which viral DNA was transfected into MT-2 cells, and the other was a competition culture assay in which MT-2 cells were infected with recombinant viruses. The results of the two methodologies were precisely in line with one another, and from these results we ascertained the three important features of mutations in the Gag region and the PR region.

First, we found that not only CSMs but also non-CSMs contribute to the recovery of fitness in PI-resistant viruses. Clones of the GP type, which had both CSMs and non-CSMs, demonstrated the highest level of viral growth for all three patient-derived *gag-pol* sequences. Interestingly, in clones 1-1 and 1-2, GP^{-c}-type recombinants did not grow, suggesting that the non-CSMs accumulated after PI treatment (probably the T371 deletion and the I401V, E368A, S473P, Q474L, and A487S mutations) were deleterious to viral growth, and these mutations were functional only in the context of CSMs. In contrast, clone 2-2 of the GP^{-c} type grew well and grew even better than clone 2-2 of the P^{+c} type. The non-CSMs observed in clone 2-2 were different from those observed in clones 1-1 and 1-2, suggesting that a wide variety of Gag mutations may emerge with PI treatment and that non-CSMs can have different impacts on viral growth, depending on the corresponding Gag and PR mutation patterns.

Second, we ascertained that two PR types are differentiated by Gag sequence independence. One is the Gag-independent PR that can be functional without any mutations in its target Gag region. The PR of clone 1-2 can be classified as this type. In clone 1-2, although the GP and P^{+c} types demonstrated better viral replication abilities, the P-type recombinant could also replicate. It appears that the mutations that accumulated in this PR could allow the viral replication capacity to recover. The second type of PR was Gag dependent, which requires Gag mutations to be functional PR. The PRs of clones 1-1 and 2-2 were of this type. In these clones, P-type recombinants had the lowest level of virus growth, and mutations in Gag, especially CSMs, were required to achieve better replication. We noted with interest that the PR sequences of clones 1-1 and 1-2, which were isolated from samples from the same patient, were quite similar to each other. The PR of clone 1-1 had additional L23I, K43T, M46I, I54V, and I62V mutations compared to the sequence of clone 1-2; and the additional mutations found in clone 1-1 made the activity of the PR dependent on the Gag mutations. This finding is an important issue with regard to drug resistance phenotyping with recombinant virus technology. Our findings indicate that the inclusion or exclusion of the HIV-1 Gag sequence may affect the nature of subsequent virus populations recovered by recombination procedures and may affect drug resistance levels. In this study, we focused on PR activity and not drug resistance phenotypes. Further studies that include phenotypic analysis and other PIs should be performed to obtain a better understanding of the nature of PI resistance and to improve treatment protocols in a practical manner.

Third, we confirmed that the A431V CSM is sufficient as a compensatory Gag mutation in certain mutation patterns. According to the results of previous studies and from our results, it appears that the M46IL mutation in the PR has a key relationship to the A431V CSM (8, 25). The ternary structures of the p7/p1 cleavage site and the PR also support the significant interaction of A431V and M46IL, as A431V is located at the S2 position of the p7/p1 cleavage site and M46I is located within the P2 site of the PR (35).

Thus, all three findings indicate that PR and mutations in its substrate, Gag, are vitally linked. In conclusion, our study demonstrates that non-CSMs are as important as CSMs for the recovery of viral fitness in drug-resistant HIV-1 with impaired PR activities. This essential relationship is the result of the survival competition evolution process of the virus during antiretroviral treatment in vivo.

ACKNOWLEDGMENTS

We thank the patients and physicians who were involved in our study. We also thank Yumi Kanekama and Mary Phillips for help in preparing the manuscript.

This study was supported by a grant from the Organization of Pharmaceutical Safety and Research of Japan and the Ministry of Health, Labour, and Welfare of the Japanese Government.

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JAIDS Journal of Acquired Immune Deficiency Syndromes
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Patterns of Point Mutations Associated With Antiretroviral Drug Treatment Failure in CRF01_AE (Subtype E) Infection Differ From Subtype B Infection

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Summary: An increasing number of HIV-1–infected patients living in developing countries now have access to antiretroviral drugs. Information regarding the drug-resistant mutations of non-B subtype HIV-1 remains limited, however. The authors cross-sectionally compared patterns of the drug-resistant point mutations in patients infected with either subtype B or CRF01_AE (subtype E) among patients who acquired HIV by sexual transmission in Japan. Protease sequence data were available from 216 patients with a detectable level of RNA copies in plasma. Based on phylogenetic analysis of the protease and the C2V3 regions, 162 subtype B and 45 CRF01_AE cases were identified; 82 subtype B and 24 CRF01_AE patients had a treatment failure with nucleoside reverse transcriptase inhibitors; and 69 subtype B and 19 CRF01_AE patients had a treatment failure with a protease inhibitor. Antiretroviral drug history was similar in subtype B-infected and CRF01_AE-infected patients. The mutations T69N and V75M in reverse transcriptase and L10F, K20I, L33I, and N88S in protease were seen more frequently in patients infected with CRF01_AE than in patients with subtype B. The mutations, D30N, A71V, and N88D were found exclusively in patients with subtype B. Most of the characteristic mutation patterns were associated with a history of receiving nelfinavir. The pattern of drug resistance mutations differs between the subtypes. Data derived from subtype B drug-resistant genotypes may not always be applicable to non-B subtypes. **Key Words:** HIV, antiretroviral drug, CRF01_AE, drug resistance, genotype

An increasing number of HIV-1–infected patients living in developing countries now have access to antiretroviral drugs for the prevention of mother-to-child HIV-1 transmission and for improving the quality and length of patients' lives as prices of antiretroviral drugs rapidly fall. The majority of patients living in such countries were infected with non-B subtype HIV-1. In Japan, the proportion of heterosexually transmitted patients has been rising, and we have previously reported that the non-B subtype, particularly CRF01_AE (subtype E), is becoming common among the heterosexually infected population.[1]

Amino acid sequence diversity in the *pol* gene is 10% to 15% between subtypes.[2] Even a single amino acid mutation can dramatically change the susceptibility or the resistance of the virus to an antiretroviral drug.[3,4] Thus, given this genetic diversity, it is plausible that non-B HIV-1 subtypes evolve differently from B subtypes, and this may be reflected in different patterns and pathways of resistance to antiretroviral drugs. Studies on antiretroviral drug resistance of non-B subtypes have been limited, however, in comparison with studies done on subtype B. We believe that it will be important to have more data on non-B subtype drug resistance mutations to monitor the inevitable emergence of drug-resistant HIV-1 in these countries and to optimize anti-

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Manuscript received December 30, 2003; accepted April 3, 2003.

retroviral drug treatment of patients infected with non-B subtype. In this article, we show results of *pol* sequence analysis among drug-naive and antiretroviral-treated patients infected with CRF01_AE in Japan in comparison with subtype B–infected patients. Some patterns of drug resistance mutations in patients infected with CRF01_AE, who were clinically resistant to antiretroviral drug therapy, were different from those observed in patients infected with subtype B.

MATERIALS AND METHODS

Study Design and Sample Selection

Since November 1996, Japan's AIDS Research Center, National Institute of Infectious Diseases, has been providing a genotyping service for clinicians in referral hospitals throughout the country. A retrospective cross-sectional study was conducted to analyze the pattern of drug resistance-associated mutations among all samples that we have received from sexually transmitted HIV-1–positive patients between November 11, 1996 and September 7, 2000.

The patients were first stratified clinically according to their viral load reduction in response to drug therapy. If the viral load was reduced to less than 400 copies/mL within 3 months of antiretroviral therapy, the patients were classified as drug sensitive. If the viral load remained more than more than 1000 copies/mL after 3 months of antiretroviral therapy, the patients were classified as having treatment failure. Patients were unclassified if they had a viral load more than 400 copies/mL and less than 1000 copies/mL on antiretroviral therapy, if they were on antiretroviral drugs for less than 3 months of therapy, or if they had taken antiretroviral therapy but were off drugs at the time of sample collection. If multiple samples existed from 1 patient, only the most recent sample was analyzed. There were 27 cases in which patients responded to a salvage therapy, including nonnucleoside reverse transcriptase inhibitors (NNRTIs). In these cases, we selected the last sample before the start of salvage therapy. Patients who had not had antiretroviral drugs prior to sample collection were regarded as drug naïve. These samples were included for cross-sectional observation to analyze baseline amino acid residues at drug resistance-associated sites. If multiple samples were available from drug-naive patients, we selected the earliest samples before therapy. There was no overlap between patients with drug treatment failure and drug-naive patients. The sample selection was independent of subsequent sequence analysis.

Sequencing Method

Sequencing HIV-1 reverse transcriptase (RT) and protease regions has been undertaken in accordance with a previously published method.[5] Briefly, viral RNA was extracted from 200 μL of plasma with an RNA extraction kit (High Pure Viral RNA Kit; Boehringer Mannheim GmbH, Mannheim, Germany); a 464-base pair (bp) protease fragment (base number of nucleotide: 2148–2611) and an 888-bp RT fragment (2485–3372) were separately amplified by PCR after a reverse transcription reaction from extracted RNA by means of an RNA-PCR kit (One Step RNA PCR Kit [AMV]; TaKaRa, Osaka, Japan). Primary PCR products were further amplified with a high-fidelity DNA polymerase (KOD DNA Polymerase; TOYOBO, Osaka, Japan).

For C2/V3 subtyping, DNA was extracted from peripheral blood mononuclear cells (PBMCs) with a DNA extraction kit (QIAamp DNA Blood Mini Kit; QUIAGEN, Germany) and a 380-bp C2/V3 fragment (7001–7380) was amplified with nested PCR using a high-fidelity PCR system (Expand High Fidelity PCR System, Boehringer Mannheim). Sequencing analysis was performed using dye terminators (BigDye Terminator Cycle Sequencing Ready Reaction Kit; Applied Biosystems, Foster City, CA) and an autosequencer (ABI PRISM 377 DNA Sequencer; Applied Biosystems). Sequence results were then analyzed by computer software (Sequence Navigator version 1.0.1, Applied Biosystems). For protease and RT drug resistance genotyping, sequence results were compared with the HXB2 reference sequence and mutation points were determined. Subtypes were determined by phylogenetic analysis; C2V3 and protease sequence results were aligned by means of the clustal-W program with a set of reference sequences recommended by the Los Alamos sequence database. The results of the alignment were then analyzed by the neighbor-joining method. This analysis also confirmed that each sequence was unique; the possibility of sequence contamination was ruled out.

Statistical Method

Proportions of mutations at each codon were first compared between drug-naive subtype B and CRF01_AE patients and between subtype B and CRF01_AE patients with treatment failure by the χ^2 test. If any significant difference was noted at $p < .05$, we compared proportions of an individual amino acid substitution with the χ^2 test or Fisher exact test if an expected cell value was less than 5. The Mantel-Haenszel method was applied to adjust for the pattern of drug usage; if a patient had a drug for at least 3 months, the patient was regarded as being significantly exposed to the drug. Continuous data were analyzed using a nonparametric test, the Kruskal-Wallis (Wilcoxon) test. The analysis was conducted using Epi Info version 6.04.

RESULTS

During the study period, we received 745 samples from 261 sexually transmitted HIV-1–positive patients from 25 referral hospitals: of these, 393 samples were taken from 115 heterosexual patients, 307 samples from 127 homosexual patients, and 45 samples from 19 patients with unknown sexual behavior. Single samples were received from 112 patients, whereas multiple samples were received from 149 patients, where the median (interquartile range [IQR]) number of samples per patient was 3,[2,5] ranging up to 35 samples per patient.

Subtyping

Protease sequence data were available from 216 patients with a detectable level of RNA copies in plasma and subjected to phylogenetic analysis for determining subtype. Protease sequence was not available in 45 patients; this group was characterized by a low level of viral load and a high CD4 count: 74% had an undetectable viral load (<50 copies/mL), the median (IQR) CD4 count was 391 (222, 612) cells/μL, and most patients (96%) had received antiretroviral drugs. One hundred

sixty-two of 216 (75.0%) patients had subtype B sequences in the protease region, 45 of 216 (20.8%) patients had CRF01_AE, 3 of 216 (1.4%) patients had F subtype, 1 of 216 patients had C subtype, 1 of 216 patients D subtype, and 4 of 216 (1.9%) patients had an unknown subtype. Sequencing of the V3 region was performed in 34 protease subtype B and 27 protease subtype CRF01_AE samples. All patients with subtype B or CRF01_AE in the protease region had concordant subtypes in the V3 region. This result supported an assumption that subtype CRF01_AE can be differentiated from subtype B on the basis of phylogenetic analysis of the protease region in this study population.

Comparison of Subtype B and CRF01_AE Patients

Table 1 summarizes sexual behavior, sex, age, and clinical response to antiretroviral drugs in 162 subtype B and 45 subtype CRF01_AE patients. The proportion of male patients was significantly higher in subtype B patients than in CRF01_AE patients ($p < .0001$). The median age was similar in both groups. Sexual behavior was reported in 191 patients; CRF01_AE infection was strongly associated with a heterosexual route of acquisition ($p < .0001$). The trend for median viral load to be higher in subtype B patients than in CRF01_AE patients was seen but not significant. The median CD4 count was significantly lower in CRF01_AE patients than in subtype B patients ($p = .0001$), and the proportion of AIDS cases (C1, C2, and C3) was significantly higher in CRF01_AE patients ($p = .038$). Patients were classified according to their history of taking antiretroviral drugs and their viral load profiles. The distribution of clinical responses to nucleoside reverse transcriptase inhibitors (NRTIs) or protease inhibitors (PIs) among CRF01_AE patients was similar to that among subtype B patients.

We further compared the history of NRTI use in 86 subtype B and 24 CRF01_AE patients with NRTI drug treatment failure (Table 2*A*). There were no significant differences in the history of NRTI regimens in terms of drug selection or median duration in months on NRTI treatment. We also compared the history of PI use in 68 subtype B and 19 CRF01_AE patients with PI drug treatment failure (see Table 2*B*). There were similarly no significant differences in the history of PI regimen in terms of drug selection or median duration in months on PI treatment.

TABLE 1. *Background information of patients*

	Subtype B	CRF01_AE	Total (N = 207)
Sex			
Male	149	30	179
Female	13	15	28
Age Median (range) years	36.5 (21, 78)	36.0 (21, 62)	36.0 (21, 78)
Sexual behavior			
Heterosexual	45	40	85
Homosexual	105	1	106
Unknown	12	4	16
RNA copies/mL			
Median (IQR)	17,050 (2400)	36,300 (5900)	23,200 (3000)
CD4 count[a]			
Median (IQR)	294 (158.5, 432)	115 (39, 317)	264 (100, 393)
Proportion of AIDS (C1, C2, and C3)[b]	38/148 (25.7%)	18/41 (43.9%)	56/189 (29.6%)
Response to NRTI			
Failure	86 (53%)	24 (53.3%)	110
NRTI naive	36 (22.2%)	8 (17.8%)	44
Sensitive	13 (8%)	5 (11.1%)	18
Unclassified	23 (14.2%)	5 (11.1%)	28
Data not available	4 (2.5%)	3 (6.7%)	7
Response to PI			
Failure	68 (42%)	19 (40%)	87
PI naive	59 (36.4%)	14 (33.3%)	73
Sensitive	11 (6.8%)	4 (8.9%)	15
Unclassified	20 (12.3%)	5 (11.1%)	25
Data not available	4 (2.5%)	3 (6.7%)	7

IQR, interquartile range; NRTI, nucleoside reverse transcriptase inhibitor; PI, protease inhibitor.
[a] $p = .0001$.
[b] $p = .038$.

TABLE 2. *Drug history of patients with drug treatment failure*

A. Nucleoside reverse transcriptase inhibitory history	Subtype B (N = 86)	n	CRF01_AE (N = 24)	n
AZT	23.5 (14, 34)	44	29 (14, 36)	14
Off AZT	15 (8, 26)	41	21 (10, 26)	9
3TC	21 (9, 31)	51	24 (17, 34)	17
Off 3TC	10 (6, 16)	26	9 (1, 9)	6
D4T	16 (12, 21)	43	22.5 (17, 27)	10
Off d4T	8 (5, 12)	9	9.5 (5, 14)	2
ddC	22 (18, 27)	7	12	2
Off ddC	9.5 (6, 17)	14	12 (4, 18)	3
ddI	16 (7, 24)	25	19 (12, 30)	5
Off ddI	9 (6, 12)	22	8 (3, 13)	8
Nevirapine	4.5	2	19	1
Off nevirapine	14	1	—	—
Efavirenz	—	—	3	1

B. Protease inhibitor history	Subtype B (N = 68)	n	CRF01_AE (N = 19)	n
Nelfinavir	16 (10, 22)	42	20 (12, 27)	11
Off nelfinavir	8 (5, 12.5)	8	5 (1, 10)	4
Indinavir	25 (12, 29)	14	15.5 (12.5, 23.5)	4
Off indinavir	12.5 (5.5, 14)	16	9 (3, 12)	7
Saquinavir	11 (8, 15)	7	18 (13.5, 22.5)	4
Off saquinavir	6 (4, 13)	9	6 (5, 6)	3
Ritonavir	11 (8.5, 14.5)	8	12	1
Off ritonavir	5 (1, 12)	7	—	—

The proportions of patients who were receiving each drug at the time of blood sampling (upper row) and the proportion of patients who had been exposed but were not receiving the drug at the time of blood sampling (lower row) are shown. Median (interquartile range) durations in months of the exposure to each drug are also shown.

AZT, azidothymidine; 3TC, lamivudine; D4T, stavudine, ddC, didoxycytidine; ddI, didanosine.

Comparison of *pol* Gene Sequences Among Drug-Naive Patients

We first compared frequencies of amino acid residues in the RT region of 34 NRTI-naive subtype B patients and 8 NRTI-naive CRF01_AE patients. Figure 1 shows amino acid residues at 16 sites where a significant difference was noted. None of the 16 sites was known as an NRTI drug resistance–associated site.[6]

A significant difference in frequencies of amino acid residues was noticed between 57 subtype B and 14 CRF01_AE PI-naive patients at nine sites (Fig. 2), where three sites were known as PI drug resistance-associated sites.[6]

Comparison of Drug Resistance–Associated Mutations Among Patients With Drug Treatment Failure

We then compared the frequencies of amino acid residues among patients with treatment failure between subtypes B and CRF01_AE. Figure 1 shows the frequencies of amino acid residues among the patients with NRTI drug treatment failure. Most of the significant amino acid variations found in drug-naive patients remained significant in the patients with NRTI treatment failure, except for two positions: I31 and G196. The frequency of Gly-to-Glu mutation at position 196 was higher in patients with NRTI treatment failure than in drug-naive patients with CRF01_AE infection, but the trend was the opposite in subtype B infections. Figure 1 also shows the frequencies of amino acid residues at the known NRTI resistance-associated sites. There were no significant differences in the frequencies of amino acid mutations between patients with subtype B and subtype CRF01_AE, except the Thr-to-Asn mutation at position 69 (T69N) and the Val-to-Met mutation at position 75 (V75M) were seen more commonly in CRF01_AE than in subtype B. This difference remained significant even after adjusting for the pattern of NRTI usage, although the difference was marginal. The relation between these mutations and a history of at least 3 months of a specific NRTI was analyzed; no specific drug was significantly associated with T69N; however, the V75M mutation was found to be significantly associated with stavudine (d4T) treatment in CRF01_AE patients ($p = 0.02$) but not in subtype B patients.

Figure 2 summarizes the frequencies of amino acid residues in the protease region among patients with PI treatment failure. It also shows the frequencies of mutations in the amino acid residues at the nine sites where significant differences were found in the drug-naive patients. The significant difference in the frequency of the Ile-to-Leu mutation at position 93 (I93L), which was detected in PI-naive patients, disappeared in PI-resistant patients, whereas the rest of the amino acid variations remained significant. Figure 2 shows the frequencies of mutations in amino acid residues at the other known sites associated with PI resistance; differences in the frequencies of the six amino acid mutations at five sites were detected in PI-resistant patients: Leu-to-Phe at position 10 (L10F), Lys-to-Ile at position 20 (K20I), Leu-to-Ile at position 33 (L33I), and Asn-to-Ser at position 88 (N88S) were more commonly seen in CRF01_AE infection than in subtype B infection. Conversely, the Asp-to-Asn at position 30 (D30N), Ala-to-Val at position 71 (A71V), and Asn-to-Asp at position 88 (N88D) were exclusively seen in subtype B infection. These differences remained significant even after adjusting for the pattern of PI usage. Analysis of specific drug use showed that six mutations (L10F, D30N, L33I, A71V, N88S, and N88D) were significantly associated with a history of nelfinavir therapy but that the K20I mutation was not associated

Codon / Consensus B	6 E	11 K	31$ I	35 I	37 T	[illegible]	43 K	[illegible]	122 K	123 D	135 I	[illegible]	173 K	174 Q	177 D	178 I	[illegible]	196$ G	207 R	[illegible]	211 R	[illegible]
B (naïve) N=34#	D(4)	R(1)	-	V(17) L(1) M(1) T(1)	-	[illegible]	-	[illegible]	E(9) Q(1)	E(8) N(2)	L(11) T(8) V(4) S(1)	[illegible]	E(3) N(2) R(1)	K(2) R(1)	E(5) G(1)	V(3) L(2)	[illegible]	E(17)	Q(29) E(3) H(1) K(1)	[illegible]	K(9) G(2) E(1) S(1)	[illegible]
AE (naïve) N=8	D(5)	N(1) T(4)	T(2)	T(8)	K(7) R(1)	[illegible]	E(7)	[illegible]	E(6)	S(6) N(1)	-	[illegible]	I(3) T(2) V(2) A(1)	K(8)	E(7)	M(2)	[illegible]	-	A(7) N(1)	[illegible]	S(7) K(1)	[illegible]
B (failure) N=82	D(16) G(1)	R(1) T(1)	L(1)	V(55) M(4) T(4) R(1)	A(5) T(4)	[illegible]	E(4) K(3) N(2)	[illegible]	E(29) K(2) Q(1)	E(17) N(4) S(1)	T(21) L(15) V(12) S(1) R(1) K(1)	[illegible]	E(1) R(1) T(1)	K(3) R(1)	E(15)	M(5) L(3)	[illegible]	E(26)	Q(67) E(13) K(2) H(1)	[illegible]	K(32) G(2)	[illegible]
AE (failure) N=24	D(13) N(2)	T(12)	T(1)	T(22) M(1) P(1)	K(23) R(1)	[illegible]	E(24)	[illegible]	E(23)	S(20) N(4)	T(4) V(1)	[illegible]	K(17) R(2) T(2) M(1) V(1)	K(24)	E(24)	M(14)	[illegible]	E(4)	A(24)	[illegible]	S(19) N(2)	[illegible]

FIGURE 1. Amino acid variations in the reverse transcriptase (RT) region. This figure shows amino acid variation at the known nucleoside reverse transcriptase inhibitor (NRTI) resistance-associated sites (shaded) and other amino acid substitutions, where a significant difference was noted among the NRTI-naive group between subtype B and CRF01_AE ($p < .05$). The frequency of each substitution is shown in brackets. #RT sequence was not available in 2 patients with subtype B. The difference was not significant among NRTI treatment failure group. Ins, insertion. Shown in bold is the site where the frequency remained significantly different among the treatment failure groups between subtype B and CRF01_AE after adjusting for NRTI drug usage patterns ($*p = .04$, $**p = .03$).

Codon / Consensus B	[illegible]	13 I	[illegible]	35 E	[illegible]	41 R	[illegible]	69 H	[illegible]	89 L	[illegible]	93 I
B (naïve) N=57	[illegible]	V(6)	[illegible]	D(6)	[illegible]	K(24)	[illegible]	K(5) N(3) R(1) Y(1)	[illegible]	-	[illegible]	L(29)
AE (naïve) N=14	[illegible]	V(12)	[illegible]	D(12)	[illegible]	K(14)	[illegible]	K(14) Q(1)	[illegible]	M(14)	[illegible]	L(1)
B (failure) N=68	[illegible]	V(27)	[illegible]	D(11)	[illegible]	K(26)	[illegible]	K(8) N(1) R(1)	[illegible]	M(2)	[illegible]	L(32) M(1)
AE (failure) N=19	[illegible]	V(15)	[illegible]	D(18)	[illegible]	K(17)	[illegible]	K(19)	[illegible]	M(13) V(4) I(2)	[illegible]	L(6)

FIGURE 2. Amino acid variation in the protease region. This figure shows amino acid variation at the known protease inhibitor (PI) resistance-associated sites (shaded) and other amino acid substitutions, where a significant difference was noted among the PI-naive group between subtype B and CRF01_AE ($p < .01$). #PI resistance-associated sites where a significant difference was noted among the PI-naive group ($p < .01$). Shown in bold is the site where the frequency remained significantly different among the treatment failure groups between subtype B and CRF01_AE after adjusting for PI drug usage patterns ($*p < .001$, $**p = .01$).

with the use of any particular drug. Interestingly, the A71V mutation was significantly associated with nelfinavir therapy in CRF01_AE patients ($p = .005$), but it was associated with indinavir therapy in subtype B patients ($p = .05$). Similarly, the N88S mutation was significantly associated with nelfinavir therapy in CRF01_AE patients ($p = .004$), whereas this mutation was significantly associated with indinavir therapy in subtype B patients ($p = .002$).

DISCUSSION

Studies have shown that naturally occurring polymorphisms in drug-naive HIV-1–positive individuals are also sites associated with resistance to antiretroviral drugs. These studies found that the RT and the protease sequences of the non-B subtype viruses were highly diverse. Amino acid sequences at positions of known NRTI resistance mutations in subtype B viruses are highly conserved between different subtypes.[7–10] Conversely, amino acids at positions of known PI resistance in subtype B viruses are highly variable in different subtypes.[10,11] The drug susceptibility of non-B subtype viruses has also been studied in phenotypic assays, but the susceptibility to various NRTIs and PIs appears to be similar across the different subtypes.[12,13] Likewise, clinical responses to antiretroviral drug therapy were found to be similar in patients infected with different subtypes.[14] Little has been reported about the characteristics of drug resistance mutation patterns of non-B subtype viruses among patients who do not respond to antiretroviral drug therapy, however. Recently, it has been reported that the prevalence of known drug resistance mutations significantly differs between subtypes B and C.[15] To our knowledge, this is the first report showing that the patterns of drug resistance mutations of CRF01_AE patients also significantly differ from those of subtype B patients.

Our observations show that many amino acid substitutions commonly seen in CRF01_AE patients with drug treatment failure were previously identified drug resistance mutations in subtype B infection. Substitutions associated with drug resistance to azidothymidine (AZT), lamivudine (3TC), and didanosine (ddI) in subtype B infections were also frequently found in CRF01_AE drug-resistant patients as previously reported.[16] A two-amino acid insertion at position 67, which we have previously demonstrated as conferring multiple RT inhibitor resistance in subtype B infection, was also seen in CRF01_AE viruses.[17] Our data showed that the two mutations, T69N and V75M, in the RT differ in frequency, however. It is worth noting that the V75M mutation was more commonly seen in CRF01_AE infection and significantly associated with d4T therapy in CRF01_AE infection but not in subtype B infection. The difference was marginal, and further investigation will be required to confirm the clinical significance of this finding. Furthermore, we have shown that several drug resistance mutations in the protease region appear to be characteristic of CRF01_AE infection. In particular, the known mutations at L10F, K20I, L33I, and N88S were found more frequently in CRF01_AE infection than in subtype B infection. Our data suggest that N88S is an important drug resistance mutation presumably against nelfinavir in CRF01_AE infection. The N88S has been reported to confer drug resistance against nelfinavir, indinavir, and BMS-232632 in subtype B[18]; however, due to the rarity of subtype B clinical samples, further studies are still required to clarify the implication of the N88S mutation.[19]

Interestingly, the N88S mutation was strongly linked with the L10F mutation in CRF01_AE patients ($p < .0001$); all 6 CRF01_AE patients with the N88S mutation also had the L10F mutation. Conversely, there were 3 subtype B patients with L10F and 4 subtype B patients with N88S, none of who had both mutations. Surprisingly, none of the PI-resistant CRF01_AE patients had D30N, A71V, or N88D, which are often found in PI-resistant subtype B patients. As previously reported, our observation also showed a strong link between D30N and N88D in subtype B patients ($p < .0001$); 21 of 22 subtype B patients with the N88D mutation also had the D30N mutation.[20]

A number of studies have shown the benefit of having genotype results for determining the optimal drug regimen in subtype B infections.[21,22] Our data suggest that the knowledge acquired from subtype B drug resistance genotypes cannot always be applied to interpret subtype CRF01_AE genotype results, however, especially when interpreting genotypic results from patients receiving nelfinavir. Accumulation of data and analysis of drug resistance mutations in non-B subtype infections are urgently needed to improve the selection of the optimum drug regimen specific for each subtype.

Our results were derived from a cross-sectional and retrospective observation with rather small data sets and do not exclude the possibility that these drug-resistance mutations existed as naturally occurring polymorphisms prior to antiretroviral treatment. Nevertheless, there were no such polymorphisms in the drug-naive patients in our data and previous reports of the *pol* gene analysis of non-B subtypes,[11] suggesting that this case is highly unlikely. Another limitation is that in the current study, we did not have information about drug adherence, which may have caused a significant proportion of treatment

failure. Further studies using prospective cohort patients with drug adherence data will be essential to confirm these preliminary findings. With much larger data sets, we may be able to tease out more minor differences. We found that these patients with CRF01_AE infection had significantly more advanced HIV disease than the subtype B patients and that the sex ratio was significantly different. We believe that these differences do not account for the difference in the patterns of drug resistance mutation, however. We were also concerned about a potential bias induced by a group of patients for whom protease sequence data were not available. The proportion of female patients in this group was 20% and that of heterosexual transmission was 49%. These proportions were higher than those for subtype B infection and lower than those for CRF01_AE infection. We suspected that this group is a mixture of both subtype B and CRF01_AE patients who were successfully treated; thus, it should not affect the results among treatment failure groups.

In summary, we have observed some unique patterns of mutations in the *pol* gene of CRF01_AE-infected patients who failed to respond to antiretroviral drug treatment. Our data strongly suggest that CRF01_AE viruses evolve differently from subtype B viruses under the selection pressure of combination antiretroviral therapy, particularly in relation to nelfinavir. It is now important to expand our knowledge of drug-resistant genotypes in widely prevailing non-B subtype HIV viruses.

Acknowledgments: This study was supported by Japan Health Science Foundation and the Organization for Pharmaceutical Safety and Research of Japan. The authors thank H. Yanai for statistical advice, H. Abumi for technical assistance, A. Suzukawa for data management, and Jonathan Weber for reading the manuscript. They also thank M. Ishikawa, T. Miura, K. Fukutake, A. Ajisawa, K. Gouchi, M. Taki, M. Koike, A. Iwamoto, H. Hanabusa, J. Mimaya, J. Takamatsu, N. Takata, E. Kakishita, S. Kashiwagi, and A. Shirahata for providing clinical information.

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among WT viruses and a persistent virus carrying the 215D mutation. Our results show that viruses with unique patterns of NAMs including D67N and/or K219Q/E are commonly found among newly diagnosed patients. The low fitness cost conferred by D67N in the absence of drug supports persistence of this mutation in the untreated population and highlights the potential for secondary transmission of viruses carrying these unique RT genotypes. The faster evolution of these mutants toward zidovudine resistance is consistent with the higher viral fitness observed in the presence of zidovudine and may have clinical implications. Our findings demonstrate that transmitted HIV-1 strains with D67N and/or K219Q/E are phenotypically different from WT viruses.

ABSTRACT 83

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Male genital tract compartmentalization and transmission of 215L revertant

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INTRODUCTION: HIV develops resistance to zidovudine (ZDV) with the primary mutation of 215F/Y, which is a two-base mutation in the codon. In the absence of ZDV selective pressure HIV often undergoes a single base mutation to a 'revertant' (215D, C, N, D, S, E, L). These revertants have a greater fitness advantage in the absence of ZDV than the 215F/Y virus and only require a one-base change in the codon from the 215F/Y virus. They are long-lived in the blood in the absence of ZDV and are associated with rapid emergence of 215F/Y when ZDV is re-instated. Transmission of ZDV resistance has been documented both with 215F/Y and 215 revertants. To investigate the nature of revertant transmission, we evaluated the first documented case of 215L transmission.

METHODS: A transmission partner pair was identified in the UCSD Primary Infection cohort. Population (Viroseq, Applied Biosystems) and dye-primer sequencing of HIV *pol*, length polymorphism analysis of V12 and V45 HIV *env* regions (GeneScan, Applied Biosystems), and phenotypic resistance testing (Phenosense, Virologic) were performed on HIV RNA isolated from blood and seminal plasma of both subjects.

RESULTS: The source subject was chronically infected with HIV and had a long antiretroviral treatment history but had not been treated for 16 months when identified with a CD4 count of 139 cells/ml and HIV viral load of 50702 copies/ml. The index subject was acutely infected and naive to antiretroviral therapy with an initial CD4 of 472 cells/ml and HIV viral load of 36962 copies/ml. Population sequencing identified a 215F/L mixture in the blood of the source partner but only 215L was found in the blood of the receptive partner. Using both population and dye-primer sequencing (10% detection of minor species) only 215L virus was identified in the semen of both subjects. Phenotype testing revealed high-level resistance to ZDV in the blood of the source but only moderate-level resistance in the index subject. Genotypic and phenotypic high-level resistance was also noted to non-nucleoside reverse transcriptase inhibitors and nelfinavir in all samples. Length polymorphism analysis of HIV derived from the blood and semen of the index subject revealed multiple HIV quasispecies, which were more similar to the quasispecies detected in the semen of the source partner than the quasispecies detected in the blood of the source subject.

CONCLUSIONS: These investigations show compartmentalization of 215 revertants in the male genital tract. This may be explained by the isolation of a founding virus or the selection of such variants in the genital tract. Since antiretrovirals differentially penetrate the blood and male genital compartments, it may facilitate the production and/or selective retention of revertants. This has significant public health implications, as these revertants represent highly fit viruses that can become resistant to ZDV more readily than wild-type virus.

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Analysis of virion morphology and assembly process in protease inhibitor-resistant HIV-1

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BACKGROUND AND OBJECTIVE: Drug-resistant viruses often demonstrate reduced viral replication compared to wild-type viruses. In protease inhibitor (PI)-resistant viruses, this reduced replication is due to impaired Pr55^{Gag} processing into mature proteins by the protease with drug-resistant mutations within its active site. In this study, to understand the pathogenesis of PI-resistant viruses, we analysed the impact of impaired Gag processing on the virion assembly process and virion morphology.

METHODS: A PI-resistant case was selected from patient samples sent to NIID for routine drug resistance genotyping. A *gag-protease* fragment derived from the patient virus was amplified by RT-PCR and inserted into an HXB2-based virus expression vector. The following four types of recombinant viruses were prepared: GP-type (patient *gag* and *protease*), P-type (HXB2 *gag* and patient *protease*), GP^{-c} type (*gag* cleavage site mutations removed from GP-type) and P^{+C} (patient *protease* and the cleavage site mutations found in the patient *gag*). Four approaches were employed to analyse characteristics of the recombinant viruses. First, replication capacities of the four recombinant viruses were evaluated using competition cultures. Second, the Pr55^{Gag} processing pattern of each recombinant virus was analysed using western blot analysis. Third, the morphology of the virus particles was analysed by electron microscopy (EM). COS7 cells or 293T cells were transfected with the recombinant virus DNA, and 48 h after the transfection, cells were harvested carefully and subjected to EM analysis. Fourth, the localization of Gag proteins inside the host cells was analysed by confocal microscopy. Gag proteins were stained by anti-p17 and/or anti-p24 monoclonal antibodies (mAbs), and cellular organelles were stained by anti-ER, anti-Golgi, anti-late endosome, or anti-Tsg101 mAbs.

RESULTS: A case with D30N/M46I/N88D/L90M PI-resistant mutations and A431V /L449F *gag* cleavage site mutations was selected for the study. There were significant differences in the replication capacities of the four recombinant virus types constructed from the *gag-protease* fragment of this patient, and the replication competence was, in order from most active to least active, GP>P^{+c}>P>GP^{-c}. We observed prominent unprocessed Pr55^{Gag} and 41 Kd intermediate *gag*-product in replication reduced virus types P^{+c}, P and GP^{-c}. In EM analysis, immature viruses were observed in P^{+c}, P and P^{-c}. Interestingly, we found intracellular virus budding in the P-type as well as the P^{+c} type virus. Consistent with this EM results, unprocessed Gag protein was found prominently at the perinuclear area in P and P^{+c} type transfected cells when analysed by confocal microscopy.

CONCLUSION: Immature virion morphologies and intracellular budding were observed in PI-resistant viruses with impaired Gag processing. Our results suggest aberrant interaction between virus proteins and host factors in PI-resistant viruses.

JOURNAL OF CLINICAL MICROBIOLOGY, Nov. 2003, p. 4971–4979 Vol. 41, No. 11
0095-1137/03/$08.00+0 DOI: 10.1128/JCM.41.11.4971–4979.2003
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Novel Enzyme-Linked Minisequence Assay for Genotypic Analysis of Human Immunodeficiency Virus Type 1 Drug Resistance

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Received 8 January 2003/Returned for modification 23 April 2003/Accepted 12 August 2003

We constructed a novel tool for genotypic analysis of human immunodeficiency virus type 1 (HIV-1) drug resistance by using an enzyme-linked minisequence assay (ELMA). ELMA is a combination of hybridization and a 1-base extension reaction, and we designed the assay to detect five mutations conferring nucleoside analogue resistance (M41L, D67N, K70R, T215Y, and M184V) and six mutations conferring protease inhibitor resistance (D30N, M46I, G48V, V82A, I84V, and L90M). At all detection points, ELMA demonstrated high sensitivity and specificity, sufficient for clinical use. Compared to that obtained by direct sequencing, the genotypic information obtained by ELMA is limited to the targeted loci for which it was designed. However, ELMA proves advantageous in several respects. The assay does not require expensive equipment, such as an autosequencer, and can be performed in regular clinical diagnostic laboratories. Therefore ELMA can be a candidate for a drug resistance monitoring assay to be introduced in developing countries. In addition, ELMA demonstrated higher sensitivity in the detection of minor resistant populations. We successfully detected a minor virus population (10%) by the assay. The high sensitivity and specificity of the assay recommend it as a first screening assay for drug resistance surveillance.

One of the major causes of human immunodeficiency virus type 1 (HIV-1) treatment failure is the emergence of drug-resistant viruses (10, 12, 18). Each anti-HIV-1 drug induces a specific amino acid mutation pattern responsible for drug resistance expression in its target enzyme, protease or reverse transcriptase (RT) (4). Therefore, the level of drug resistance can be evaluated by nucleotide sequencing of the part of the genome encoding the target enzyme. Several clinical cohort studies have shown that monitoring of the drug resistance genotype during treatment is beneficial to treatment outcome and prognosis—indeed, such testing appears to be necessary in order to proceed with high-quality treatment (1, 6). Nucleotide sequencing technology based on the Sanger method has advanced greatly in the past decade. Fluorerescein-labeled deoxynucleoside triphosphates (dNTP) and the capillary-type autosequencer have made it possible to analyze samples more easily and faster than previously possible. However, the expensive equipment required for such analysis limits its availability, especially in developing countries, where the need for drug resistance genotyping is increasing together with the introduction of generic anti-HIV-1 drugs. Thus, the development of inexpensive and rapid genotypic assays other than those using direct sequencing is eagerly anticipated.

To reduce the cost and increase the availability of drug resistance genotyping, several simplified mutation detection assays, such as line probe assays (19), oligonucleotide ligation assays (7), and mutagenically separated PCR (8, 16), have been developed. Hybridization is the technology commonly used to identify specific nucleotide sequences, by using short complementary oligonucleotide probes, and specificity is controlled by a delicate probe-target annealing interaction. Therefore, unexpected mutations in the target sequence region may cause false results, and standard hybridization may not be a suitable strategy to apply to genes with high polymorphism. To minimize the effect of mutations within probe-targeted sequences and at the same time preserve the simplicity and availability of hybridization, we constructed an HIV-1 genotypic assay, named the enzyme-linked minisequence assay (ELMA), based on a modified hybridization procedure. In ELMA, two modifications to the standard hybridization method were introduced. First, a relatively low annealing temperature was selected for the hybridization reaction. The less-restricted hybridization condition minimized the effect of unexpected mutations within the target sequence and decreased the risk of false-negative results. Second, a 1-base extension reaction of the probe with tagged deoxynucleotide was added after the hybridization step. By this enhanced process, it became possible to control the reaction performance by the 3′ end of the probe. False-positive results due to probe-target misannealing during the hybridization step were eliminated by this additional 3′-end control. By these modifications, we successfully constructed a genotyping assay designed to detect representative drug resistance mutations of zidovudine (AZT) and lamivudine (3TC) and primary mutations of the protease inhibitors.

MATERIALS AND METHODS

Basics of ELMA. The basic technology of ELMA is a combination of DNA hybridization and point mutation detection by 1-base elongation with a biotinylated deoxynucleotide, i.e., a minisequence (11). The assay consists of four major steps: (i) extraction and amplification, (ii) hybridization, (iii) extension, and (iv) visualization. In the first step, extraction and amplification, target DNA

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TABLE 1. Primers used for amplification of the first-strand protease and RT DNA fragments

Enzyme targeted	Primer designation (orientation)	Sequence
Protease	PRO5 (sense)	5'-AGA CAG GYT AAT TTT TTA GGG A
	PRO2L (antisense)	5'-TAT GGA TTT TCA GGC CCA ATT TTT GA
RT	RT1L (sense)	5'-ATG ATA GGG GGA ATT GGA GGT TT
	RT4L (antisense)	5'-TAC TTC TGT TAG TGC TTT GGT TCC

fragments with one or more detection points are amplified *from* patient plasma viral RNA. In the second step, hybridization, the denatured amplified target DNA fragments are captured by the corresponding oligonucleotide probe applied to an enzyme-linked immunosorbent assay plate. The third step is an extension step. A biotinylated dNTP is incorporated on the 3' end of each oligonucleotide probe. The final step is visualization, in which the incorporated biotinylated dNTP is visualized by using horseradish peroxidase (HRP)-conjugated avidin and HRP as substrates.

Extraction of viral RNA and amplification of target fragments. HIV-1 RNA was extracted from 200 μl of patient plasma by using a commercially available viral RNA extraction kit (Roche Diagnostics, Basel, Switzerland). Reverse transcription and the outer PCR were performed by using a one-step RT-PCR system (Takara, Osaka, Japan) with a 30-min reverse transcription step at 60°C, followed by 30 cycles of three-step PCR as follows: 95°C for 30 s, 55°C for 30 s, and 72°C for 30 s. With this outer PCR, 480-bp protease fragments and 888-bp RT fragments were amplified independently. The primers used in this outer PCR are shown in Table 1. In the inner PCR step, short target DNA fragments, three in the protease region (Fig. 1, fragments a to c) and four in the RT region (Fig. 1, fragments d to g), were amplified with AmpliTaq DNA polymerase (Applied Biosystems, Foster City, Calif.) and the primers listed in Table 2. The outer PCR products were denatured by 5 min of incubation at 95°C, followed by 40 cycles of three-step PCR as follows: 95°C for 30 s, 60°C for 30 s, and 72°C for30 s. Immediately after PCR termination, 50 μl of denaturation buffer (0.4 M NaOH) was added to the PCR tubes to keep the amplicons as single-stranded DNA and to inactivate residual *Taq* enzyme. These amplified DNA fragments included 11 drug resistance mutations, as follows: 6 mutations conferring resistance to major protease inhibitors (D30N, M46I, G48V, V82A, I84V, and L90M) (3, 9, 17), 4 mutations conferring resistance to nucleoside analogues (M41L, D67N, K70R, and T215Y) (13), and the 3TC resistance mutation M184V (21). The details of the fragments are summarized in Table 2.

Hybridization of amplified targets and determination of alleles by 1-base extension reaction. Key to the present assay are the designs of the hybridization probe and the minisequence step following hybridization. Two types of detection strategy were employed (Fig. 2). The first strategy, type A, was to determine the nucleoside pattern of the detection point by annealing of the 3' end of the hybridization probe. In this strategy, a mutation point locates exactly on or 1 base upstream of the 3' end of the probe. If the 3' end of the probe exactly matches the target DNA, then a biotinylated dNTP, complementary to the target locus, will be incorporated in the subsequent extension step. On the other hand, if the 3' end of the probe does not match the target sequence, the biotinylated dNTP will not be incorporated. Thus, two probes are required in this strategy, one for the wild type and the other for the mutant, and the nucleoside pattern is determined by ascertaining whether the biotinylated dNTP is incorporated or not. This type A strategy is used for determining six protease inhibitor resistance mutations (D30N, M46I, G48V, V82A, I84V, and L90M) and four RT inhibitor resistance mutations (M41L, D67N, K70R, and M184V). In the second strategy, type B, probes were designed to reach exactly 1 base before the detection point, and the nucleoside pattern was defined by analyzing the type of biotinylated dNTP taken up during the extension reaction. Therefore, only one common probe is required for the type B assay. This type B strategy is employed for the T215Y assay, and the wild type and the mutant are distinguished by incorporation of biotinylated dATP or dTTP. Figure 3 shows the alignment of the probes in a 96-plate format.

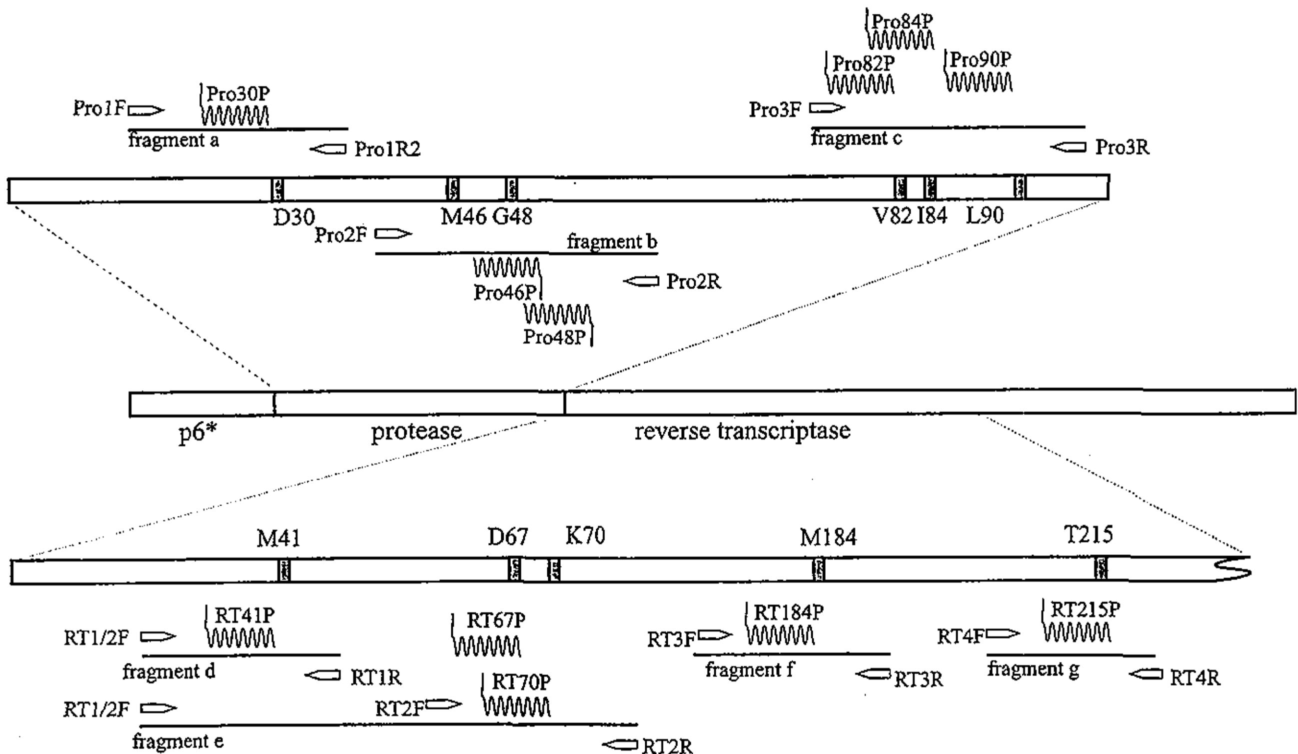

FIG. 1. PCR primers, amplified target fragments, and detection probes. A total of seven target fragments are amplified in the assay. There are six detection points in the protease region and five detection points in the RT region. Open arrows, PCR primers; solid lines, target fragments, wavy lines, detection primers; shaded boxes, target sites.

TABLE 2. Primers used for amplification of target DNA and probes for hybridization

Enzyme targeted	Position	ELMA type	Fragment	Primer for amplification			Probe for hybridization		
				Name	Direction[a]	Sequence	Pattern	Name	Sequence
Protease	30	A	a	PRO1F	Senses	ATA GAC AAG GAA CTG TAT CC	D30	PRO30P-W	Amine-AGG AAG CTC TAT TAG ATA CAG GAG CAG ATG
				PRO1R2	Antisense	AAA TTC ATT TCT TCT AAT ACT GT	N30	PRO30P-M	Amine-AGG AAG CTC TAT TAG ATA CAG GAG CAG ATA
	46	A	b	PRO2F	Sense	GCC AGG AAG ATG GAA ACC AA	M46	PRO46P-WC	Amine-TTT GAT AAA ACC TCC AAT TCC CMC TAC CAT
				PRO2R	Antisense	TGT AGG TCC TAC TAA TAC TG	M46	PRO46P-WA	Amine-TTT GAT AAA ACC TCC AAT TCC CMC TAA CAT
							146	PRO46P-MTTG	Amine-TTT GAT AAA ACC TCC AAT TCC CMC TAT CAA
							146	PRO46P-MATA	Amine-TAC TTT GAT AAA ACC TCC AAT TCC CMC TAT
	48	A					G48	PRO48P-W	Amine-TCT TAC TTT GAT AAA ACC TCC AAT TCC CCC
							V48	PRO48P-M	Amine-TCT TAC TTT GAT AAA ACC TCC AAT TCC CAC
	82	A	c	PRO3F	Sense	ATA CCC ATA GAA ATC TGT GG	V82	PRO8P-2W	Amine-GGT ACA GTA TTA GTA GGA CCT ACA CCT GTC
				PRO3R	Antisense	GGA AAA TTT AAA GTG CAA CCA A	A82	PRO82P-M	Amine-GGT ACA GTA TTA GTA GGA CCT ACA CCT GCC
	84	A					I84	PRO84P-W	Amine-CAG TAT TAG TAG GAC CTA CAC CTG TCA AYA
							V84	PRO84P-M	Amine-CAG TAT TAG TAG GAC CTA CAC CTG TCA AYG
	90	A					L90	PRO90P-W	Amine-CAC CTG TCA ACA TAA TTG GAA GAA ATC TGT
							M90	PRO90P-M	Amine-CAC CTG TCA ACA TAA TTG GAA GAA ATC TGA
RT	41	A	d	RT1/2F	Sense	GTT AAA CAA TGG CCA TTG ACA GA	M41	RT41P-W	Amine-TAA AAG CAT TAG TAG AAA TTT GTA CAG AAA
				RT1R	Antisense	GTA TGG ATT TTC AGG CCC AAT T	L41	RT41P-M	Amine-TAA AAG CAT TAG TAG AAA TTT GTA CAG AAC
							L41	RT41P-MT	Amine-TAA AAG CAT TAG TAG AAA TTT GTA CAG AAT
	67	A	e	RT1/2F	Sense	GTT AAA CAA TGG CCA TTG ACA GA	D67	RT67P-W	Amine-ATA CTC CAG TGT TTG CCA TAA AGA AAA ARG
				RT2R	Antisense	TGA ACT TCC CAG AAG TCT TGA G	N67	RT67P-M	Amine-ATA CTC CAG TGT TTG CCA TAA AGA AAA ARA
	70	A					K70	RT70P-W	Amine-GTT CTC TGA AAT CTA CTA ATT TTC TCC ATT
							R70	RT70P-M	Amine-GTT CTC TGA AAT CTA CTA ATT TTC TCC ATC
	184	A	f	RT3F	Sense	AGC ATG ACA AAA ATC TTA GAG CC	M184	RT1840P-W	Amine-AAA ATC CAG ACA TAG TTA TCT ATC AAT ACA
				RT3R	Antisense	TAT TTC TAA GTC AGA TCC TAC ATA	V184	RT184P-M	Amine-AAA ATC CAG ACA TAG TTA TCT ATC AAT ACG
	215	B	g	RT4F	Sense	GCA GCA TAG AAC AAA AAT AGA GG	T215Y	RT215-P	Amine-CTG AGA CAA CAT CTG TTG AGG TGG GGA TTT
				RT4R	Antisense	TAT CAG GAT GGA GTT CAT AAC C			

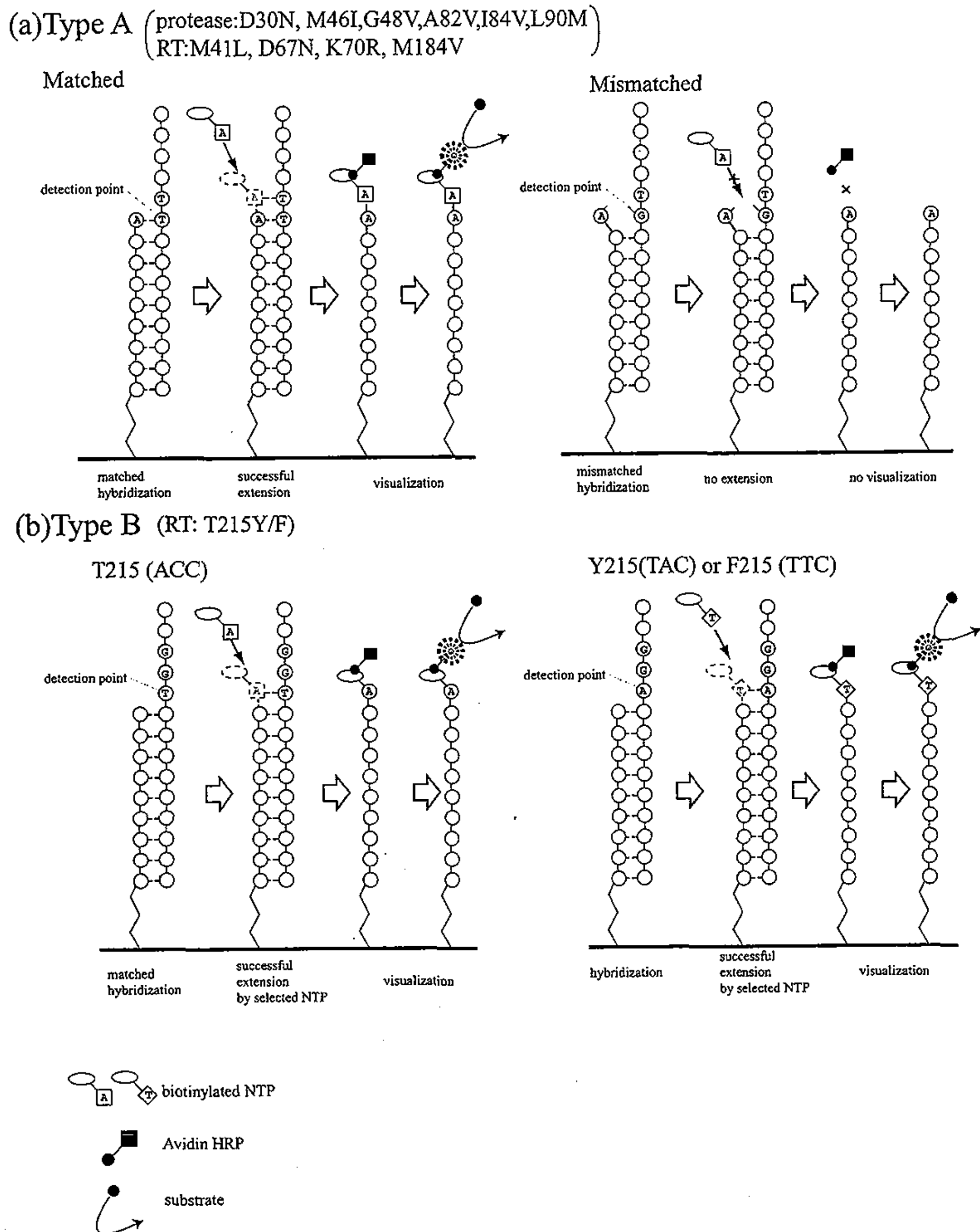

FIG. 2. Two ELMA detection strategies. (a) Type A. The 3′ end of the detection primer is designed to reach exactly the detection point. (Left) The corresponding biotinylated dNTP is incorporated at the 3′ end of the probe if the 3′ end of the probe matches with the target DNA. (Right) There will be no incorporation in the case of mismatch. Thus, two probes, a wild-type-specific and a mutant-specific probe, are used for the assay. (b) Type B. The 3′ end of the detection primer is designed to reach 1 base before the detection point. The mutation pattern is determined by the type of biotinylated dNTP (dATP or dTTP) incorporated at the detection point. Therefore, the probe in the type B strategy is not type specific.

Oligonucleotide probes were covalently bound to 96-well DNA-binding plates (Corning Costar Corp, Cambridge, Mass.) by a 3-h incubation at 37°C. The wells coated with oligonucleotide probes were filled with 100 μl of hybridization buffer (6× SSPE [pH 7.4] [1× SSPE is 0.18 M NaCl, 10 mM NaH_2PO_4, and 1 mM EDTA {pH 7.7}], 1% {wt/vol] Tween 20, 0.03 M HCl), and subsequently each PCR amplicon was applied to the appropriate well and incubated for 1 h at 55°C. After the hybridization step, AmpliTaq DNA polymerase (Applied Biosystems) and the appropriate biotinylated dNTP were added to each well and incubated

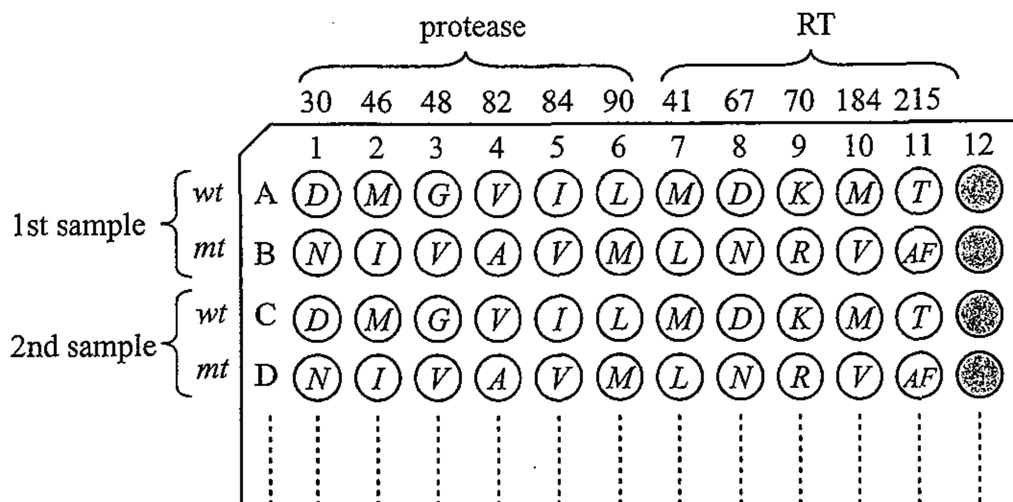

FIG. 3. Alignment of hybridization probes in a 96-plate format. There are two rows for each sample. The first row of each sample is coated with wild-type (wt) detection probes, and the second row of each sample is coated with mutant (mt) probes. The italicized letter in each well demonstrates the detectable amino acid pattern.

for 1 h at 37°C. By this reaction, the corresponding dNTP was incorporated into the 3′ end of the probe. Subsequently, hybridized target DNA was completely detached from the oligonucleotide probes and removed from the wells by three washes with a washing buffer (0.5× phosphate-buffered saline–0.1% Tween 20). Following this step, the covalently linked oligonucleotide probe with or without the corresponding biotinylated dNTP remained in the well. The final step was the determination step. Streptavidin-HRP (Roche Diagnostics, Mannheim, Germany) was added to the well and incubated for 30 min at room temperature. Each well was again washed three times with the washing buffer, followed by the addition of the substrate TMBlue (Cytech) to visualize incorporation of the dNTP at the 3′ ends of the probes.

Evaluation of the sensitivity of the assay by limiting dilution. The sensitivity of ELMA at each detection point was evaluated by limiting dilution of the template DNA. An HXB2 wild-type clone and 11 recombinant clones, each with a single drug resistance mutation, which were selected as detection points of the assay, were used. The 11 recombinant clones were constructed on an HXB2 backbone as described previously (20). The copy numbers of the fragments were calculated according to the concentration and size of the plasmid DNA. Serial 10-fold dilutions ranging from 10^6 to 10^3 were made for each plasmid clone, and ELMA genotyping was performed for all of the dilutions. In these analyses, hybridization cutoff levels were evaluated from the mismatched pairs of target DNA and hybridization probe, i.e., mutant target versus wild-type probe or wild-type target versus mutant probe.

Evaluation of the assay sensitivity for detection of minor mutant populations. The ability of the assay to detect minor mutant populations was evaluated by analyzing the mixture of wild-type and mutant templates. The ratios of the wild type to the mutant in the mixtures were 1:1, 10:1, and 100:1. The total DNA template amount was fixed at 10^5 copies. The test was performed for all 11 detection points. The analyses were repeated four times with independently prepared serial dilutions each time.

Evaluation of assay performance against patient samples. To evaluate the reliability of ELMA, patient samples were analyzed both by ELMA and by standard sequencing, and the results of the two assays were compared. Forty-five samples were chosen randomly from the HIV-1-infected patient samples sent to the National Institute of Infectious Diseases for routine drug resistance genotyping from November 1996 to November 2000.

The details of in-house sequencing have been described elsewhere (15). In brief, HIV-1 RNA was extracted from 200 μl of patient plasma and reverse transcribed to cDNA by using murine leukemia virus RT (Takara). Subsequently, a 480-bp fragment, which covers the whole protease region, and an 888-bp RT fragment including all the known drug resistance mutation points were amplified individually by nested PCR. The nucleotide sequence of each DNA fragment was analyzed by cycle sequencing using Big-Dye terminator chemistry (Applied Biosystems) and an ABI-377 autosequencer (Applied Biosystems). Electropherograms were carefully analyzed using Sequence Navigator (Applied Biosystems).

RESULTS

Evaluation of assay sensitivity and end point level of the assay. The ELMA data for each detection probe against wild-type and mutant target DNAs are summarized in Tables 3 and 4. In order to determine the limit of the copy number that could be detected by the assay, each target DNA was serially diluted in the range of 10^6 to 10^1 copies. Average optical densities (OD) with standard deviations (SD) based on quadruplicated data are shown.

The data of mismatched target DNA and probe pairs, i.e., wild type probe versus mutant target and mutant probe versus wild type target, were used to define the cutoff OD for each probe. The cutoff was calculated as the average OD + 3 SD. As shown in Tables 3 and 4, each of the probes has a unique cutoff value, which probably reflects the melting temperature of the probes. The highest cutoff value was 1.455 for the protease position 48 wild-type probe, and the lowest cutoff value was 0.125 for the RT position 70 wild-type probe. The detection end point (the lowest copy number for which an OD higher than the cutoff was obtained) for each detection point was determined by using the cutoff values listed in Tables 3 and 4.

Most of the probes were sensitive enough to detect templates of $<10^2$ copies. However, one wild-type probe (position 215) and three mutant probes (positions 215, 82, and 84) demonstrated lower sensitivities, with copy numbers at the 10^3 level. At most detection points, the sensitivities were at the same level for mutant and wild type detection. Three loci, positions 48, 82, and 84, showed different detection limits. The mutant probe was 1 log unit more sensitive than the wild-type probe at position 48, whereas the wild-type probes were 2 log units more sensitive than the mutant probes at positions 82 and 84.

TABLE 3. Detection end points of wild-type ELMA probes

Codon	Target	OD[a] of probe-target pair at the following copy number of the target:						Mean[c]	SD[d]	Cutoff[b] (mean + 3 SD)
		10^6	10^5	10^4	10^3	10^2	10^1			
41	Wild type	>2.000	>2.000	>2.000	>2.000	>2.000	1.942 ± 0.117			0.212
	Mutant	0.103 ± 0.010	0.103 ± 0.073	0.121 ± 0.063	0.096 ± 0.005	0.096 ± 0.009	0.105 ± 0.007	0.104	0.036	
67	Wild type	>2.000	>2.000	>2.000	>2.000	>2.000	1.952 ± 0.096			0.311
	Mutant	0.193 ± 0.027	0.210 ± 0.016	0.225 ± 0.022	0.249 ± 0.009	0.233 ± 0.015	0.165 ± 0.024	0.212	0.033	
70	Wild type	1.553 ± 0.344	1.673 ± 0.385	1.587 ± 0.349	1.828 ± 0.344	1.903 ± 0.195	1.736 ± 0.260			0.125
	Mutant	0.093 ± 0.007	0.059 ± 0.009	0.075 ± 0.007	0.056 ± 0.001	0.070 ± 0.012	0.094 ± 0.010	0.074	0.017	
184	Wild type	>2.000	>2.000	>2.000	>2.000	>2.000	1.840 ± 0.321			0.560
	Mutant	0.088 ± 0.008	0.078 ± 0.002	0.088 ± 0.007	0.091 ± 0.027	0.061 ± 0.007	0.256 ± 0.369	0.110	0.150	
215	Wild type	>2.000	>2.000	1.949 ± 0.067	0.585 ± 0.097	0.085 ± 0.041	0.041 ± 0.006			0.171
	Mutant	0.041 ± 0.007	0.078 ± 0.067	0.082 ± 0.065	0.044 ± 0.004	0.029 ± 0.007	0.034 ± 0.005	0.051	0.040	
30	Wild type	>2.000	>2.000	>2.000	>2.000	1.810 ± 0.275	0.410 ± 0.131			0.521
	Mutant	0.305 ± 0.031	0.269 ± 0.027	0.273 ± 0.025	0.310 ± 0.033	0.150 ± 0.029	0.056 ± 0.017	0.227	0.098	
46	Wild type	>2.000	>2.000	>2.000	>2.000	>2.000	1.464 ± 0.681			0.156
	Mutant	0.084 ± 0.011	0.094 ± 0.008	0.096 ± 0.007	0.103 ± 0.018	0.076 ± 0.011	0.034 ± 0.009	0.081	0.025	
48	Wild type	>2.000	>2.000	>2.000	>2.000	>2.000	1.464 ± 0.659			1.455
	Mutant	0.448 ± 0.014	0.411 ± 0.041	0.415 ± 0.041	0.435 ± 0.057	0.735 ± 0.170	1.123 ± 0.216	0.594	0.287	
82	Wild type	>2.000	>2.000	1.682 ± 0.637	1.937 ± 0.120	0.379 ± 0.376	0.602 ± 0.981			0.328
	Mutant	0.184 ± 0.034	0.164 ± 0.013	0.166 ± 0.014	0.171 ± 0.029	0.041 ± 0.005	0.036 ± 0.007	0.127	0.067	
84	Wild type	>2.000	>2.000	1.756 ± 0.489	>2.000	0.611 ± 0.509	0.677 ± 0.957			0.284
	Mutant	0.141 ± 0.019	0.134 ± 0.014	0.135 ± 0.013	0.162 ± 0.004	0.229 ± 0.018	0.201 ± 0.012	0.167	0.039	
90	Wild type	>2.000	>2.000	>2.000	1.936 ± 0.129	1.261 ± 0.863	0.082 ± 0.055			0.213
	Mutant	0.134 ± 0.013	0.126 ± 0.009	0.125 ± 0.011	0.086 ± 0.010	0.039 ± 0.006	0.048 ± 0.005	0.093	0.040	

[a] Average ± SD based on quadruplicated data.
[b] Determined from OD of mismatched probe-target pairs (wild-type probe and mutant target). The lowest copy number with an OD higher than the cutoff is designated the end point of the detection (indicated by boldfaced OD).
[c] Mean OD of mismatched probe-target pairs.
[d] SD of mismatched probe-target pairs.

Evaluation of assay sensitivity for detection of mutant populations mixed with wild-type populations. In Tables 3 and 4 the sensitivities of the probes were evaluated with a clonal DNA target amplified from HXB2 clones. However, virus populations in patients exist as mixed populations in clinical samples. Therefore, the probe sensitivity was evaluated by testing a *mixture* of wild-type and mutant targets. The same wild-type and mutant target templates used in the end point assay were mixed in three different wild-type/mutant ratios: 1:1, 10:1, and 100:1. All of the mixtures were adjusted to 10^5 copies of DNA so that the 100:1 mixture would contain more than 10^3 copies of the mutant template, a number sufficient to be detected at all detection points. Each test was repeated four times. Although the 10^3 copy level was a sufficient template number for

TABLE 4. Detection end points of mutant ELMA probes

Codon	Target	OD[a] of probe-target pair at the following copy number of the target:						Mean[c]	SD[d]	Cutoff[b] (mean + 3 SD)
		10^6	10^5	10^4	10^3	10^2	10^1			
41	Wild type	0.145 ± 0.026	0.182 ± 0.025	0.167 ± 0.004	0.172 ± 0.011	0.171 ± 0.119	0.191 ± 0.015	0.171	0.048	
	Mutant	>2.000	>2.000	>2.000	1.531 ± 0.938	>2.000	1.786 ± 0.251			
67	Wild type	0.164 ± 0.019	0.191 ± 0.016	0.194 ± 0.017	0.211 ± 0.029	0.149 ± 0.008	0.176 ± 0.071	0.181	0.036	0.289
	Mutant	>2.000	>2.000	>2.000	>2.000	>2.000	1.995 ± 0.010			
70	Wild type	0.169 ± 0.029	0.173 ± 0.069	0.145 ± 0.022	0.208 ± 0.005	0.221 ± 0.029	0.287 ± 0.071	0.200	0.061	0.383
	Mutant	1.143 ± 0.209	0.653 ± 0.248	0.659 ± 0.248	0.310 ± 0.031	0.526 ± 0.015	0.632 ± 0.072			
184	Wild type	0.096 ± 0.022	0.131 ± 0.015	0.123 ± 0.004	0.119 ± 0.006	0.061 ± 0.008	0.079 ± 0.013	0.101	0.028	0.185
	Mutant	>2.000	>2.000	>2.000	>2.000	>2.000	1.614 ± 0.772			
215	Wild type	0.160 ± 0.024	0.204 ± 0.013	0.198 ± 0.030	0.115 ± 0.005	0.028 ± 0.006	0.035 ± 0.004	0.123	0.074	0.345
	Mutant	1.157 ± 0.152	1.929 ± 0.143	1.928 ± 0.144	0.716 ± 0.050	0.121 ± 0.019	0.043 ± 0.013			
30	Wild type	0.106 ± 0.023	0.107 ± 0.007	0.130 ± 0.010	0.137 ± 0.010	0.054 ± 0.006	0.046 ± 0.005	0.096	0.037	0.207
	Mutant	1.963 ± 0.074	1.862 ± 0.213	1.864 ± 0.208	1.800 ± 0.231	0.859 ± 0.137	0.194 ± 0.085			
46	Wild type	0.343 ± 0.035	0.295 ± 0.015	0.334 ± 0.022	0.319 ± 0.022	0.380 ± 0.273	0.089 ± 0.059	0.293	0.141	0.716
	Mutant	>2.000	>2.000	>2.000	>2.000	>2.000	0.843 ± 0.457			
48	Wild type	0.642 ± 0.068	0.526 ± 0.056	0.576 ± 0.054	0.668 ± 0.130	0.539 ± 0.095	0.500 ± 0.368	0.575	0.162	1.061
	Mutant	>2.000	>2.000	>2.000	>2.000	>2.000	>2.000			
82	Wild type	0.309 ± 0.045	0.238 ± 0.050	0.222 ± 0.068	0.193 ± 0.030	0.051 ± 0.012	0.172 ± 0.129	0.197	0.099	0.494
	Mutant	>2.000	>2.000	>2.000	1.923 ± 0.154	0.064 ± 0.043	0.036 ± 0.005			
84	Wild type	0.160 ± 0.009	0.164 ± 0.039	0.199 ± 0.028	0.191 ± 0.015	0.104 ± 0.010	0.168 ± 0.021	0.164	0.037	0.275
	Mutant	>2.000	>2.000	>2.000	1.784 ± 0.302	0.150 ± 0.014	0.145 ± 0.022			
90	Wild type	0.368 ± 0.026	0.263 ± 0.068	0.310 ± 0.033	0.164 ± 0.060	0.053 ± 0.023	0.040 ± 0.004	0.200	0.132	0.596
	Mutant	>2.000	>2.000	>2.000	>2.000	0.603 ± 0.664	0.050 ± 0.020			

[a] Average ± SD based on quadruplicated data.
[b] Determined from OD of mismatched probe-target pairs (mutant probe and wild-type target). The lowest copy number with an OD higher than the cutoff is designated the end point of the detection (indicated by boldfaced OD).
[c] Mean OD of mismatched probe-target pairs.
[d] SD of mismatched probe-target pairs.

TABLE 5. Summary of comparison between ELMA and sequencing results for clinical samples

Enzyme targeted	Codon	No. of samples	No. of samples for which the following combination of results[a] was obtained:									Sensitivity[b]	Specificity[c]
			a	b	c	d	e	f	g	h	i		
RT	41	43	21	14	1	3	0	2	1	1	0	0.947	0.955
	67	44	20	10	3	5	0	6	0	0	0	1.000	1.000
	70	44	36	4	0	0	0	3	0	0	1	0.800	1.000
	184	42	20	18	0	1	1	1	0	2	0	0.909	1.000
	215	31	10	18	0	0	1	1	0	1	0	0.950	1.000
Protease	30	38	22	11	0	0	0	2	0	1	2	0.786	1.000
	46	45	30	8	3	0	1	1	1	1	0	0.923	0.968
	48	45	44	0	1	0	0	0	0	0	0	1.000	1.000
	82	43	36	5	1	0	0	0	0	1	0	0.857	1.000
	84	45	41	3	0	0	0	0	1	0	0	1.000	0.976
	90	45	23	18	1	1	1	0	1	0	0	1.000	0.958

[a] a, wild type by both ELMA and sequencing; b, mutant by both methods; c, mixture by both methods; d, mixture by ELMA and mutant by sequencing; e, mutant by ELMA and mixture by sequencing; f, mixture by ELMA and wild type by sequencing; g, mutant by ELMA and wild type by sequencing; h, wild type by ELMA and mutant by sequencing; i, wild type by ELMA and mixture by sequencing.

[b] Calculated as (b + c + d + e)/(b + c + d + e + h + i).

[c] Calculated as a/(a + g).

all mutant probes, only three mutants, M41L, V82A, and L90M, were successfully detected in four reproduced tests with a wild-type/mutant ratio of 100:1. For the other eight loci (D67N, K70R, M184V, T215Y, D30N, M46I, G48V, and I84V), the test was not sensitive enough to detect a 1% mutant population in the mixture (detection was consistently unsuccessful at this 100:1 ratio); however, the mutant population was successfully detected at a 10:1 ratio (10%). For single-population detection, the results show that the lowest detectable level was 10^2 copies. However, with a mixed viral population, 10^2 copies of mutant clones were not detected when mixed with 10^6 copies of wild-type clones. In that case, the lowest level of the minor population which could be detected was 10^3 copies/ml. This discrepancy between the detectable copy number of clonal and mixed target populations may be due to competition between HIV-1 mutant and wild-type target DNAs.

Evaluation of assay performance against patient samples. The performance of ELMA with clinical samples was evaluated by testing 45 HIV-1 patient samples. The RNA copy number of the 45 patients ranged from $10^{2.6}$ to $10^{6.3}$ copies/ml (average, $10^{5.3}$; median, $10^{4.3}$). In this study, HIV-1 RNA was extracted from patient plasma, and target DNA was prepared by reverse transcription and nested PCR. The first PCR product was also analyzed by the direct sequencing method, and the result was compared with the ELMA result. The comparison of the direct sequencing results with the ELMA results is summarized in Table 5. The sensitivity and specificity of ELMA were calculated for each detection point, using the sequencing results as the standard. Because ELMA may be used for the first screening of drug resistance, it should capture all possible resistant cases. Therefore, in the calculations of specificity and sensitivity for which formulas are given in the footnotes to Table 5, "mutant" sequencing results and "mixture" ELMA results were considered concordant, as were "mixture" sequencing results and "mutant" ELMA results. In addition, "mixture" sequencing results and "wild-type" ELMA results were considered discordant. Further, cases in which "wild-type" sequencing results and "mixture" ELMA results were obtained were excluded from the calculations, because we could not rule out the possibility that ELMA had detected a minor mutant fraction that the sequencing failed to detect.

As further shown in Table 5, more than 93% of the samples were analyzed successfully by ELMA at all detection points except position 215. At position 215, ELMA failed to successfully analyze 14 of 45 samples (31%). For some reason the PCR products of these failed samples did not respond to either the wild-type or the mutant probe in the hybridization step. To understand the reason for the unresponsiveness to the codon 215 probes, we compared the target sequences of the position 215 probes of the 31 successfully analyzed samples with those of the 14 failed samples. We found that the frequency of polymorphisms in the target sequences, especially in the 3′ half, was significantly higher in samples for which ELMA analysis failed. High mutation frequencies were observed at the 10th, 11th, 13th, 19th, 20th, and 27th bases of the target sequences in these failed cases.

As shown in Table 5, there were 14 discordant results in total. Among these, we were able to specify the reason for the discordance for three results (two results for M184V and one result for T215Y). For position 184, the assay was constructed to distinguish between methionine (encoded by <u>A</u>TG) (underlining indicates a point targeted by the ELMA probe) and valine (<u>G</u>TG) by targeting the first base of the triplet. However, in the two failed cases, the substitution resulted not in valine (<u>G</u>TG) but in isoleucine (<u>A</u>TA). Thus, in this mutation pattern, the assay could not detect the substitutions. We observed a similar pattern in a position 215 discordant case. The assay was designed to distinguish between threonine (<u>A</u>CC) and tyrosine or phenylalanine (<u>T</u>AC or <u>T</u>TC) by targeting the first base of the triplet. The mutation pattern of the discordant case was isoleucine (<u>A</u>TA); therefore, our assay failed to detect the mutant.

For the other 11 discordant cases, we could not explain the discordance either by substitution pattern or by sequence polymorphisms in the target regions. The most likely explanation is population deviation caused by PCR primer selectivity. In these cases, the DNA population different from that of direct sequencing was preferentially amplified in the nested PCR.

DISCUSSION

Our newly constructed genotyping assay, ELMA, a combination of hybridization and 1-base extension reaction, demonstrated high sensitivity and specificity, sufficient to detect 11 different drug resistance mutations. The most critical point in developing the assay was optimizing the common hybridization condition for 11 different probe-target bindings. Appropriate annealing temperature and hybridization buffer conditions differ according to the length and sequence of the probes, and ideally these should be chosen specifically for each probe. However, as our assay was constructed in a 96-well format, the same buffer and temperature were required for all the probes in order to keep the assay procedure simple. The melting temperatures of the probes ranged from 74 to 88°C according to the targeted sequence, and the final hybridization temperature used for the assay was 74°C, adjusted to the lowest melting temperature of all the probes.

Because the probe-targeted regions of protease inhibitor-resistant mutants had higher GC contents than those of RT inhibitor-resistant mutants, the hybridization condition was less restrictive for the protease inhibitor resistance mutations. This condition is reasonable, because generally protease is highly polymorphic and is expected to have multiple mutations in the probe target regions. In ELMA, the goal of the hybridization step is to capture the target DNA, and the determination of wild type or mutant is made through the binding of the probe 3′ end and the subsequent extension step. Therefore, the 3′-end nucleotide sequence pattern of the probes was critical for assay performance, and the balance between the attractive force of the matched nucleotide pairs and the repulsion force of the mismatched pairs appeared to affect the cutoff OD of the probes. In fact, each probe had a different cutoff value, as shown in Tables 3 and 4. The probes for G48V detection demonstrated significantly high cutoff values: 1.455 for the wild type and 1.061 for the mutant. These high cutoff values can be explained by examination of the sequences of the 3′ ends of the probes. As shown in Table 2, the wild-type and mutant probe sequences were TCCCCC-3′ and TCCCAC-3′, respectively. In the case of a mismatch between a wild-type probe and a mutant target, or between a mutant probe and a wild-type target, the nucleotide pair at the underlined position would be T-C or A-G, respectively. The repulsion forces produced by G-C and A-G mismatches (which may cause the 3′ end of the probe to become detached) are relatively weak compared to the attraction force caused by the surrounding four G-C matched pairs. Therefore, 3′-end cysteine tends to bind to the target even though the next nucleoside does not match with the target, and the high probability of misbinding resulted in a high OD cutoff. Although G48V probes demonstrated high cutoffs, this did not affect assay performance: as shown in Table 5, both the sensitivity and the specificity of ELMA for G48V scored 1.000.

More than the cutoff values, the polymorphisms observed in the target regions are critical for the assay. If there are too many polymorphisms in the target region, probes may not detect the amplified target DNA. In particular, we experienced this problem in designing the probe for the position 215 mutation. Only 31 out of 45 test samples were successfully analyzed by ELMA at position 215. When comparing the sequences of the probe target regions of the 31 successful samples and 14 failed samples, we noted that a significantly higher number of mutations accumulated in the failed samples. To improve the success rate of the assay, it may be necessary to design another probe, taking into consideration the frequency of the accumulated mutations in the probe target region. The limitation of the present probe design can be observed at other detection points as well. There were only four detection points (protease positions 46, 48, 84, and 90) at which all 45 test samples were successfully analyzed. The data suggest a requirement of multiple probes for each detection point to overcome nucleoside polymorphisms in the probe target regions.

Thus, compared to direct sequencing, ELMA is limited in the quality and quantity of the results. Still, the assay is attractive in several respects.

One interesting aspect of ELMA is that the test can detect a minor drug-resistant population equivalent to 10% of the total virus population according to the mixture analyses performed with recombinant clones. This number compares favorably to that for standard direct sequencing, which generally can detect a minor population equivalent to 30 to 50% of the total virus population (22). In the comparison of ELMA and direct sequencing for 45 patient samples, 16 samples tested "wild type" by direct sequencing and "mixture" by ELMA. The data suggest that minor drug-resistant mutant populations might have been detected by ELMA. To confirm the mixture result by ELMA, we performed multiple cloning for the same sequenced samples. Seventeen to 26 clones were sequenced in each sample, and we successfully detected drug-resistant mutant clones in 6 out of 16 samples. The frequencies of the mutant clones ranged from 11.7 to 47.6%. We could not find mutant clones in the remaining 10 samples, but we cannot conclude that these were false-positive results, as a possibility remains that ELMA detected minor populations of <5% in these samples. Another attractive feature of the assay is that the test can be performed in a few hours without the use of expensive equipment.

Taking these qualities into consideration, ELMA can be utilized in a practical manner in the following situations and for the following uses. First, as there is no requirement for expensive equipment such as autosequencers, and considering the high sensitivity of the assay, ELMA is an excellent candidate for drug resistance genotyping to be used in developing countries, where, with the greater availability of generic antiretroviral treatment, the introduction of a drug resistance monitoring system has been an urgent issue. Although specialized training is required to run the assay, a clinical diagnostic laboratory can introduce the assay without an investment in additional equipment. Second, ELMA can be used as a tool for drug-resistant population surveillance. Today, with regard to primary HIV-1 infection, there is an obvious risk of transmission of drug-resistant HIV-1 (2, 23). Because some of the drug-resistant HIV-1 strains demonstrate reduced viral replication activity compared to that of the wild-type virus (14), the resistant viruses can become the minor population upon termination of anti-HIV-1 treatment (5). This is an important issue in understanding the effect of preexisting resistant populations on antiretroviral treatment outcome and in the prognosis of infected patients. Therefore, it is imperative that minor hidden resistant virus populations in treatment-naïve

patients be detected, and ELMA as we have described it here has an advantage in the survey.

In conclusion, we successfully constructed a new assay for genotypic analysis of drug resistance, which can be performed in a standard PCR laboratory. However, improvement of the assay through further simplification of the assay procedure, and addition of other important drug resistance mutation points which we have not yet designed, is required for use in clinical studies.

ACKNOWLEDGMENTS

We thank the patients and physicians who were involved in our study. We thank the technical staff of Genome Science Inc for assisting in the experiments. We also thank Yumi Kanekama and Mary Phillips for help in preparing the manuscript.

This study was supported by a grant from the Organization of Pharmaceutical Safety and Research (OPSR) of Japan and by the Ministry of Health, Labor and Welfare of the Japanese Government.

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