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Emergence of Protease Inhibitor Resistance Mutations in Human Immunodeficiency Virus Type 1 Isolates from Patients and Rapid Screening Procedure for Their Detection

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Patient human immunodeficiency virus type 1 (HIV-1) isolates that are resistant to protease inhibitors may contain amino acid substitutions L10I/V, M46L/I, G48V, L63P, V82A/F/T, I84V, and L90M in the protease gene. Substitutions at positions 82 and/or 90 occur in variants that display high levels of resistance to certain protease inhibitors. Nucleotide substitutions at these two sites also lead to the loss of two *Hin*dII restriction enzyme digestion sites, and these changes make possible a rapid procedure for the detection of drug-resistant variants in patients on protease inhibitor therapy. This procedure was used to detect the emergence of mutated viruses at various times after the initiation of therapy with the HIV-1 protease inhibitor indinavir. The method includes viral RNA isolation from plasma and reverse transcription PCR amplification of the protease gene with fluorescence-tagged primers. The PCR product is digested with *Hin*dII, the cleavage products are separated on a urea-acrylamide gel in a DNA sequencer, and the extent of cleavage is automatically analyzed with commercially available software. In viruses from 34 blood samples from four patients, mutations leading to an amino acid change at residue 82 appeared as early as 6 weeks after the start of therapy and persisted throughout the course of the study period (48 weeks). Mutations leading to double substitutions at residues 82 and 90 were seen at a lower frequency and appeared later than the change at position 82. The changes detected by restriction enzyme cleavage were confirmed by DNA sequencing of the cloned protease genes by reverse transcription PCR amplification of viral RNA from isolates in plasma. In addition to the changes at positions 82 and 90, we have identified M46L/I, G48V, and I54V substitutions in isolates derived from indinavir-treated patients. *Hin*dII analysis of uncloned, PCR-amplified DNA offers a rapid screening procedure for the detection of virus isolates containing mutations at amino acid residues 82 and 90 in the HIV-1 protease gene. By using other restriction enzymes, the same method can be used to detect additional protease drug-resistant variants and is generally applicable for the detection of mutations.

Recent clinical trials of human immunodeficiency virus (HIV) protease inhibitors have revealed that these drugs can significantly reduce the level of HIV in blood and, for ritonavir, delay the onset of disease and prolong survival (3, 5, 17, 29, 31). Protease inhibitor-resistant virus isolates emerge during therapy, although the time at which they are detected varies and may depend on the dose administered. Long-term data on the persistence of resistant isolates and their effect on the clinical outcome are not yet available. These drug-resistant HIV type 1 (HIV-1) isolates contain a variety of amino acid substitutions in the protease region, either singly or in combination (1, 6–9, 11, 13, 14, 18–20, 22, 23, 27, 28, 30, 32, 34–39, 41–44). Consistent patterns of substitutions have not always been seen in patients receiving the same drug, and mutations vary for different protease inhibitors. Additional substitutions have been identified in resistant virus variants that were derived by passaging HIV in susceptible cell cultures in the presence of protease inhibitors. The predominant substitutions occur at amino acid positions 8, 10, 32, 36, 46, 48, 50, 54, 63, 71, 75, 82, 84, 88, and 90. Generally, as the number of mutations increases, so does the level of drug resistance (6, 7, 23, 35, 44). However, mutations at codon positions 82 and/or 90 in the protease gene were found to be consistently associated with drug resistance in patients treated with saquinavir (Ro 31-8959; Invirase; Hoffmann-La Roche) (9), ritonavir (ABT-538; Norvir; Abbott) (11), and indinavir (MK-639; L-735,524; Crixivan; Merck) (6, 7). In this report, we describe the mutations that emerged in patients receiving indinavir and a simple assay system for identifying amino acid substitutions at two critical positions: codons 82 and 90.

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MATERIALS AND METHODS

Samples. The cryopreserved plasma samples used in the present study were obtained from patients infected with HIV who were participating in a clinical trial with (i) indinavir at a dose of 600 mg four times a day (patients H and P) or (ii) the same therapy combined with interleukin-2 that is given by continuous infusion for 5 days every 2 months (patients A and L) (15). The patients had previously received nucleoside analogs. Patients on the first regimen were permitted to switch to combination therapy with interleukin-2 and other antiretroviral agents after 12 weeks. Samples obtained from 10 patients before receiving indinavir therapy were used to analyze the preexisting mutations. All subjects provided written informed consent and the protocol was approved by the institutional review board of the National Institute of Allergy and Infectious Diseases.

RNA isolation and cDNA synthesis. HIV-1 RNA was isolated from 260 μl of plasma by using the QIAamp HCV kit (Qiagen Inc., Chatsworth, Calif.). HIV-1 RNA was reverse transcribed to cDNA by using oligonucleotide primer A and avian myelobastosis virus reverse transcriptase (Invitrogen Corporation, San Diego, Calif.). The primers used for cDNA synthesis and PCR amplification are listed in Table 1.

PCR. The HIV-1 protease gene sequence was amplified by PCR with a mixture of Klentaq1 (Ab Peptides, Inc., St. Louis, Mo.) and *Pfu* (Stratagene, La Jolla, Calif.) DNA polymerases (2) and the B-C primer pair. Nested PCR was carried

TABLE 1. Primers used for cDNA synthesis and PCR

Procedure	Primer	Position (strand)	Sequence
cDNA	A	3626–3649 (−)	5′-TTG TTT TAC ATC ATT AGT GTG GGC-3′
First PCR	B	1881–1904 (+)	5′-GAA GCA ATG AGC CAA GTA ACA AAT-3′
	C	3543–3566 (−)	5′-GAT ATG TCC ATT GGC CTT GCC CCT-3′
Nested PCR	D	1965–1988 (+)	5′-TTC AAT TGT GGC AAA GAA GGG CAC-3′
	E	3500–3523 (−)	5′-TAA GTC TTT TGA TGG GTC ATA ATA-3′
Nested Fam-labeled PCR	F	2295–2316 (+)	5′-Fam-TAG GGG GGC AAC TAA AGG AAG C-3′
	G	2591–2610 (−)	5′-Hex-ACT TTT GGG CCA TCC ATT CC-3′

out with the D-E primer pair by using the enzymes mentioned above. The PCR product was then purified by using the QIAquick-spin PCR purification kit (Qiagen, Inc.), ligated with the pCR II vector (Invitrogen Corporation) and was transformed into competent *Escherichia coli* INVαF′ cells. DNA prepared from recombinant clones was sequenced by using ABI PRISM dye-labeled terminators and AmpliTaq DNA polymerase, FS (Perkin-Elmer/Applied Biosystems Division, Foster City, Calif.).

Fluorescence labeling of DNA. A nested PCR was carried out with AmpliTaq DNA polymerase (Perkin-Elmer/Applied Biosystems Division) preincubated with monoclonal antibody to *Taq* polymerase (Chimerx, Madison, Wis.) and the F-G primer pair; primers F and G were 5′ end labeled with fluorophores: Fam and Hex, respectively. The cycling conditions were as follows: 1 cycle of pre-PCR (1 min at 94°C, 1 min at 60°C, and 5 min at 72°C), 30 cycles of PCR (30 s at 94°C, 30 s at 60°C, and 2 min at 72°C), and finally, 10 min at 72°C. The PCR product was digested with the *Hin*dII restriction enzyme. An aliquot was dried and redissolved in deionized formamide-EDTA solution. After adding internal lane standards (Genescan-350 Tamra), the samples were heated to 92°C for 2 min, cooled rapidly on ice, and applied onto a 6% urea-acrylamide (sequencing) gel. Electrophoresis was carried out in an ABI 373 sequencing apparatus, and the data were collected and analyzed by using Genescan and Genotyper application software, respectively. The intensities of the fluorescent bands were measured as peak areas under the curve, and each band was expressed as a percentage of the total lane fluorescence.

RESULTS

Identification of mutations at amino acid codons 82 and 90. By using the MacVector computer program, all of the known nucleotide substitutions specific for protease inhibitor resistance (32) were inserted in the protease region and screened to determine if these resulted in changes in restriction enzyme digestion patterns. Two *Hin*dII sites overlapped with amino acid positions 82 and 90; thus, virus variants with mutations at these sites would not be cleaved. On the basis of this observation, we generated fluorescence-labeled PCR products of the protease gene from HIV isolates obtained from patients being treated with indinavir. The PCR products were digested with *Hin*dII and separated on a polyacrylamide gel. The predicted sizes of the cleavage products associated with different patterns of resistance are shown in Fig. 1A, and the restriction enzyme digestion patterns obtained for patient H during the course of therapy are presented in Fig. 1B. DNA that was amplified with the Fam-labeled 5′ primer showed blue bands of 203 bp (wild type at residue 82), 226 bp (residue 82 mutant [82mt]), and 316 bp (residue 82 and 90 mutant [82mt+90mt]). DNA amplified with the Hex-labeled 3′ primer showed yellow bands of 90 bp (wild type at residue 90) and 316 bp (82mt+90mt). Virus with 82mt+90mt generate bands at 316 bp with both Fam- and Hex-labeled primers, and the band at this position in Fig. 1B is therefore a mixture of both bands.

*Hin*dII analysis of Fam-labeled PCR products from virus isolates from patient H showed drug resistance mutations by 6 weeks after the start of indinavir therapy, when 58% of the viral species showed 82 mt, and the population was composed of 100% mutant variants after 10 weeks. Although 4% of the

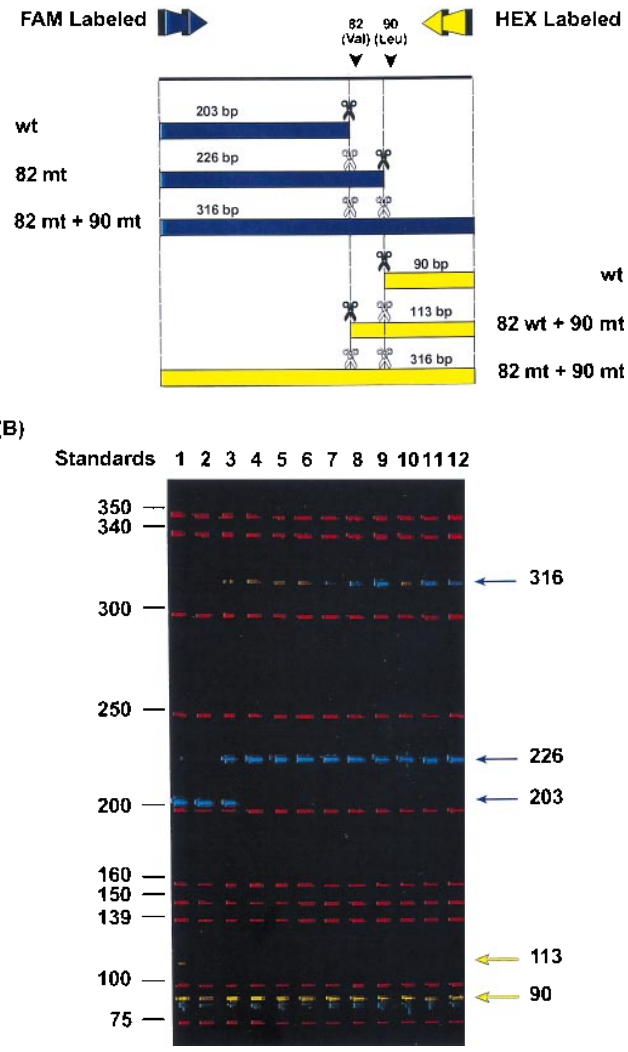

FIG. 1. Detection of protease mutations by restriction enzyme analysis. (A) PCR amplification and *Hin*dII digestion of the protease gene. The blue and yellow bars indicating the 5′ and 3′ primers used in the PCR were labeled with Fam and Hex, respectively. Wild-type sequences at residues 82 and 90 allowed digestion with *Hin*dII restriction enzyme (denoted as ✂), and drug resistance mutations at these positions led to the loss of restriction sites (denoted as ✂). (B) *Hin*dII analysis of fluorescence-labeled PCR products. Lanes 1 through 12, sequential plasma samples obtained from patient H before drug administration and at 3, 6, 10, 16, 20, 24, 27, 32, 36, 40, and 44 weeks of indinavir administration. The sizes of the denatured fragments of the internal lane standards are given on the left side (in base pairs).

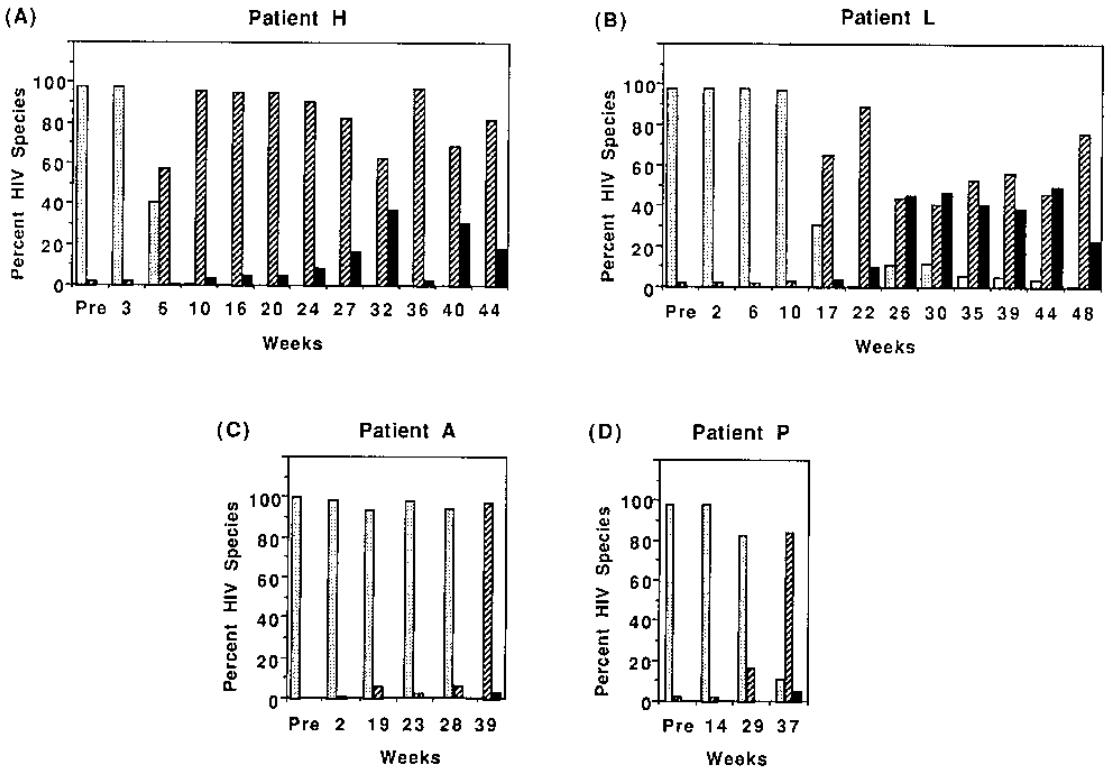

FIG. 2. *Hin*dII analysis of uncloned PCR-amplified DNA derived from plasma samples from four patients (A to D). The intensities of the fluorescent DNA bands corresponding to the wild type (203 nucleotides [ ]), 82mt (226 nucleotides [▨]), and 82mt+90mt (316 nucleotides [■]) were measured, and their percentages of the total lane fluorescence were plotted during indinavir therapy. All experiments were repeated at least two times, and similar results were obtained in each experiment.

viral population showed 82mt+90mt at week 10, higher levels (17%) occurred only at 27 weeks after therapy (Fig. 2A).

In patient L the dominant 82mt first appeared at 17 weeks of therapy, and a mixture of single and double mutants persisted for 48 weeks (Fig. 2B).

In patients A and P, mutations at position 82 were observed only at later time points: 39 and 29 weeks, respectively, after the initiation of therapy (Fig. 2C and D). In neither of these patients did we observe detectable levels of 82mt+90mt virus during these time periods.

Low frequencies (~1%) of 82mt and 90mt (data not shown) were seen in samples from patients even before the initiation of indinavir therapy. Similar findings have been reported previously by others (24, 45, 46), and their relevance is discussed later in this report.

Comparison of restriction enzyme analysis with sequence analysis. At each time point that a sample was analyzed by fluorescence analysis, a PCR product lacking the fluorescent ends was generated and cloned, and DNA purified from selected clones was sequenced. The data presented in Table 2 provide a profile of all of the changes in the protease enzyme that were generated during drug administration. The fraction of clones showing mutations leading to drug-resistant specific amino acid substitutions V82A/F and L90M for patient H is shown graphically in Fig. 3. Sequence data are based on the analyses, on average, of 10 clones (range, 7 to 14 clones), and so they primarily provide a qualitative picture of the species of virus that are present. However, even with this small sample size, there was general agreement between the data for 82mt and 82mt+90mt obtained by sequence analysis and by restriction enzyme analysis (compare Fig. 3 and Fig. 2A). In addition to amino acid substitutions at positions 82 and 90, amino acid substitutions M46I/L, G48V, and I54V were frequently observed (Table 2). L10I/V and L63P were observed in samples obtained both before and after the indinavir treatment. None of the samples examined had mutations specific for a I84V substitution.

The nucleotide changes leading to amino acid substitutions for 82mt (Val to Ala, Thr, and Phe) and 90mt (Leu to Met) result in the loss of the *Hin*dII restriction site [GT(T/C) ↓ (A/G)AC]. Mutations that change the neighboring amino acids, at positions 83, 89, and 91, and silent mutations at positions 82 and 90 can also lead to the loss of the *Hin*dII restriction site. These mutations would not contribute to drug resistance but would affect the interpretation of data obtained by restriction enzyme analysis. To determine if this is a relevant concern, we first measured the substitutions that occurred in the protease gene in isolates from 10 patients prior to exposure to any protease inhibitor (Fig. 4). Only one change was seen among a possible 445 substitutions (at amino acid positions 82, 83, 89, 90, and 91) in 89 clones. In the 302 clones from four patients sequenced during the course of therapy, 5 changes among a possible 1,510 substitutions were noted. This would not introduce a significant error by the procedure that we have described.

Mutations exclusive to residue 90 were observed at a very low frequency in our sequence data, and we did not observe bands corresponding exclusively to 90mt virus variants in the *Hin*dII analysis. To determine the sensitivity of the assay, we mixed various concentrations of 82mt+90mt DNA with wild-type DNA before carrying out PCR, and could detect the mutant-specific bands on the gel when 1% mutant DNA was

TABLE 2. Amino acid substitutions in protease region of viral isolates[a]

Patient	Wk	No. of clones analyzed	No. of clones showing amino acid substitutions						
			L10I/V	M46L/I	G48V	154V	L63P	V82A/F	L90M
H	Pre[b]	8					8		
	3	9	1	2			9		
	6	10		8			10	5	
	10	10		10			10	10	
	16	9		9			9	9	1[c]
	20	10		10		6	10	10	1
	24	10		9		6	10	9	
	27	8	1	8		4	8	8	1
	32	10	4	10		7	10	10	2[d]
	36	10	10	10		10	10	10	
	40	10	9	10		7	10	10	1
L	Pre	10	10				10		
	2	10	10				10		
	6	8	8				8		
	10	10	10	1			10		
	17	10	10	2	7		10	7	
	22	10	10	1	8	6	10	9	1
	26	8	8		7	8	8	8	
	30	9	9	1	8	7	9	9	1
	35	9	9	3	6	1	9	6	3
	44	10	10	2	8	1	10	8	2
	48	10	10	2	6	3	10	8	2
A	Pre	10						1[e]	
	2	7							
	19	10		3			1		
	23	10							
	28	13							
	39	12	1	12			2	12	
P	Pre	8	8						
	14	14	14						
	29	10	10	10					

[a] Amino acid sequences were deduced from the nucleotide sequences of the protease-coding region. Substitutions compared with the consensus sequence are shown. Exceptions to the amino acid substitutions were observed, as indicated in footnotes *c* to *e*.
[b] The sample was obtained pretreatment.
[c] One clone L90W.
[d] One clone L90S.
[e] One clone V82I.

present (data not shown). We have recently shown that the procedure described in the Materials and Methods section can be further simplified by using a single round of PCR amplification of cDNA under the conditions described for the fluorescence labeling of DNA. The pattern of products obtained by enzyme digestion were identical, and the calculated percentages were similar to those obtained from nested PCR amplifications (unpublished data).

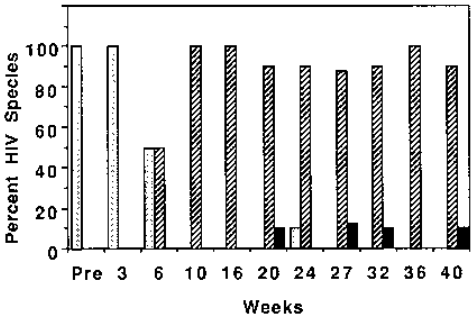

FIG. 3. Nucleotide sequence analysis of cloned DNA derived from reverse transcription PCR of viral RNA from the plasma of patient H. The percentage of wild-type clones (▨) or clones showing 82mt (▨) or 82mt+90mt (■) among the total number of clones whose sequences were determined during indinavir therapy were plotted.

DISCUSSION

The conventional method of genotype analysis for identifying drug resistance mutations involves reverse transcription PCR amplification of viral RNA and nucleotide sequencing of a selected number of recombinant clones. Although sequencing provides precise information about the nucleotide changes, the procedure involves a heavy commitment of time and labor, and the information obtained describes only the predominant viral populations. Techniques such as nucleotide-specific probe hybridizations (10, 12, 21, 25, 40) and sequencing of uncloned PCR products (16, 46, 47) have been specifically designed to detect the true in vivo existence of a large number of viral quasispecies. The screening assay described here offers a simple procedure for identifying amino acid substitutions at codons 82 and 90 of the protease gene in uncloned viral species. Other amino acid mutations associated with resistance to protease inhibitors can also be detected by restriction enzyme

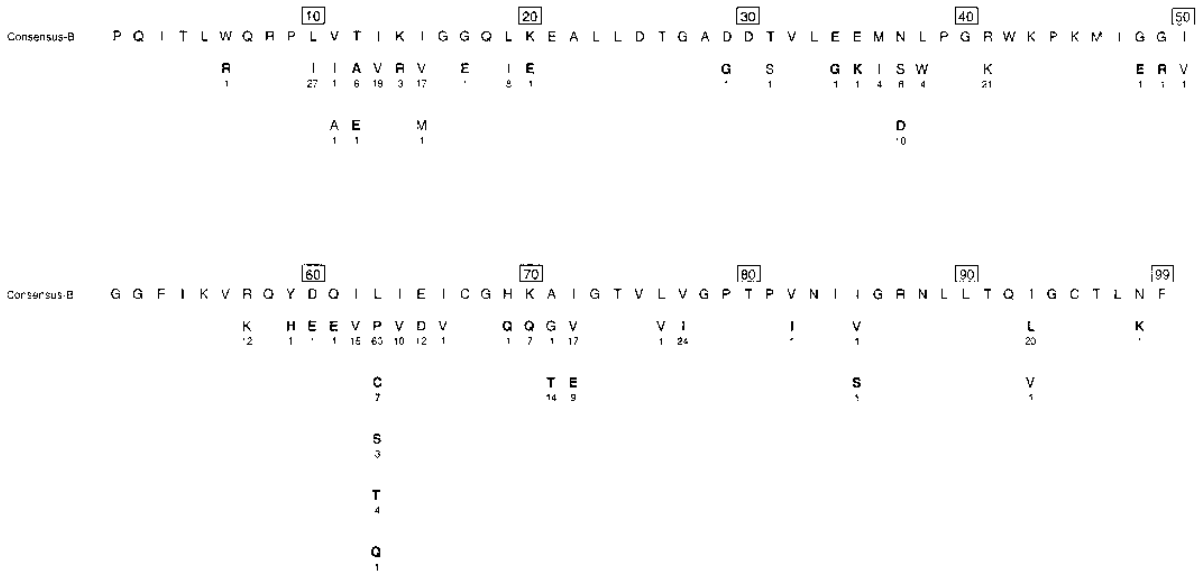

FIG. 4. Deduced amino acid variation in 89 sequences of the HIV-1 protease derived from virus in plasma obtained from 10 patients prior to indinavir therapy. The consensus sequence of the clade B subtype (33) listed on the top line is used for comparison. The single-letter amino acid code is used, and the variations from the consensus sequence at each position are listed below the consensus sequence. The numbers below the amino acid code letter represent the number of times that a particular variant appeared. Nonconservative substitutions are given in boldface type.

analysis: for example, R8Q by *Asp*EI, L10R by *Mvn*I, V32I and V75I by *Ssp*I, M46L by *Tsp*509I, M46F by *Apo*I or *Tsp*509I, K45I+M46F by *Ssp*I, I50V by Tsp509I, A71V by *Sfc*I, V82A by *Bsp*MI, I84V by *Mae*II, N88D by *Dpn*I or *Dpn*II, and I93L by *Alu*I. While these changes are not consistently observed in the resistant HIV isolates obtained from patients treated with protease inhibitors, the procedure that we have described can readily be accommodated to measure genotypic changes at these sites.

Possible complicating factors in applying this procedure would be the occurrence of silent mutations that affected restriction enzyme cleavage without altering the gene product, or changes in amino acids adjacent to positions 82 or 90 that are part of the enzyme recognition site and that would block restriction enzyme cleavage but would not confer resistance. These substitutions occurred at a frequency of less than 1% in the 391 clones that we examined (see Results). While mutations at positions 82 and 90 appear to be critical in conferring drug resistance, the absence of mutations at these positions, on the other hand, does not preclude the existence of an alternate resistance pathway.

Investigators may find patients in whose HIV isolates a fraction of the protease sequences present prior to therapy is not cleaved by a particular restriction enzyme. By sequence analysis, we observed one clone with a V82I substitution prior to therapy. A similar low frequency of a preexisting V82I substitution in clones from clinical samples has been reported earlier (24, 45, 46). In contrast, an unusually high frequency of V82I substitutions was reported by Lech and coworkers (26) in their studies of the protease gene. If such a population had been present in our studies, more than 20% of the molecules would have been resistant to cleavage prior to therapy. In these latter studies (26), sequence analysis was carried out by examining proviral DNA, whereas virion RNA was used in the present experiments. It is likely that this is the basis for the different findings. Genetic information provided by the examination of proviral DNA is derived in part from the analysis of noninfectious or replication-incompetent forms of the virus (48). The observation that 6% of the clones containing the protease gene contain stop codons when proviral DNA is examined (26), while none are seen in the protease gene of HIV derived from viruses from plasma, is consistent with this interpretation.

In addition to the V82A/F and L90M substitutions in the protease gene, M46L/I, G48V, and I54V changes were also observed. Substitutions at these sites that are associated with drug resistance have been reported previously (7, 13, 20, 30). L10I/V and L63P changes were observed in virus isolates from plasma samples obtained both before and after indinavir treatment, and both leucine and proline have been detected at position 63 in wild-type isolates in other studies (33). Their role in drug resistance is unclear. Mutations at V82 and I84 decrease protease activity, while second-site mutations at M46I and L63P have been reported to alter the protease conformation further and to compensate for the deleterious effects of mutations in the active site at residues 82 and 84 (4, 28). The I84V substitution that has been identified in several drug-resistant isolates was not seen in the present study.

While the mutant with double mutations at positions 82 and 90 may confer a higher degree of protease inhibitor resistance than either single mutant, in each of the four patients studied, variants with the altered genotype at position 82 were detected first. Even when low levels of the double mutant were seen at as early as 10 to 17 weeks after the start of therapy, the mutant with a single mutation at position 82 persisted as the dominant variant. The failure of the double mutant to emerge as the dominant species may reflect the fact that the double mutation confers decreased replication competency on the virus.

*Hin*dII analysis of uncloned, PCR-amplified DNA offers a rapid screening procedure for the detection virus isolates containing mutations at amino acid residues 82 and 90 in the HIV-1 protease gene. While the method can only be used to provide indirect evidence of drug resistance, the advantages of this procedure include its simplicity and speed and its use of virion RNA rather than cloned proviral DNA. By using other enzymes, this technique can be applied to other mutations, and

it will be useful for the rapid detection of mutations in clinical isolates.

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