	L10I, M46IM, A71TA, 184V, L90M	27
	K20MI, N37DN, I62V, L63P, K70R, I72LI, G73S	K20MI, N37DN, I62V, L63P, K70R, I72TI, G73S	K20I, D29VD, N37D, R41KR, I62V, L63P, K70R, I72TI, G73S	
624	D30N, N88D	D30N, N88D	D30N, I54V, A71TA, N88D, L90M	29
	I13V, E35D, M36I, L63P	I13V, E35D, M36I, L63P	I13V, K20R, L33IL, E35D, M36I, L63P	
1124	L10I	L101, I54VI, V82AV	L101, I54V, A71V, V82A, L90M	46
	I62V, L63P, V77I, 193L	D60ED, Q61EQ, I62V, L63P, V77I, I93L	K43T, D60E, Q61E, I62V, L63P, V77I, I93L	
1157	L101, M46L, A71V, L90M	L101, M46LI, A71V, I84VI, L90M	L101, M461, A71V, I84V, L90M	43
	I13V, K14R, N37D, L63P, G73S, V77I, I85V	I13V, K14R, N37D, L63P, G73S, V77I, I85V	I13V, K14R, N37D, L63P, I72LI, G73S, V77I, 185V	
1168	L90M	V82AV, L90M	I84VI, L90M	30
	N37H, R41K, I64V, G73CG, V77I	N37H, R41K, I64V, G73CG, V77I	N37H, R41K, I64V, H69RH, G73CG, V77I	
1174		A71VA, L90ML	A71VA, L90ML	16
	L63PL, V77I, I93L	L63PL, V77I, I93L	L63PL, V77I, I93L	
1317	N88S	N88S	A71X, N88S, L90M	21
	I15V, K20T, E35D, M36I, R57K, L63P, I93LI	I15V, K20T, E35D, M36I, R57K, L63P, I93LI	I15V, K20T, E35D, M36I, R41KR, R57K, I62V, L63P, C67YC	
1391		I84VI, L90ML	A71V, I84V, L90M	45
	R57K, L63P	R57K, L63P	R57K, L63P	
1434	V32I, M46IM, V82A	V32IV, M46IM, I54VI, A71VA, V82A	L10IL, M46L, I54VI, A71V, V82A, L90ML	46
	T12S, N37S, R41K, I47VI, L63P, I93L	T12S, N37S, R41K, I47VI, L63P, I93L	T12S, N37S, R41K, L63P, T74ST, I93L	
1590	L10I, I54V, A71V, L90M	L10I, I54V, A71V, V82AV, L90M	L10I, I54V, A71V, V82AV, L90M	34
	T12E, L19IL, L63P, G73S, V77I, I93L	T12E, L19IL, L63P, G73SG, V77IV, I93L	T12E, L19I, L63P, G73SG, I93L	
1624	I54V, A71V	I54VI, A71V, I84VI	I54LI, A71V, I84VI	51
	I15V, L24I, L33F, E34Q, M36L, N37SN, L38W, P39SP, F53L, R57K, D60E, I62V, L63P G73C, N83D	I15V, L24I, L33F, E34Q, M36L, N37SN, L38W, P39SP, F53L, R57K, D60E, I62V, L63P G73C, N83D	I15V, A22VA, L24I, L33F, E34Q, M36L, L38W, P39S, F53L, R57K, D60E, I62V, L63P, G73C, N83D	
1799		V82AV	V32I, M46I, A71V, V82A	27
	K14R, R41K, L63P	K14R, R41K, L63P	K14R, R41K, L63P	
1808	L10I, M46I, L90M	L10I, M46I, L90M	L10I, M46I, I84V, L90M	28
	I15V, K20I, M36I, R41K, I62V, L63P, I72L, G73S, I93L	I15V, K20I, M36I, R41K, I62V, L63P, I72LI, G73S, I93L	I15V, K20I, M36I, R41K, I62V, L63P, I72L, G73S, I93L	
1855	L10I, I54V, A71V, V82A	L10I, I54V, A71V, V82A	L10I, M46L, I54V, A71V, V82A, L90ML	33
	I62V, L63P, G73S, V77I, I93L	I62V, L63P, G73S, V77I, I93L	I62V, L63P, G73S, V77I, I93L	
3910			L10I, M46L, I54V, A71V, V82A, I84V, L90M	50
	P39A, L63P	P39A, L63P	L23I, P39A, D60ED, I62VI, L63P, V77I,	
4014		L10FL, M46IM, I54VI, V82AV, L90ML	M46I, I54V, V82A	50
	I64V	I64VI	R41SR, I64V	
4334	A71T	L10IL, I54VI, A71IT, V82AV, I84VI, L90ML	L10IL, I54V, A71IT, I84V, L90ML	24
	E35D, N37D, L63P, I66MI	E35D, M36IM, N37D, L63P, I66MI	L19IL, K20KIM, L33IL, E35D, M36IM, N37D, L63P, Q92KQ	
4426	L10I, M46LM, I54AV, A7IV, V82A	L10I, M46LM, I54AV, A71V, V82A	L10I, I54V, A71V, V82A, L90M	15
	L19T, N37S, F53LF, L63PL	L19T, N37S, F53LF, L63PL	L19T, N37S, F53LF, L63P	
4795			D30N, N88D	30
	L63PS	L63PS	L63P	
5371	L10FL	L10LF	V82VAIT	13
	I15MI, K20R, L24FL, L63P, I93L	I15MI, K20R, L24FL, L63P, I93L	K20R, L63P, I93L	
8026	L10V, A71V, L90M	L10V, A71V, L90M	L10X, A71V, I84V, L90M	49
	K20I, E35D, M36I, N37S, R41K, D60E, I62V, L63P, I64V, I93L	K20I, E35D, M36I, N37S, R41K, D60E, I62V, L63P, I64V, I93L	K20IR, E35D, M36I, N37S, R41K, D60E, I62V, L63P, I64V, P79A, I93L	

[a] The first line of each genotype lists the detected drug resistance mutations targeted by the RNA-HTA probe. The second and third lines list all mutations from the subtype B consensus. Targeted drug resistance mutations in 11 patients that were not detected by direct PCR population sequencing in the baseline sample are underlined.

of the slower-mobility minority variant. At a 1% frequency, the purified minority variant was amplified pure (Fig. 2B).

Persons receiving protease inhibitors. The treatment histories and plasma viremia levels of 21 persons in whom new primary protease inhibitor resistance mutations emerged in between two episodes of virologic failure are shown in Fig. 3. The plasma samples at the two time points shown by vertical lines are referred to here as the baseline and the follow-up samples. The mean interval between the paired plasma samples tested was 31.4 weeks (4 to 51 weeks). Thirteen persons were receiving their first protease inhibitor at the time of the baseline sample; eight persons had received two or more protease inhibitors before the baseline sample. Fourteen persons were treated with a new protease inhibitor between the baseline and follow-up samples; seven persons continued receiving their initial protease inhibitor.

Fractionation of protease variants within patients' quasispecies. The protease locus was amplified by reverse transcrip-

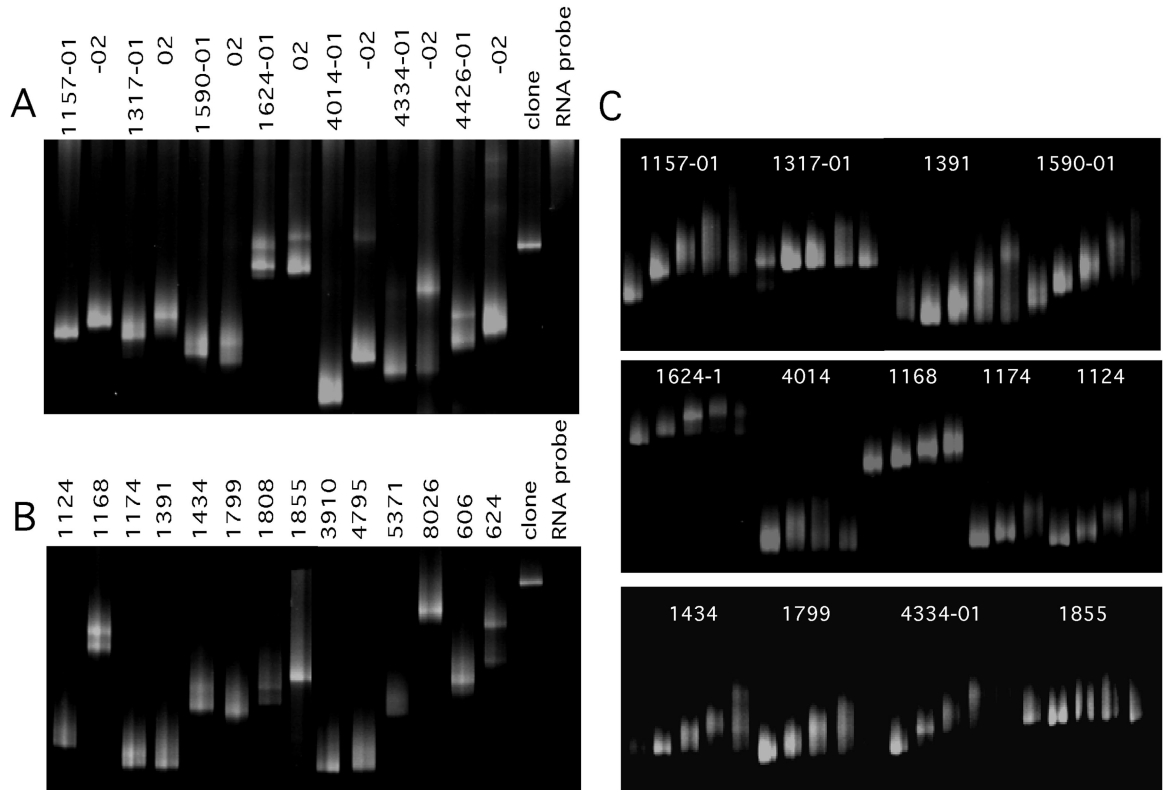

FIG. 4. RNA-HTA of clinical samples. Patient identifications are similar to those in Fig. 3. (A) RNA-HTA of samples collected at baseline and follow-up are labeled −01 and −02, respectively. (B) RNA-HTA of samples from patient baseline only. (C) RNA-HTA analysis of the PCR products derived from RNA-HTA gel pieces. A clonal PCR target (PCPXM010) and RNA probe-alone lanes are included.

tion-nPCR from the baseline and when available from the follow-up plasma samples and directly sequenced (see Materials and Methods) (Table 1). The drug resistance genotypes derived by direct PCR population sequencing were identical to those originally determined at Stanford University within the range of interlaboratory reproducibility of drug resistance genotyping (<1% difference for all base calls, with 90% of these difference being due to mixed base differences) (20, 34, 66). The same PCR amplicons were then analyzed by RNA-HTA (Fig. 4). When both the baseline and follow-up plasma sample from the same subject were available, different RNA-HTA mobility variants were seen at both time points (Fig. 4A). Such mobility differences were expected following the acquisition of at least one primary drug resistance mutation detected by direct PCR population sequencing (Table 1). RNA-HTA from patients for which only the baseline plasma sample was available for analysis are also shown (Fig. 4B). In contrast to the sharp bands produced by clonal PCR products, the PCR products of these clinical samples generated RNA-HTA patterns consisting of one major band flanked by a diffuse smear of DNA or two or more distinct RNA-HTA bands (Fig. 4A and B).

Protease gene variants with different RNA-HTA mobilities were isolated by taking five small gel plugs along the length of each RNA-HTA lane (see Materials and Methods) (Fig. 2A). The DNA strands of the RNA/DNA heteroduplexes in the gel plugs were amplified by PCR. For a few representative patients, the multiple PCR products derived from gel pieces were again reannealed with the pro1 RNA probe and separated using RNA-HTA (Fig. 4C). Some series of PCR products from the same gel lanes showed little or no RNA-HTA mobility differences (Fig. 4C, 1317 and 1855), while others showed clear mobility differences (Fig. 4C, 1157-01 and 1391). This result indicated that sequence variants within the same quasispecies could be isolated from different locations of an RNA-HTA gel lane even when it consisted of a single diffuse band.

Sequence analysis of RNA-HTA-isolated protease variants. The five PCR products derived from each of the RNA-HTA lanes of the 21 baseline plasma samples were then directly sequenced. The sequencing electropherograms of each of the five RNA-HTA gel pieces-derived PCR products and the electropherogram of the original, unfractionated PCR product were aligned. Both complete and partial (i.e., mixed) nucleotide base differences (see Materials and Methods) were then identified. The presence of a drug resistance (or subtype B consensus-deviating) mutation was defined as its detection at a level of ≥25% in one or more of the five electropherograms obtained per sample (see Materials and Methods). Examples of differences seen between the direct (population) and the RNA-HTA-assisted sequencing electropherograms, showing the detection of low-frequency drug-resistant variants, are shown in Fig. 5. Newly detected drug resistance mutations were often seen in more than one of the RNA-HTA plugs (Fig. 5). Minority drug resistance and other mutations relative to subtype B consensus were typically seen in the PCR from the RNA-HTA gel pieces with the slowest mobilities (Fig. 5). The

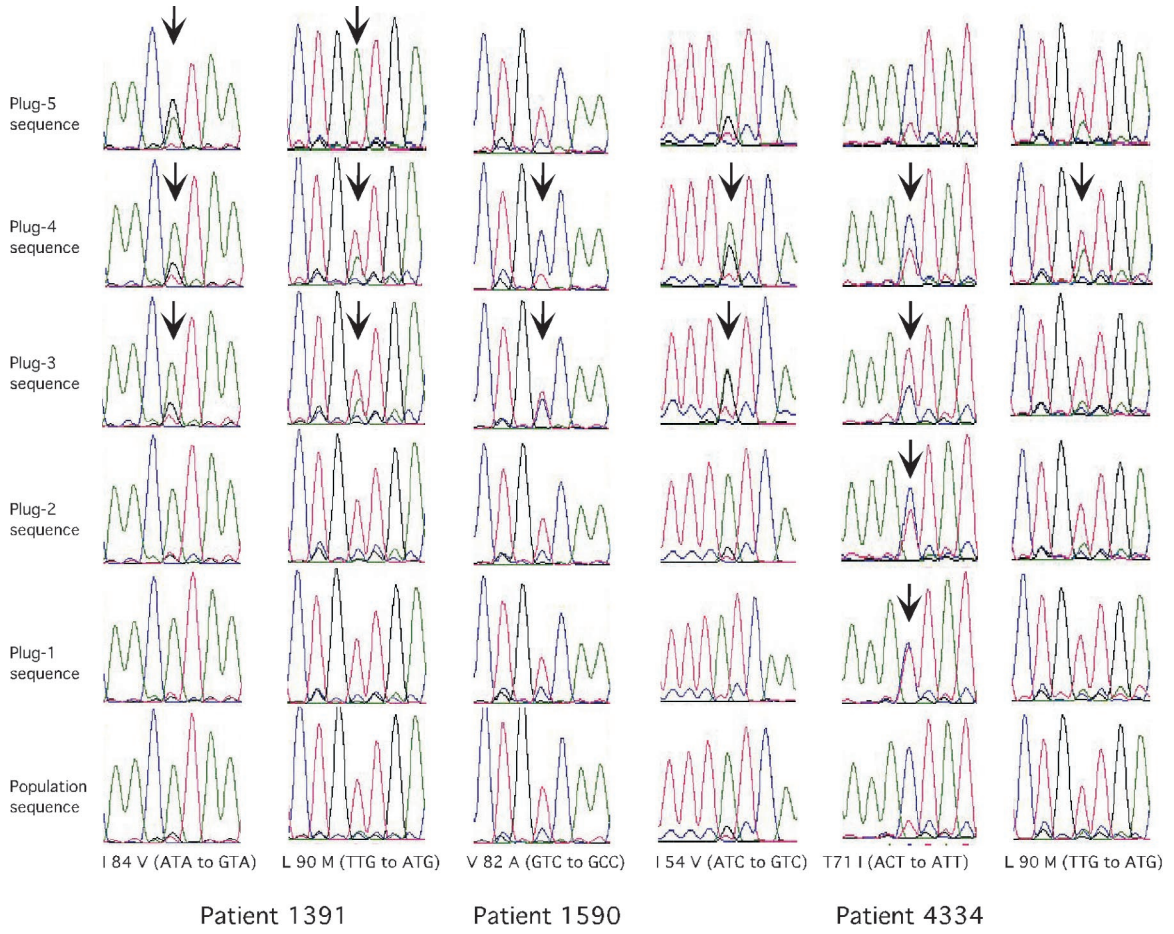

FIG. 5. Comparison of direct PCR population sequencing and RNA-HTA-enhanced sequencing. Representative electropherograms show the detection of drug resistance mutations (arrows) in PCR products derived from RNA-HTA-derived gel plugs, while such mutations were not detected in the directly sequenced PCR product (population sequence).

extra RNA/DNA heteroduplex structure distortions (and mobility retardation) induced by sequence differences between the subtype B consensus-based probe and annealed protease sequence variants likely account for this observation.

Mixed base calls were often detected in the RNA-HTA gel piece-derived electropherograms. Four possible mechanisms may account for such mixed, rather than pure, nucleotide base calls. The size of the gel piece used and RNA/DNA heteroduplex gel streaking from electrophoretically faster bands (Fig. 2B, lane 7) may result in coamplification of distinct variants in the same gel piece. Two variants differing at a particular base may also comigrate on RNA-HTA due to the inability of the RNA probe and gel condition used here to cause sufficient mobility differences (Fig. 1B). Lastly, other protease gene differences may result in incidental comigration of different variants.

For each baseline plasma sample, the drug resistance mutations (and other mutations resulting in non-subtype B consensus amino acids) from each of the five RNA-HTA gel piece-derived electropherograms were assembled into a single composite RNA-HTA genotype (Table 1). When compared with the population sequencing-based genotype of the same plasma sample, previously undetected low-frequency drug resistance mutations were detected in 11 of 21 persons (Table 1). In 8 of these 11 persons, every drug resistance mutation detected at baseline using RNA-HTA was detected at the follow-up bleed (16 to 51 weeks later) using traditional direct PCR population sequencing (Table 1). This result indicates that emerging drug-resistant variants could be identified earlier using RNA-HTA than by direct population sequencing. In 4 of 11 persons in whom primary drug resistance mutations were detected only using RNA-HTA, the baseline samples tested were from persons who had temporarily discontinued either all protease inhibitors (persons 1391 and 4014) or all ARV drugs (patients 1124 and 4334) in their regimens (Fig. 3).

Absence of minority drug-resistant variants in drug-naive subjects. Using RNA-HTA, we analyzed the plasma quasispecies from nine HIV-infected persons with no previous history of ARV therapy that were identified during testing of voluntary blood donations. No primary drug resistance mutations were detected as either majority or minority variants. Only common polymorphisms (K20R, L63P, and A71V), which can also serve as accessory drug-resistant mutations, were detected as majority variants by both direct PCR population and RNA-HTA sequencing (data not shown).

Linkage between different drug resistance mutations. Direct PCR population sequencing provides a quasispecies consensus sequence of all the coamplified HIV variants. When multiple drug resistance mutations are detected as mixed base peaks in

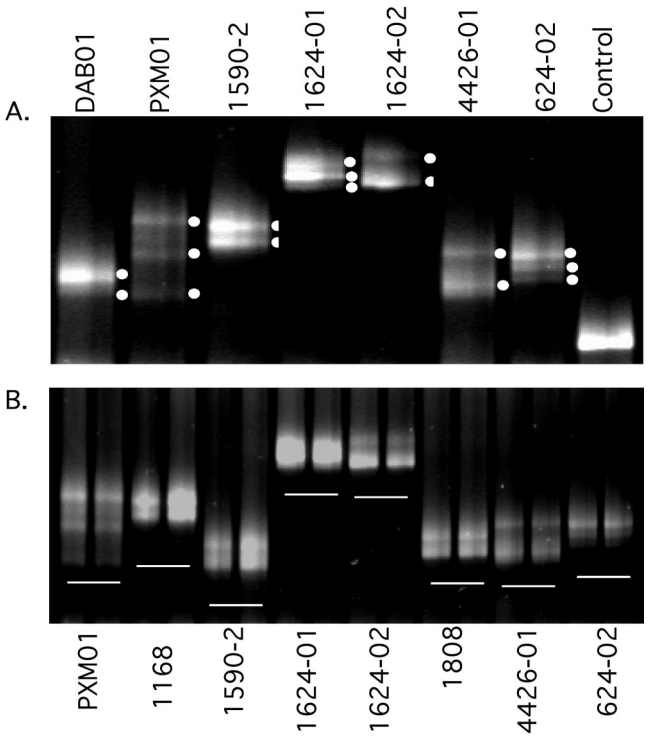

FIG. 6. Mutation linkage analysis and quasispecies sampling reproducibility. (A) Analysis of samples with distinct RNA-HTA bands. RNA/DNA heteroduplex bands labeled by a white dot were separately purified and sequenced. (B) Independently generated PCR products were analyzed by RNA-HTA in neighboring gel lanes to test for reproducible viral population sampling.

direct PCR population sequencing, it is not possible to determine if they are present on the same or different genomes. Samples from seven persons whose plasma quasispecies generated distinct RNA-HTA bands were further analyzed (Fig. 6A). The distinct RNA-HTA bands were first quantified by fluorescence gel scanning to determine the relative frequencies of these protease gene variants in plasma (Table 2). RNA-HTA bands were then isolated from gels, reamplified by PCR, and sequenced. The different RNA-HTA band-derived protease variants from the same patients all showed one or more base differences at one or more of the 12 targeted drug resistance codons as well as at other amino acid positions (Table 2). In sample PXM01, primary protease inhibitor-resistant mutations D30N and L90M appeared to be present on different genomes, in keeping with a report that these two mutations may evolve along independent pathways (72), while in sample 624-2 D30N and L90M mutations were both present on each of the three major linkage groups. Mixed bases were also occasionally detected when distinct RNA-HTA bands were isolated and sequenced, indicating that different variants may comigrate (for example, PXM01 linkage group II K20TK and D30ND [Table 2]). The presence of mixed bases in a distinct RNA-HTA band may therefore still require analysis of plasmid subclones to establish linkage, but the process can be made more efficient using RNA-HTA.

Reproducible quasispecies population sampling. An accurate genetic analysis of virus populations requires that enough genomes be sampled (i.e., reverse transcribed and amplified by PCR) so that the resulting mixture of amplified variants reflects their original frequency in the blood sample. A consequence of insufficient population sampling is therefore the generation of different variant frequencies in independent PCRs generated from the same sample (16, 21, 33, 50). For example, if only a few genomes are PCR amplified from a highly diverse population because of its low viral load (i.e., low cDNA input into the PCR), then different genomes may be amplified in independent PCRs. In order to test the ability of the protocol used here to reproducibly sample persons' protease quasispecies, we generated and compared by RNA-HTA duplicate PCRs initiated with different aliquots of the same cDNA (i.e., independent samplings) (Fig. 6B). The RNA-HTA patterns from independent samplings were identical, indicating that the protease quasispecies samplings were sufficiently high to reproducibly generate the same mixed populations of genetic variants.

DISCUSSION

Techniques used for HIV drug resistance genotyping can be divided into sequencing and non-sequencing-based methods (13). Nonsequencing methods include the line probe assay,

TABLE 2. Linkage analysis of protease mutations on different genomes

Patient	Linkage groups[a]	% Variants
DAB01	I. I15V, M36I, N37E, L63P, I64V, T74S, L90M	32
	II. I15V, M36I, N37E, L63P, A71T, N88D, L90M	68
PXM01	I. I15V, K20T, E35D, N37S, R41K, R57K, I62V, L63P, I64V, A71V, V77I, L90M, I93L	17
	II. I15V, K20TK, D30ND, E35D, N37S, R41K, R57K, I62V, L63P, I64V, A71V, V77I, I93L	21
	III. I15V, D30N, E35D, N37S, R41K, R57K, I62V, L63P, I64V, A71V, V77I, I85VI, I93L	44
1590-2	I. L10I, T12E, L19I, I54V, L63P, A71V, V82A, L90M, I93L	43
	II. L10I, T12E, L19I, I54V, L63P, A71V, G73S, V77I, L90M, I93L	57
1624-1	I. V11I, I15V, L24I, L33F, E34Q, M36L, L38W, P39S, F53L, R57K, D60E, I62V, L63P, A71V, G73CS, N83D, I84VI	12
	II. I15V, L24I, L33F, E34Q, M36L, L38W, P39S, F53L, I54V, R57K, D60E, I62V, L63P, A71V, G73C, N83D	52
	III. I15V, L24I, L33F, E34Q, M36L, N37S, L38W, F53L, I54V, R57K, D60E, I62V, L63P, A71V, G73C, N83D	34
1624-2	I. I15V, A22V, L24I, L33F, E34Q, M36L, L38W, P39S, F53L, R57K, D60E, I62V, L63P, A71V, G73C, N83D, I84V	70
	II. I15V, L24I, L33F, E34Q, M36L, N37SN, L38W, P39S, F53L, I54L, R57K, D60E, I62V, L63P, A71V, G73C, N83D	30
4426-1	I. L10I, L19T, N37S, M46LM, F53LF, I54VI, A71V, V82A	61
	II. L10I, L19T, N37S, I54AV, L63P, A71V, V82A	39
624-2	I. I13V, K20R, D30N, E35D, M36I, I54VI, L63P, A71TA, I84IV, N88D, L90M	8
	II. I13V, K20R, D30N, E35D, M36I, L63P, A71V, I84V, N88D, L90M	20
	III. I13V, K20R, D30N, L33IL, E35D, M36I, I54V, L63P, A71T, N88D, L90M	71

[a] Differences between linkage groups are underlined.

oligonucleotide ligation assay, microarrays, and PCR with mutation-specific primers (1, 2, 23, 27, 29, 37, 40, 41, 53, 61, 67, 71, 77). DNA sequencing methods include direct PCR sequencing and sequencing of multiple plasmids from subcloned PCR products.

Non-sequencing-based methods are generally highly sensitive for detecting minority drug resistance mutations (65, 76, 77), but they suffer from several limitations. The exact sequence context of any mutation cannot be determined without sequencing the entire gene. The growing number of drug resistance-related mutations requires the optimization and simultaneous use of an increasingly large number of highly sensitive single-nucleotide querying assays. Polymorphisms in the sequence immediately neighboring the queried nucleotide positions may decrease annealing of the probe or primer, potentially leading to decreased sensitivity or false-negative results (44). Linkage of mutations present at low frequency on the same genome is not possible using single-nucleotide querying assays.

Direct PCR population sequencing can overcome all but the last of these problems, but at the cost of decreased sensitivity for low-frequency variants. A PCR subcloning-sequencing approach allows the linkage of different mutations on the same genomes and, if sufficient numbers of subclones are analyzed, may be highly sensitive to low-frequency variants but is extremely labor-intensive (39, 43, 49, 55, 57, 58, 63). The RNA-HTA assay was developed to overcome the low sensitivity of direct PCR population sequencing without resorting to generating and sequencing large numbers of subclones.

Heteroduplex mobility assays and HTAs can provide information on the presence of specific low-frequency variants, overall genetic diversity, or changes in quasispecies variant composition within HIV quasispecies (5, 15, 17–19, 21, 33, 35, 43, 55, 58–60) and have been used for the study of other variable viral species (4, 8, 31, 35, 45, 52, 68, 69, 73, 75, 78, 81, 84). Using heteroduplex mobility assays or HTAs, the electrophoretic separation of variants differing by only single-nucleotide substitutions is not possible unless special electrophoretic conditions or HTA probes are used (21, 60), and these approaches have not been used to directly isolate low-frequency variants for sequencing.

The UHG RNA probe used was designed to enhance the electrophoretic separation of wild type from drug-resistant variants at six common primary codons (D30N, G48V, I54V, V82A, I84V, and L90M) and six secondary or accessory drug resistance codons (L10I, V32I, M46I, 150V, A71V, and N88D). Using a panel of subcloned protease variants and quasispecies from clinical samples, we showed that the pro1 RNA probe allowed the separation of sequence variants at these targeted sites. Reconstitution experiments indicated that variants could be quantified at frequencies of $\geq$5% and PCR amplified at frequencies of $\geq$0.5%.

For some quasispecies PCR amplicons, distinct RNA-HTA bands were detected. Such quasispecies appeared to consist of multiple high-frequency viral lineages, and their purification on RNA-HTA gels allowed mutation linkage groups to be generated (Fig. 4 and 6 and Table 2). Other quasispecies produced a single but diffuse RNA-HTA band. When different regions of such diffuse bands were isolated and the variants were reamplified and sequenced, different drug-resistant variants were detected. These viral populations therefore appeared to be less structured than those producing distinct RNA-HTA bands and to consist of a multitude of low-frequency variants distributed around a quasispecies consensus variant in a more typical theoretical quasispecies viral "cloud" fashion (24).

Using RNA-HTA-based sequencing on 21 patient samples, we were able to detect primary drug resistance mutations at an earlier date than was possible using direct PCR population sequencing. These results indicate that RNA-HTA, by virtue of identifying low-frequency drug resistance mutations, may reduce the likelihood of treatment failure by helping to exclude potentially inactive drugs from salvage regimens. This study may overestimate the actual frequency with which such low-frequency mutations occur in genotyped samples, as persons were selected on the basis of acquiring one or more new protease inhibitor resistance mutations at the follow-up time point. Further studies are under way to determine how often additional mutations may be detected by RNA-HTA in less-heavily treated persons who are not selected on the basis of a second virologic failure showing additional mutations. The approach used here for the study of the HIV-1 protease gene should be readily applicable to the sequence analysis of other highly variable loci.

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