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Mutations Associated with Failure of Raltegravir Treatment Affect Integrase Sensitivity to the Inhibitor In Vitro▽

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Raltegravir (MK-0518) is a potent inhibitor of human immunodeficiency virus (HIV) integrase and is clinically effective against viruses resistant to other classes of antiretroviral agents. However, it can select mutations in the HIV integrase gene. Nine heavily pretreated patients who received salvage therapy including raltegravir and who subsequently developed virological failure under raltegravir therapy were studied. For each patient, the sequences of the integrase-coding region were determined and compared to that at the beginning of the treatment. Four different mutation profiles were identified in these nine patients: E92Q, G140S Q148H, N155H, and E157Q mutations. For four patients, each harboring a different profile, the wild-type and mutated integrases were produced, purified, and assayed in vitro. All the mutations identified altered the activities of integrase protein: both 3′ processing and strand transfer activities were moderately affected in the E92Q mutant; strand transfer was markedly impaired in the N155H mutant; both activities were strongly impaired in the G140S Q148H mutant; and the E157Q mutant was almost completely inactive. The sensitivities of wild-type and mutant integrases to raltegravir were compared. The E92Q and G140S Q148H profiles were each associated with a 7- to 8-fold decrease in sensitivity, and the N155H mutant was more than 14-fold less sensitive to raltegravir. At least four genetic profiles (E92Q, G140S Q148H, N155H, and E157Q) can be associated with in vivo treatment failure and resistance to raltegravir. These mutations led to strong impairment of enzymes in vitro in the absence of raltegravir: strand transfer activity was affected, and in some cases 3′ processing was also impaired.

Retrovirus integrase (IN), a *pol* gene product, is responsible for the integration of retrovirus DNA into the host cell genome. Integration is an essential step of human immunodeficiency virus type 1 (HIV-1) replication.

HIV-1 IN (32 kDa) is a 288-amino-acid (aa) protein consisting of three independent structural domains (8, 18, 21). The N-terminal domain (aa 1 to 49) contains an HHCC motif, which binds zinc, thereupon promoting multimerization to tetramers (6, 26, 27), a protein state required for strand transfer activity (6, 26, 27). The central catalytic core domain (aa 50 to 212) contains the D, D(35)E catalytic motif (Asp64, Asp116, and Glu152) in a highly conserved spatial arrangement (22). The C-terminal domain (aa 213 to 288) has DNA-binding activity (3, 28).

IN catalyzes two reactions. The first is 3′-end processing, during which the terminal GpT dinucleotide is cleaved from the 3′ end of each long terminal repeat, producing CpA 3′-hydroxyl ends (9). This reaction takes place in the cytoplasm within a nucleoprotein complex referred to as the preintegration complex (30). The preintegration complex is transported through the nuclear pore to the nucleus, where the second activity—strand transfer—occurs. During this second step, IN transfers both newly exposed 3′ extremities of the viral DNA into the target DNA by a one-step transesterification; the viral genome is thereby inserted and covalently linked into the host genome (2, 7).

IN inhibitors, a new class of antiretroviral agents, block HIV-1 IN activity (25, 33). Two classes of inhibitors, interfering either with 3′-end processing in the cytoplasm (1, 32) or with strand transfer in the nucleus (10, 11, 13, 15, 38), have been described. Although both classes have been proven to be able to block HIV replication in cell culture (1, 15), only the second class was found to possess antiviral activity in vivo (5, 16, 29, 34). Due to its mechanism of action, this novel class of antiretroviral agents (ARV) is potentially valuable; it is active against viruses resistant to other classes of antiretrovirals such as nucleoside reverse transcriptase inhibitors (NRTI), nonnucleoside reverse transcriptase inhibitors (NNRTI), protease inhibitors (PI), and entry inhibitors. Raltegravir (MK-0518) is a member of this class of novel HIV-1 inhibitors interfering with the strand transfer stage. When used in combination with an optimized regimen, it is active in patients infected with drug-resistant viruses: a decrease of approximately 2 log units in HIV RNA levels is observed by week 24. However, it has been suggested that virological failure in patients under treatment with this compound is due to mutations in the IN gene (4a, 35a).

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TABLE 1. Baseline characteristics of patients

Patient	HIV-1 load (log_{10} copies/ml)[a]	No. of CD4 cells/mm^3	Mutations[b] mediating resistance to:			GSS[c]
			NRTI	NNRTI	PI	
1a	4.9	65	M41L, A62V, ins69SSS, F77L/F, M184V, T215C	G190S/G	L10F, K20I/A, V32I, M46I, I47V, F53L, I54L, A71V, V77I, I84V, L90M/L	0
1b	5	6	A62V, K65R, V75I, F77L, Y115F, F116Y, Q151M	V106I, Y188L	L10V, I15V, K20R, V32I, L33F, M36I, M46L, I54L, I62V, L63P, A71V, G73S, V82A, I84V, L89V, L90M	1
1c	4.3	370	M41L, V118I, M184V, L210W, T215Y	K103N	L10V, L33F, M46L, F53L, I54S, L63P, A71I, V82A, L90M	0
2a	4.3	2	M41L, D67N, T69N, L74I, V75S, V118I, M184V, L210W, T215Y	V108I, Y181C, G190A	L10F, V11I, I15V, V32I, L33F, M36L, M46I, I47V, I54L, Q58E, I62V, L63P, A71V, G73S, I84V, I85V, L89V, L90M	0
2b	4.7	238	D67N, T69D, K70R, Y115F, F116Y, Q151M, M184V, T215F, K219Q	Y181C, Y188L	L10V/F, V11I, V32I, L33F, M46I, I47V, I54L, I62V, L63P, A71I, G73A, I84V, N88D, L90M	0
2c	5.2	17	M41L, K65R, T215Y		L10V, K20R, L33F, M36I, M46I, F53L, I54A, Q58E, D60E, L63P, H69K, V77I, V82A, L89M, L90M	1
2d	5.4	39	M41L, ins69SVT, L74V, V118I, M184V, L210W, T215Y	A98G, K101E, V108I, Y181C, G190A	L10I, V11I, I15V, M36I, M46I, I47V, I54M, I62V/I, L63P, A71V, L76V/I, I84V, I85V, L89I, L90M	0
3	5.4	5	M41L, E44A, D67N, L74V, V75T, V118I, M184V, L210W, T215Y	Y181C, G190S	L10I, I47V, G48V/I, F53L, I54M, L63P, A71V, G73A, L76V, V77I, V82C, L89V, L90M	0
4	5.4	1	M41L, L74I/V, V118I, M184V, L210W, T215Y	K101Q	L10I, L33F, M46L, I54L, Q58E, I62V, L63P, A71V, V82A, I84V, L90M	1

[a] Levels of HIV RNA in plasma were determined by using the Cobas AmpliPrep/Cobas TaqMan HIV-1 test.
[b] ins, insertion.
[c] GSS, genotypic sensitivity score of the optimized background regimen associated to raltegravir, according to genotypic resistance testing interpreted with the French ANRS AC11 algorithm, version 16 (www.hivfrenchresistance.org).

Here we describe the genetic changes in the IN gene for nine heavily pretreated patients who received salvage therapy including raltegravir and who subsequently suffered virological failure. This study identified four different profiles. The catalytic activities of the corresponding wild-type (pre-salvage treatment) and mutated recombinant INs derived from four patients, each harboring a different genetic change in the IN gene, were assayed in vitro, and their sensitivities to raltegravir were compared.

MATERIALS AND METHODS

Study design and subjects. Nine patients who received raltegravir as part of an expanded access program in France and who suffered virological failure while on this regimen were studied. The genotypic analysis of IN resistance was performed following the French National Guidelines for resistance testing (www.hivfrenchresistance.org). All these patients received at least one NRTI and one boosted PI either with or without enfuvirtide in their optimized regimens. The optimized regimen associated with raltegravir was selected according to previous antiretroviral exposure, and genotypic resistance testing was interpreted with the French ANRS AC11 algorithm, version 16 (www.hivfrenchresistance.org).

RNA isolation, cDNA synthesis, and PCR. Plasma (500 μl) was centrifuged at 19,000 × *g* for 1 h at 4°C, and viral RNA was extracted from the pellet using the Cobas Amplicor test (Roche). Ten-microliter aliquots of RNA were used for reverse transcription-PCR (Titan One-Tube RT-PCR kit; Roche Applied Science) by following the manufacturer's instructions and using 0.4 μM (final concentration) each primer (IN12 and IN13) located in the IN gene (17). The reverse transcription step was carried out at 50°C for 30 min, and PCR involved 40 repeat cycles (94°C for 30 s, 56°C for 30 s, and 68°C for 1 min) followed by incubation at 68°C for 7 min. The second-round PCR using AmpliTaq DNA polymerase (Applied Biosystems) was performed by following the manufacturer's instructions and using each primer, IN1 and BH4 (17), at a final concentration of 1 μM. PCR involved 40 repeat cycles (94°C for 1 min, 55°C for 1 min, and 72°C for 1 min) followed by incubation at 72°C for 7 min. The PCR product was purified either with an Amicon Microcon-100 centrifugal filter device (Millipore) for sequencing or on an agarose gel for cloning.

Sequencing. The IN gene was sequenced using a cycle sequencing reaction with the BigDye terminator kit (Applied Biosystems). A set of four primers was used for complete coverage of both strands of the IN gene. Primers were as follows: two forward primers, IN1 (17) (nucleotides [nt] 4137 to 4157 in the HIV-1 HxB2 sequence) and IN4542S (5′-GCAGGAAGATGGCCAGT-3′; nt 4542 to 4558), and two reverse primers, IN4764AS (5′-CCATTTGTACTGCTG TCTTAA-3′; nt 4743 to 4764) and BH4 (17) (nt 5200 to 5222). The sequences were analyzed using Sequence Navigator software.

Production and purification of IN. The PCR products corresponding to the entire IN sequences were digested with NdeI and BamHI and ligated into pET-15b, a bacterial expression vector (Novagen). The IN sequence was verified by sequencing for all constructs.

His-tagged INs were produced in *Escherichia coli* BL21(DE3) and purified under nondenaturing conditions as previously described (27). Protein production was induced at an optical density of 0.7 by addition of isopropyl-β-D-thiogalactopyranoside (IPTG) to 1 mM. Cultures were incubated for 3 h at 37°C and then centrifuged. The cells were resuspended in buffer A (50 mM Tris-HCl [pH 8], 1 M NaCl, 4 mM β-mercaptoethanol) and lysed with a French press. The lysate was centrifuged (30 min at 10,000 rpm), and the supernatant was filtered (pore size, 0.45 μM) and incubated overnight with nickel-nitrilotriacetic acid agarose beads (Qiagen). The beads were washed first with buffer A alone and then extensively with buffer A supplemented with 80 mM imidazole. His-tagged proteins were then eluted from the beads with buffer A supplemented with 1 M imidazole and 50 μM zinc sulfate. They were then dialyzed overnight against 20 mM Tris-HCl (pH 8), 1 M NaCl, 4 mM β-mercaptoethanol, and 10% glycerol. The samples were aliquoted and rapidly frozen at −80°C.

IN activity assay. For activity assays, the 21-mer oligodeoxynucleotide U5 (5′-GTGTGGAAAATCTCTAGCAGT-3′) was radiolabeled with T4 polynucleotide kinase (New England Biolabs) and [γ-^{32}P]ATP (3,000 Ci/mmol; Amersham) and purified on a Sephadex G-10 column (GE Healthcare). The double-stranded oligodeoxynucleotide substrate was obtained by mixing equimolar amounts of labeled U5B and its complementary strand (5′-ACTGCTAGAGA TTTTCCACAC-3′) in the presence of 100 mM NaCl. IN assays were carried out

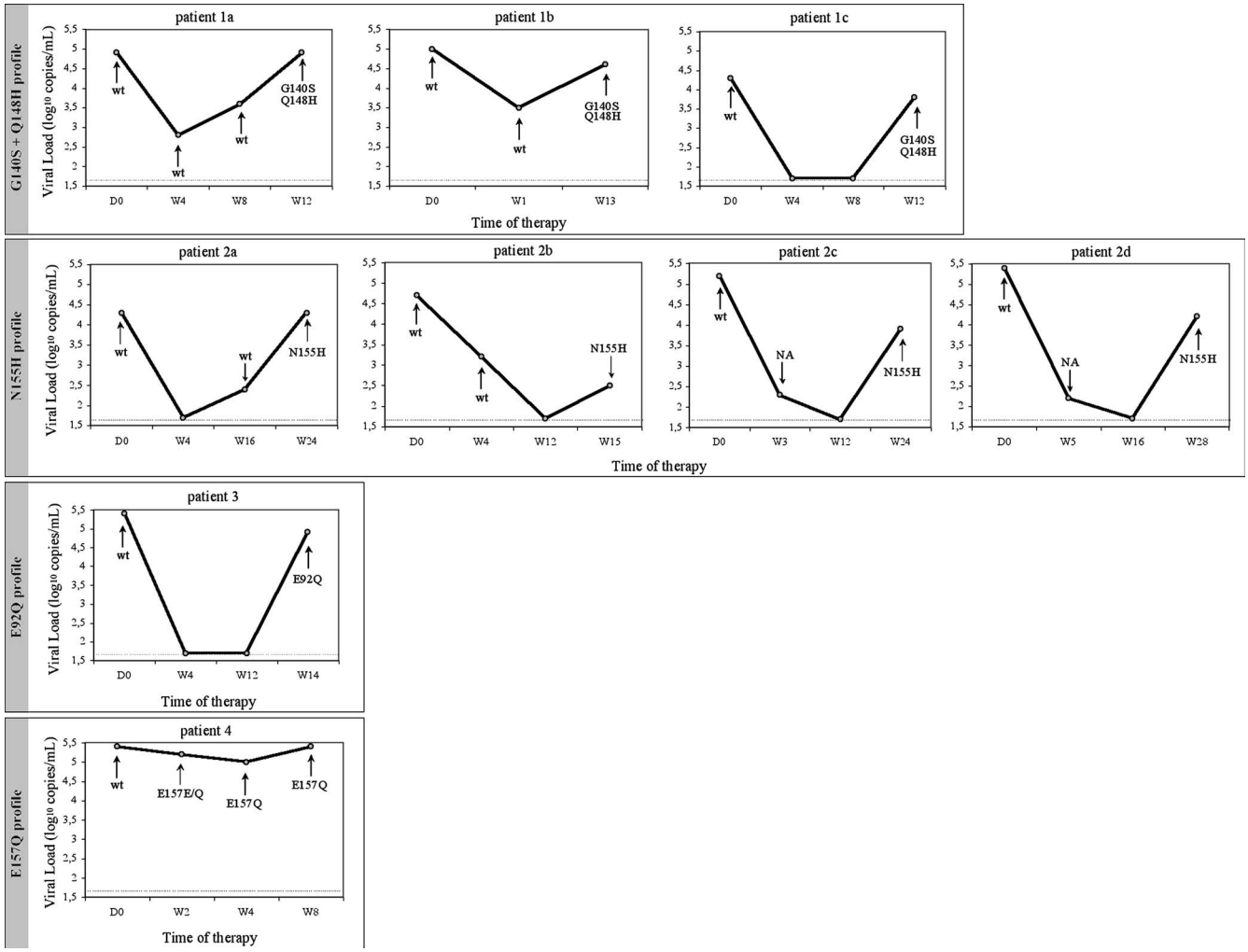

FIG. 1. Kinetics of HIV RNA copy numbers in plasma for nine patients failing raltegravir therapy. Dotted lines at 1.60 $\log_{10}$ copies/ml (40 copies/ml) show the detection limit of the viral load assay. wt, wild type; NA, nonamplifiable.

for 1 h at 37°C in a buffer containing 10 mM HEPES (pH 7.2), 1 mM dithiothreitol, and 7.5 mM magnesium chloride in the presence of 12.5 nM double-stranded DNA substrate and 200 nM recombinant IN. Products were separated by electrophoresis in denaturing 18% acrylamide–urea gels. Gels were analyzed with a Storm 840 phosphorimager (GE Healthcare Life Sciences, Piscataway, NJ) and quantified with ImageQuant (version 4.1) software. Inhibition in the presence of the drug was expressed as a fractional product (percentage of the activity of the control without drug). The 50% inhibitory concentration (IC_{50}), defined as the concentration of raltegravir that results in 50% inhibition, was calculated from inhibition curves fitted to experimental data with Prism software, version 4.0 (GraphPad Software, Inc., San Diego, CA).

RESULTS

Patients and viral load. Nine highly treatment experienced patients, infected with multidrug-resistant viruses, were treated with raltegravir at 400 mg twice daily. The baseline characteristics of these nine patients are given in Table 1. The median plasma HIV-1 RNA level was 5 $\log_{10}$ copies/ml (range, 4.3 to 5.4 $\log_{10}$ copies/ml). All nine patients harbored highly mutated viruses with resistance to NRTI, NNRTI, and PI, and their genotypic sensitivity scores (number of active ARV in the background regimen associated to raltegravir) were ≤ 5.

For six out of nine patients (patients 1c, 2a to -d, and 3), the HIV-1 load decreased from the baseline to become undetectable (<1.60 $\log_{10}$ copies/ml) in 4 to 16 weeks and then returned to baseline values, except for patient 2b (Fig. 1). For the three remaining patients (patients 1a, 1b, and 4), the HIV-1 load either showed only a slight decrease (5.0 $\log_{10}$ copies/ml for patient 4) or decreased but remained detectable (2.86 and 3.5 $\log_{10}$ copies/ml for patients 1a and 1b, respectively) (Fig. 1).

HIV-1 IN sequence analysis of clinical strains before raltegravir treatment. The complete nucleotide sequences of the 864-nt IN coding regions were determined for clinical isolates obtained from the nine patients before the initiation of anti-IN treatment (day zero). The corresponding 288-aa sequences were compared to the HxB2 IN reference sequence; the level of divergence for the whole protein was determined to be between 2.7 and 5.5%, depending on the patient (2.7% for patient 3; 3.8% for patients 1c, 2a, 2c, and 4; 4.1% for patients 1a and 2b; 5.5% for patients 1b and 2d). The G123S, R127K, and N232D mutations (with respect to HxB2) were present in all patients on day zero, and D10E, E11D, A21T, A23V, D25E,

TABLE 2. Evolution of IN amino acid substitutions during raltegravir therapy

Patient	Subtype	Anti-HIV-1 agents received at D0[a]	No. of CD4 cells/mm^3	Viral load ($\log_{10}$ copies/ml)[b]	Time of therapy[c]	IN sequence relative to the following HxB2 reference sequence[d]:																				
						N-term domain																Catalytic core domain				
						7 K	10 D	11 E	14 K	17 S	20 R	21 A	23 A	25 D	28 L	31 V	32 V	39 S	50 M	72 V	73 I	92 E	101 L	111 K	112 T
1a	B	3TC, TDF, LPV/r, fos-APV/r	65	4.9	D0	Q	E	D	-	-	-	-	-	-	-	-	-	-	L	-	-	-	I	-	-
			129	2.8	W4	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
			148	3.6	W8	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
			148	4.9	W12	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
1b	B	T20, TMC-125, DRV/r, 3TC	6	5	D0	-	E	D	-	-	-	T	-	E	-	-	-	-	-	I	-	-	-	-	-
			13	3.5	W1	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
			66	4.6	W13	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
1c	B	AZT, 3TC, IDV/r, LPV/r	370	4.3	D0	-	E	-	-	N	-	-	-	-	I	-	-	C	-	I	-	-	-	-	-
			352	1.6	W4	NA																			
			303	1.6	W8	NA																			
			299	3.8	W12	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
2a	B	3TC, TDF, ABC, fos-APV/r	2	4.3	D0	-	-	-	-	-	-	-	-	-	-	-	-	-	-	I	-	-	I	-	-
			22	1.6	W4	NA																			
			27	2.4	W16	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
			22	4.3	W24	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
2b	B	ddI, 3TC, TDF, LPV/r, T20	238	4.7	D0	-	E	-	-	-	-	-	-	-	-	-	I	-	I	I	-	-	-	-	I
			287	3.2	W4	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
			298	1.6	W12	NA																			
			267	2.5	W15	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
2c	B	ABC, 3TC, TDF, ATV/r, TMC-125	17	5.2	D0	-	E	-	E	-	-	-	-	-	-	I	-	-	-	I	V	-	-	-	-
			127	2.3	W3	NA																			
			140	1.6	W12	NA																			
			134	3.9	W24	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
2d	B	3TC, T20, TPV/r	39	5.4	D0	-	E	D	-	-	-	-	-	-	-	M	I	C	-	-	-	-	I	R	-
			127	2.2	W5	NA																			
			90	1.6	W16	NA																			
			80	4.2	W28	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
3	B	T20, ABC, 3TC, DRV/r	5	5.4	D0	-	E	-	-	-	K	-	V	-	-	-	-	-	-	-	-	-	-	-	-
			175	1.6	W4	NA																			
			139	1.6	W12	NA																			
			52	4.9	W14	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	**Q**	-	-	-
4	B	TDF, FTC, TPV/r	1	5.4	D0	-	E	D	-	-	-	T	V	E	-	-	-	-	-	I	-	-	-	-	-
			31	5.2	W2	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
			20	5.0	W4	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
			25	5.4	W8	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

[a] AZT, zidovudine; TDF, tenofovir; 3TC, lamivudine; ddI, didanosine; LPV/r, lopinavir; fos-APV, fosamprenavir; ABC, abacavir; T20, enfuvirtide; DRV/r, darunavir; FTC, emtriva; TPV/r, tipranavir; TMC-125, etravirine; IDV/r, indinavir; ATV/r, atazanavir. D0, day zero.

[b] Levels of HIV RNA in plasma were determined by using the Cobas AmpliPrep/Cobas TaqMan HIV-1 test; the detection limit of the viral load assay is 1.60 $\log_{10}$ copies/ml.

[c] W, week.

[d] Positions and amino acids for the HxB2 sequence are given. At day zero, the IN sequence was compared to the HxB2 reference sequence; the modified amino acids are given, and dashes mean that there is no modification compared to the reference sequence. At follow-up times (weeks 2 to 24), the IN sequences were compared to the day zero sequence, and dashes mean that there is no modification compared to the day zero sequence. Mutations that appeared when the viral load increased during treatment are boldfaced. term, terminal; NA, nonamplifiable.

V32I, S39C, V72I, L101I, I113V, S119P, T122I, A124T, T125A, I135V, V201I, T218S, and L234I were each present in at least two patients. Each patient also harbored specific amino acid sequence differences with respect to HxB2, including K7Q, M50L, I200L, and I220L for patient 1a; L28I, S119G, and I203M for patient 1b; S17N for patient 1c; K156N, V165I, and I220V for patient 2a; M50I, T112I, A124N, L234V, and S255R for patient 2b; K14E, V31I, and I73V for patient 2c; V31M, K111R, G193E, and S195G for patient 2d; and R20K, I204V, and T206S for patient 3 (Table 2).

Analysis of sequences of clinical isolates during raltegravir treatment. For each patient, the IN gene sequence was determined using isolates obtained at various time points during follow-up. For each of the nine patients, one or two mutations appeared when the viral load increased back toward the initial viral load; however, four different patterns of mutations were

TABLE 2—*Continued*

																							C-term domain			
113 I	119 S	122 T	123 G	124 A	125 T	127 R	135 I	140 G	148 Q	155 N	156 K	157 E	165 V	193 G	195 S	200 I	201 V	203 I	204 I	206 T	218 T	220 I	232 N	234 L	255 S
-	-	-	S	-	-	K	V	-	-	-	-	-	-	-	-	L	I	-	-	-	-	L	D	-	-
-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
-	-	-	-	-	-	-	-	**S**	**H**	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
-	G	I	S	T	A	K	-	-	-	-	-	-	-	-	-	-	I	M	-	-	S	-	D	I	-
-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
-	-	-	-	-	-	-	-	**S**	**H**	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
-	P	I	S	-	A	K	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	D	-	-
-	-	-	-	-	-	-	-	**S**	**H**	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
-	-	-	S	T	-	K	-	-	-	-	N	-	I	-	G	-	I	-	-	-	-	V	D	-	-
-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
-	-	-	-	-	-	-	-	-	-	**H**	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
V	-	-	S	N	-	K	-	-	-	-	-	-	-	-	-	-	I	-	-	-	-	-	D	V	R
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V	-	-	S	-	A	K	-	-	-	-	-	-	-	-	-	-	I	-	-	-	-	-	D	I	-
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-	P	-	S	T	-	K	V	-	-	-	-	-	-	E	-	-	I	-	-	-	S	-	D	-	-
-	-	-	-	-	-	-	-	-	-	**H**	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
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identified. Three patients (patients 1a, 1b, and 1c) showed two mutations, G140S and Q148H, that seemed to appear simultaneously, whereas four patients (patients 2a, 2b, 2c, and 2d), showed only one mutation, N155H, and patients 3 and 4 showed one mutation, E92Q or E157Q, respectively (Fig. 1; Table 2).

Four of the nine patients, each showing a different profile (patient 1a, G140S Q148H; patient 2a, N155H; patient 3, E92Q; patient 4, E157Q), were selected for extended analyses of IN activity in order to estimate, in vitro, the impact of the mutations on the 3′ processing and strand transfer activities.

Expression of wild-type INs and sensitivities of the 3′ processing and strand transfer activities to raltegravir. The wild-type recombinant INs from the four patients were produced and assayed in vitro for their sensitivities to raltegravir. All four enzymes were very sensitive to raltegravir (Fig. 2B and data not shown). IC_{50}s were determined by in vitro dose-response assays and were comparable (7 to 10 nM) and in agreement with values previously reported for laboratory strains (29a, 31).

Strand transfer activity was specifically inhibited, whereas 3′ processing was unaffected at inhibitor concentrations up to 100 times the IC_{50}, thus confirming that raltegravir acts as a strand transfer inhibitor (15).

Comparison of the sensitivities of wild-type and mutant INs to raltegravir. The mutant enzymes were first tested for their catalytic activities, and their efficiencies were compared to that of the wild-type enzyme (Table 3). All mutations diversely altered the functions of IN in vitro (Fig. 2; Table 3). The E92Q mutant was moderately impaired in both 3′ processing and strand transfer, retaining 76% and 90% of these activities, respectively (Fig. 2A; Table 3). The N155H mutant expressed active 3′ processing (72% of wild-type activity) but was impaired in strand transfer (12% of wild-type activity). Both

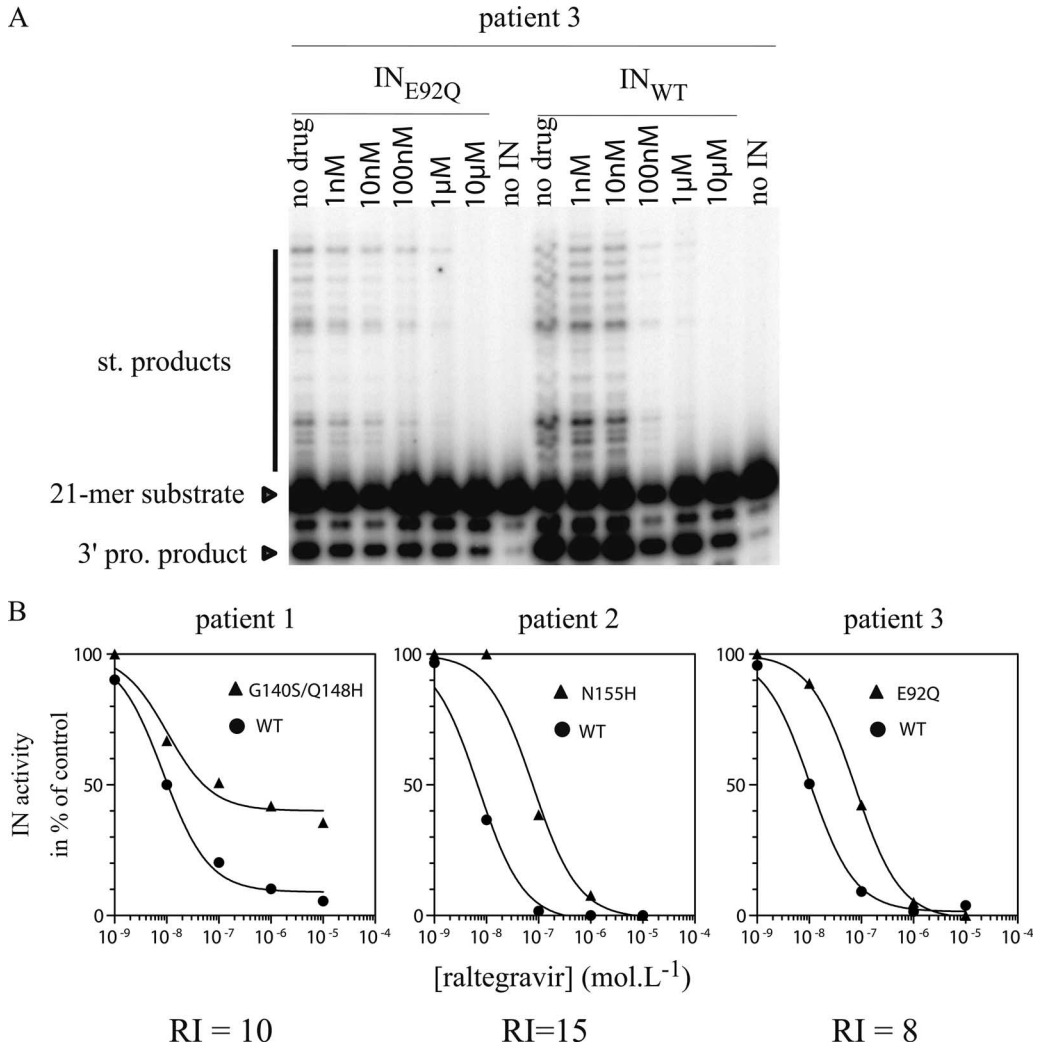

FIG. 2. 3′ processing and strand transfer activities of recombinant INs. (A) Activities of recombinant INs. INs obtained from patient 3 on day zero (IN_{WT}) and at week 14 (IN_{E92Q}) were incubated for 1 h at 37°C with 10 nM substrate (21-mer) in the presence of various concentrations of raltegravir (given above the lanes). st., strand; 3′ pro, 3′ processing. (B) Comparison of inhibition curves obtained for isolates from patients 1, 2, and 3. RI, resistance index. Symbols: ●, data obtained for wild-type virus at day zero; ▲, data obtained for resistant viruses.

activities were strongly impaired in the G140S Q148H mutant, which retained only residual activity (3% of wild-type activity). The E157Q mutant was almost completely inactive in both 3′ processing and strand transfer. The G140S Q148H, N155H, and E92Q mutant enzymes retained sufficient activity for testing of their sensitivities to raltegravir (Fig. 2B). However, the in vitro activity of the E157Q mutant from patient 4 was too low for the inhibitor to be tested.

All three mutant enzymes were more resistant to raltegravir than the parental enzymes: the G140S Q148H and E92Q mutants were 7 to 8 times more resistant, and the N155H mutant was more than 14 times more resistant (Fig. 2).

TABLE 3. Activities of mutant INs

IN enzyme	% of wild-type activity	
	3′ Processing	Strand transfer
Wild type	100	100
Mutant		
E92Q	76	90
N155H	72	12
G140S Q148H	3	3

DISCUSSION

Raltegravir belongs to a new class of antiretroviral compounds that targets HIV-1 IN. It is currently undergoing late-stage clinical trials with patients infected with multidrug-resistant HIV-1. We report the sequence diversity and evolution of IN during viral escape in nine individuals infected with multidrug-resistant HIV-1.

Although viral loads were initially high, they decreased rapidly to minimum HIV-1 RNA titers, most often after 4 weeks of raltegravir treatment. For six patients, the treatment led to

a substantial decrease in the viral load, whereas for three patients the decrease was weaker.

Initially, the IN sequences showed little divergence from the HxB2 reference sequence, although there were numerous reverse transcriptase (RT) and protease mutations in these isolates from patients failing treatment. Indeed, some associations between RT mutations and IN mutations related to resistance to IN inhibitors have been shown for patients receiving highly active antiretroviral therapy. For example, in the present study, the IN mutations at baseline included V165I for one patient and T206S for another; these mutations have previously been reported to be associated with RT resistance mutations (3a). However, it is not clear whether this polymorphism can be implicated in the profile of response to raltegravir treatment.

The kinetics of viral escape from the treatment were not exactly the same for the nine patients studied, but the viral load tended to return to initial values for most of them. However, there seems to have been no link between the profile of mutations and the kinetics of the viral load. When the viruses of these nine patients escaped treatment, they harbored at least one mutation in the IN gene: E92Q, G140S Q148H, N155H, or E157Q.

The IN mutations reported were associated with phenotypic resistance of the enzyme, thus confirming that IN is the primary target of the inhibitor in vivo. The observation that the HIV-1 load was able to rebound to the pretherapeutic level for viruses harboring only a single mutation suggests that the genetic barrier to resistance to this compound is thin: the selection of only one mutation seems to be sufficient for virological failure of raltegravir treatment in these highly experienced patients (GSS $\leq$ 1). Moreover, the absence of a pharmacokinetic/pharmacodynamic relation for raltegravir in naive patients does not argue for increasing the dose of raltegravir to overcome the resistance (29).

No selection of secondary mutations was observed in this study, in contrast with the findings of a substudy of resistance conducted in a phase 2 trial, where secondary mutations seemed to appear after the selection of mutations at position 148 or 155 (13a). In the same study, it was suggested that there were two genetic pathways for the development of resistance to raltegravir: one involving Q148H and the other involving N155H mutations. N155H is known to generate resistance to the IN inhibitor naphthyridine carboxamide (L-870812) (14), and G140S is involved in resistance to chicoric acid and the DKA (diketo acids) family of IN inhibitors (23, 24). Some studies have shown that the Q148 residue is implicated in interactions with the 5′ terminal end of viral DNA that allow efficient IN strand transfer (12, 20), suggesting that a mutation at this position could reduce IN activity. However, the present study suggests that, in addition to both these resistance profiles, there are other pathways associated with raltegravir resistance, involving E92Q or E157Q mutations.

The existence of several IN resistance profiles is similar to what has been described for other ARV classes, such as NNRTI. However, the determinants of the evolution toward these different profiles are unknown. They could be related to different factors such as the genetic polymorphism of the IN gene, pharmacokinetic factors such as raltegravir trough levels, or the sequences of other genes encoding proteins that are likely to interact with IN, such as RT (36, 37).

These findings argue strongly that raltegravir should be used only in combination with other active drugs. Most of the mutations we report severely impaired the function of the enzyme. It is not clear whether this lack of activity reflects an intrinsic property of the mutated enzyme or whether it is merely due to the purification procedure. Indeed, several factors, such as concentration and the presence of cations and detergents during purification, appear to impact the ability of recombinant HIV-1 IN to perform efficient integration (35). The presence of a His tag at the N-terminal extremity may also affect the activities of the enzymes, since it is known that this sequence impacts the abilities of INs to form active oligomers (19). If it eventually turned out that the virus was able to replicate despite encoding a catalytically inactive IN, the mechanism involved would remain to be elucidated. Cellular factors that stimulate integration in vitro, such as LEDGF (lens epithelium-derived growth factor), could be involved (4). This type of phenomenon has already been described, for example, for PI: viral mutations in Gag cleavage sites are selected in order to overcome the decrease in viral fitness due to the selection of resistance mutations in the HIV-1 protease.

At least four genetic profiles (E92Q, G140S Q148H, N155H, and E157Q) can be associated with in vivo treatment failure and resistance to raltegravir. These mutations led to strong impairment of IN in vitro in the absence of raltegravir: strand transfer activity was affected, and in some cases 3′ processing was also impaired.

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