

# Fifth mutation in human immunodeficiency virus type 1 reverse transcriptase contributes to the development of high-level resistance to zidovudine

(AIDS/recombination/selective PCR/sensitivity/antiviral)

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Communicated by George H. Hitchings, December 2, 1991

**ABSTRACT** It is recognized that high-level resistance to 3'-azido-3'-deoxythymidine (AZT, zidovudine, or Retrovir) is conferred by the presence of four mutations in the human immunodeficiency virus (HIV) reverse transcriptase [RT; deoxynucleoside-triphosphate:DNA deoxynucleotidyltransferase (RNA-directed), EC 2.7.7.49] coding sequence. However, a number of clinical isolates have been observed that exhibit high-level resistance but contain only three of the four identified mutations (Asn-67, Arg-70, and Tyr-215). Construction of a molecular clone with this genotype gave rise to only a partially resistant virus, raising the possibility that an additional mutation existed in some clinical isolates. Using an HIV marker rescue system, we have mapped and identified a fifth mutation conferring resistance to zidovudine, namely, methionine to leucine at codon 41 of HIV RT. An infectious molecular clone containing this mutation together with three previously identified mutations in the RT coding sequence yielded highly resistant HIV after transfection of T cells. Direct detection of the fifth mutation in DNA samples from cocultured peripheral blood lymphocytes by the PCR revealed that it occurred relatively early in the development of zidovudine resistance. However, this mutation was only detected after the appearance of the codon 215 change in the RT coding sequence. Identification of this mutation in addition to the other known mutations conferring resistance enables rapid and direct correlation between an RT genotype and sensitivity of the virus.

The development of drug-resistant human immunodeficiency virus type 1 (HIV-1) strains occurs during the treatment of acquired immunodeficiency syndrome (AIDS) and HIV infection. Several studies have documented the appearance of resistant HIV-1 in patients receiving prolonged 3'-azido-3'-deoxythymidine (AZT, zidovudine, Retrovir) therapy (1–5). More recently, resistance to another nucleoside analogue, 2',3'-dideoxyinosine (ddI), has been reported (6). The clinical significance of resistance to either drug is not yet fully understood, although it appears in the case of zidovudine therapy that the emergence of resistant HIV is not associated with sudden clinical deterioration (2).

Resistance to zidovudine is due to the accumulation of specific amino acid substitutions in HIV reverse transcriptase [RT; deoxynucleoside-triphosphate:DNA deoxynucleotidyltransferase (RNA-directed), EC 2.7.7.49] (7). Comparative nucleotide sequence analysis of HIV isolates obtained from individuals receiving zidovudine therapy revealed that the most resistant isolates had four mutations (Asp-67 → Asn, Lys-70 → Arg, Thr-215 → Tyr or Phe, and Lys-219 → Gln) or the first three of these (7) in the RT coding sequence. This raised the possibility that the highly resistant isolates with

only the three mutations might contain an as-yet-unidentified additional mutation(s). To assess the relative contribution of the mutations to the overall level of resistance, a series of infectious molecular clones have been constructed (8). Generally, the level of resistance to zidovudine increases with the accumulation of mutations. However, the virus derived from clone HIVRTMC/F, which is mutant at the first three amino acids but wild type at codon 219, only exhibits partial resistance (8). This can be contrasted with two clinical isolates of apparently the same genotype but exhibiting high-level resistance. Again, this indicates the presence of an as-yet-unidentified mutation.

Here we report the mapping and identification of a fifth mutation (methionine to leucine at codon 41) in the RT coding sequence of HIV-1, which contributes to zidovudine resistance. The introduction of this amino acid substitution into the infectious molecular clone HIVRTMC/F showed that Leu-41, when present with Asn-67, Arg-70, and Tyr-215, produced a highly resistant virus phenotype. The polymerase chain reaction (PCR) has been applied to detect zidovudine-resistance mutations in samples from infected individuals (2, 8). This technique was extended here to analyze the Met-41 → Leu substitution in HIV samples from a series of treated patients. The significance of these results to the development of zidovudine resistance are discussed.

## MATERIALS AND METHODS

**Cells and Virus.** The human T-lymphoblastoid cell line C8166 (9) was used to propagate HIV and for electroporation experiments. These cells were routinely maintained in RPMI 1640 medium supplemented with 10% (vol/vol) fetal calf serum, plus antibiotics (RPMI 10). HT4LacZ-1 cells (HeLa cells expressing the human CD4 receptor and containing the  $\beta$ -galactosidase gene under the control of the HIV long terminal repeat) (10) were used to determine sensitivity of HIV to zidovudine (1, 11, 12). This cell line was maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum, plus antibiotics (DMEM 10). The HIV clinical isolates used were A036B, A036D, P026A, and P026B (1, 7, 8). To produce virus stocks, C8166 cells ( $2 \times 10^6$ ) were infected with HIV [at a multiplicity of  $\approx 0.1$  TCID<sub>50</sub> (tissue culture 50% infective dose) per cell] and incubated at 37°C for 7–8 days. Cell-free virus supernatants were stored at –70°C.

**Cloning and Sequencing of the RT Coding Region from Clinical Isolates.** The RT coding region [1.7 kilobases (kb)] was amplified by PCR from infected-cell DNA and from the

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Abbreviations: HIV-1, human immunodeficiency virus type 1; RT, reverse transcriptase; ddI, 2',3'-dideoxyinosine; PBL, peripheral blood lymphocyte.

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phage M13 *pol* gene clones mpRT1/H, RTMC/F, and RTMC described previously (8, 13) and was cloned into the M13 vector mptac18.1 as described (7). Single-stranded DNA from the M13 clones was used for DNA sequencing by the dideoxynucleotide chain termination method (14).

**Marker Transfer and Fine Mapping of the Resistance-Confering Mutation.** Cloned clinical isolate RT coding regions were transferred into the HXB-2D genetic background by homologous recombination with an RT-deleted proviral clone, pHIVARTBstEII, as described (6). Briefly, a molecular clone of HXB-2D was modified to delete 1430 nucleotides in the RT coding region between residues 2618 and 4111 and to introduce a *Bst*EII restriction enzyme site at the deletion junction. HIV variants were generated by cotransfection of C8166 cells with the RT coding sequence derived from M13 clones and with the RT-deleted proviral clone linearized at the *Bst*EII restriction enzyme site. Cells were maintained in RPMI 10, and after 12–14 days, virus stocks were prepared from cell-free culture supernatants.

Intertypic RT clones were made by the exchange of various restriction fragments from clinical isolate RT coding sequences into mpRTMC/F. M13 replicative-form DNA of the cloned RT coding regions was digested with combinations of enzymes according to the manufacturer's instructions (Bethesda Research Laboratories). Fragments were purified from LMP agarose by hot phenol extraction and then ligated by using T4 DNA ligase (Pharmacia) under conditions specified by the manufacturer. Ligated DNA was used to transform *Escherichia coli* strain TG1. The resulting plaques were assayed for functional RT activity as described (15). Intertypic functional RT coding regions were transferred into the HXB-2D genetic background by the homologous recombination system described above.

**Construction of Mutant Proviral HIV Clones.** Mutants containing the Met-41 → Leu amino acid change were created by site-directed mutagenesis (16) of the previously described M13 RT clones RTMF, RTMC/F, and HXB-2D (8). All mutants were verified by nucleotide sequence analysis (14). M13 replicative-form DNA was prepared, and the mutant RT coding regions were transferred into the HXB-2D genetic background by homologous recombination as described above.

**Zidovudine Sensitivity Assay.** Plaque reduction assays were performed by infection of HT4LacZ-1 cell monolayers as described (1, 11, 12). The 50% inhibitory concentration ( $IC_{50}$ ) values were derived from plots of percentage inhibition versus zidovudine concentration. Viral isolates with  $IC_{50}$  values up to  $0.05 \mu\text{M}$  were considered sensitive; with  $IC_{50}$  values between  $0.05$  and  $1 \mu\text{M}$ , partially resistant; and with  $IC_{50}$  values above  $1 \mu\text{M}$ , highly resistant (1).

**Detection of Met-41 → Leu Mutation by PCR.** Selective PCR, used to discriminate wild type (Met-41) from mutant (Leu-41) at codon 41 in cocultured peripheral blood lymphocyte (PBL) samples, was performed as described (8). Briefly, DNA was extracted from about  $2 \times 10^6$  PBLs, and an initial PCR was performed with primers A ( $5'$ -TTCCATT-AGTCCTATT- $3'$ ) and NE1 ( $5'$ -TCATTGACAGTCCAGCT- $3'$ ). After amplification, samples were extracted with chloroform and diluted 1:100. Five microliters of the diluted PCR product was then reamplified by using PCR in a mixture ( $100 \mu\text{l}$ ) that contained 25 mM KCl; 2.5 mM MgCl<sub>2</sub>; 50 mM Tris-HCl (pH 8.3); 0.1 mg of bovine serum albumin per ml; 0.2 mM each of dATP, dGTP, dCTP, and dTTP; and 0.25  $\mu\text{g}$  of each primer. To identify wild-type codon 41, the primer pair was primer A with primer 5W ( $5'$ -AATTTCCCTTCCTTTTCCTT- $3'$ ). To identify mutant codon 41, primer A was paired with either primer 5M ( $5'$ -AATTTCCCTTCCTTTTCCTTA- $3'$ ) or 5M(C) ( $5'$ -AATTTCCCTTCCTTTTCCTG- $3'$ ). The reactions were subjected to 30 cycles of 1 min at  $94^\circ\text{C}$ , 30 sec at  $50^\circ\text{C}$ , and 30 sec at  $72^\circ\text{C}$  with a Perkin-Elmer/

Cetus DNA thermal cycler. PCR products were separated through Tris borate composite gels of 3% Nusieve/1% agarose. The PCR products were scored as described (2).

## RESULTS

**Transfer of Resistance Phenotype with RT.** We wished to determine initially whether the zidovudine-resistance phenotype of the clinical isolates A036D and P026B was encoded by their respective RT coding regions, to eliminate the possibility of other viral gene products or cellular factors contributing to zidovudine resistance. Therefore, we transferred the cloned RT coding regions from clinical isolates into the HXB-2D genetic background by recombination. The virus recovered was assessed for sensitivity to zidovudine and was compared with the original clinical isolates (Fig. 1). For A036 isolates, transfer of the pretherapy RT coding region yielded a virus as sensitive to zidovudine ( $IC_{50} = 0.022 \mu\text{M}$ ) as the parent clinical isolate ( $IC_{50} = 0.025 \mu\text{M}$ ). Transfer of the post-zidovudine-therapy resistant RT gave rise to a virus that was highly resistant to the drug ( $IC_{50} = 2.5 \mu\text{M}$ ), a value very similar to the parental clinical isolate ( $IC_{50} = 2.8 \mu\text{M}$ ). A similar pattern was seen for P026 isolates (data not shown). This indicated that all of the resistance phenotype was conferred by the RT coding region of the respective clinical isolate.

**Fine Mapping of the Additional Zidovudine Resistance-Confering Mutation.** The variant HIVRTMC/F, whose RT was modified by site-directed mutagenesis, has the same genotype at codons 67, 70, 215, and 219 as the clinical isolates A036D and P026B (7, 8). However, HIVRTMC/F only exhibits partial resistance to zidovudine ( $IC_{50} = 0.31 \mu\text{M}$ ). It therefore provided a suitable genetic background for the

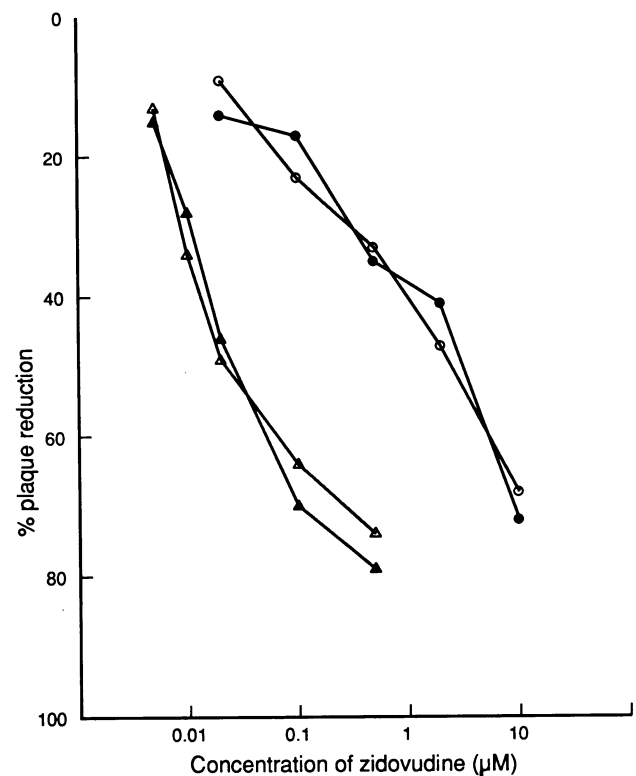


FIG. 1. Cotransfer of zidovudine resistance with RT. The RT genes of clinical isolates A036B (pretherapy) and A036D (late therapy) were transferred into the HXB-2D genetic background. Rescued virus was assessed for sensitivity to zidovudine in A036B' ( $\Delta$ ) and A036D' ( $\circ$ ) and compared with that of the parent clinical isolates A036B' ( $\Delta$ ) and A036D' ( $\bullet$ ).

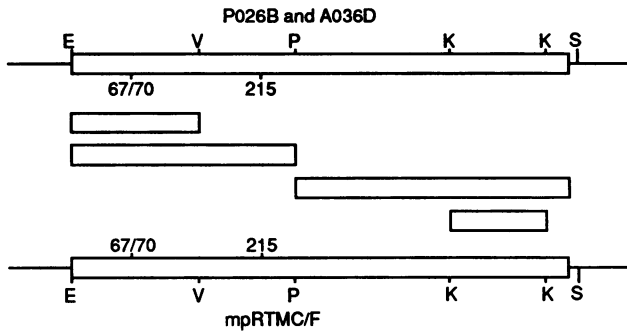


FIG. 2. RT coding sequence (open boxes) from clinical isolates P026B and A036D and from the M13 pol gene clone RTMC/F was cloned into the vector M13mptac18.1 as described. The four restriction fragments *EcoRI-EcoRV*, *EcoRI-Pvu II*, *Kpn I*, and *Pvu II-Sal I* were isolated from the RT clone mpP026B and substituted for the corresponding restriction fragments of mpRTMC/F. In addition, reciprocal substitutions of the *EcoRI-EcoRV* restriction fragment from mpRTMC/F were constructed in mpP026B and mpA036D. The RT coding sequences were transferred into the HXB2-D genetic background to yield the viruses detailed in Table 1. The positions of the known mutations conferring zidovudine resistance at codons 67, 70, and 215 are indicated. E, *EcoRI*; V, *EcoRV*; P, *Pvu II*; K, *Kpn I*; S, *Sal I*.

transfer of subfragments of the resistant clinical isolate RT coding regions to map a region conferring the increase in resistance to zidovudine. Four restriction fragments were transferred from mpP026B RT into mpRTMC/F RT (Fig. 2). Virus recovered by transfection of C8166 cells with DNA clones was assessed for sensitivity to zidovudine (Table 1). Transfer of the 3' 933-nucleotide *Pvu II/Sal I* restriction fragment or the internal *Kpn I* fragment did not cause an increase in resistance relative to HIVRTMC/F ( $IC_{50} = 0.3 \mu M$  and  $0.28 \mu M$ , respectively). However, transfer of the 5' 759-nucleotide *EcoRI/Pvu II* restriction fragment yielded virus with increased resistance to a level associated with a highly resistant phenotype ( $ID_{50} = 1.75 \mu M$ ). The increase in resistance could be further mapped to the 5' 437-nucleotide *EcoRI/EcoRV* restriction fragment, as recombinant virus containing this fragment had an  $IC_{50}$  of  $1.23 \mu M$ . Reciprocal recombinants were also created where the 5' 437-nucleotide

Table 1. Sensitivity of intertypic viruses to zidovudine

HIV variant	Restriction fragment transferred	Zidovudine $IC_{50}$ , $\mu M$	SE
HIVRTMC/F		0.31	0.039
P026B-C/F-RI/RV	<i>EcoRI/EcoRV</i>	1.23	0.14
P026B-C/F-RI/PvuII	<i>EcoRI/Pvu II</i>	1.75	0.53
P026B-C/F-Sal/PvuII	<i>Sal I/Pvu II</i>	0.30	0.035
P026B-C/F-KpnI	<i>Kpn I</i>	0.28	0.025
RTMC/F-026B	<i>EcoRI/EcoRV</i>	0.33	0.12
RTMC/F-036D	<i>EcoRI/EcoRV</i>	0.41	0.15

Sensitivity to zidovudine of rescued intertypic RT-containing viruses was assessed by plaque-reduction assay. Isolates of P026 prefix were derived from the transfer of restriction fragments from mpP026B-cloned RT gene into the mpRTMC/F background. Isolates of RTMC/F prefix were derived from the reciprocal transfer of restriction fragments from mpRTMC/F-cloned RT gene into either the mpP026B background (RTMC/F-026) or the mpA036D background (RTMC/F-036D). Mean  $IC_{50}$  values and standard errors were derived from two or three separate plaque-reduction assays.

*EcoRI/EcoRV* restriction fragment from mpRTMC/F was used to replace the corresponding fragment of both clinical isolates P026B and A036D (Table 1). As anticipated, recovered virus exhibited decreased zidovudine sensitivity, with only a partially resistant phenotype ( $IC_{50} = 0.33 \mu M$  and  $0.41 \mu M$ , respectively). These studies clearly demonstrated that the additional determinants of resistance were in the 5' 437 nucleotides of the RT coding region.

**Sequence Comparison of the 5' 437 Nucleotides of the RT Coding Region and Analysis of Site-Directed Mutants.** The complete RT DNA sequence was obtained for both the sensitive and resistant isolate pairs P026 and A036. The predicted amino acid sequence for the 5' 437 nucleotides is shown in Fig. 3. Amino acid variation between both pairs of sensitive and resistant isolates was observed. Six variant amino acids were seen in A036 isolates and three in the P026 isolates. Of these changes, only three were common to both isolates, Asp-67 → Asn, Lys-70 → Arg, and a previously unidentified amino acid substitution of Met-41 → Leu. In the case of the P026 pairs, these were the only three amino acid changes between sensitive and resistant isolates, strongly

						50	
P026A	PISPIETVPV	KLNP	GM	DGPR	VKQWPLTEEK	IKALVEICTE	MEKEGKISKI
P026B	PISPIETVPV	KLNP	GM	DGPR	VKQWPLTEEK	IKALVEICTE	MEKEGKISKI
A036B	PISPIETVPV	KLKP	GM	DGPK	VKQWPLTEEK	IKALVEICTE	MEKEGKISKI
A036D	PISPIEAVPV	KLKP	GM	DGPK	VKQWPLTEEK	IKALLEICTE	MEKEGKISKI
							100
P026A	GPENPYNTPV	FAIKKK	SNK	WRKLVDFREL	NKRTQDFWEV	QLGIPHPAGL	
P026B	GPENPYNTPV	FAIKKK	SNR	WRKLVDFREL	NKRTQDFWEV	QLGIPHPAGL	
A036B	GPENPYNTPV	FAIKKK	STK	WRKLVDFREL	NKRTQDFWEV	QLGIPHPAGL	
A036D	GPENPYNTPV	FAIKKK	STR	WRKLVDFREL	NKRTQDFWEV	QLGIPHPAGL	
							146
P026A	KKKKS	SVTVLD	VGD	AYFSVPL	DEDFRKYTAF	TIPSTINN	ETP GIRYQY
P026B	KKKKS	SVTVLD	VGD	AYFSVPL	DEDFRKYTAF	TIPSTINN	ETP GIRYQY
A036B	KKKKS	SVTVLD	VGD	AYFSVPL	DKDFRKYTAF	TIPSTINN	ETP GIRYQY
A036D	KKKKS	SVTVLD	VGD	AYFSVPL	DKDFRKYTAF	TIPSTINN	ETP GIRYQY

FIG. 3. Comparison of the predicted amino acid sequences from the 5' 437-nucleotide *EcoRI-EcoRV* restriction fragments of the cloned RT genes of clinical isolates P026A and A036B (pretherapy) and P026B and A036D (late therapy). Strain variations either between isolates from different patients or between isolates from the same patient at different times of drug therapy are shown as open boxes. The positions of amino acid substitutions Met-41 → Leu, Asp-67 → Asn, and Lys-70 → Arg are marked by shaded boxes.

Table 2. Zidovudine sensitivity of HIV variants with defined mutations in RT

HIV variant	Mutations introduced	Zidovudine IC <sub>50</sub> , μM	SE	Fold increase
HXB2-D		0.01	0.001	1
HXB 41L	L-41	0.04	0.006	4
HIVRTMF	Y-215	0.16	0.011	16
HXB 41L/215Y	L-41, Y-215	0.60	0.049	60
HIVRTMC/F	N-67, R-70, Y-215	0.31	0.039	31
RTMC/F 41L	L-41, N-67, R-70, Y-215	1.79	0.17	179
HIVRTMCY	N-67, R-70, Y-215, Q-219	1.21	0.12	121
HIVRTMC	N-67, R-70, F-215, Q-219	1.47	0.13	147

The genotypes of HIV variants constructed by site-directed mutagenesis are shown. All infectious mutant viruses appeared similar to wild-type virus, as they were recovered from transfection experiments at similar times with little variation in titer (data not shown). Zidovudine sensitivity was assessed by plaque-reduction assay. Mean IC<sub>50</sub> values and standard errors are shown, which were derived from between two and five independent sensitivity assays.

implicating Met-41 → Leu as the additional zidovudine resistance-conferring mutation.

To assess the contribution to zidovudine resistance, the amino acid change, Met-41 → Leu was introduced into the previously described M13 RT clones mpRTMF (Tyr-215), mpRTMC/F, and mpHXB-2D (wild type), and virus was recovered by transfection of T cells. Leu-41 in an otherwise wild-type background caused a modest decrease in sensitivity of 4-fold relative to wild type (Table 2). However, when present with Tyr-215, Leu-41 caused a further decrease in sensitivity (HIVRTMF 41Leu, IC<sub>50</sub> = 0.60 μM) compared with HIVRTMF (IC<sub>50</sub> = 0.16 μM). This represents a 60-fold increase in resistance over wild-type HIV. When introduced into HIVRTMC/F, Leu-41 caused an increase in resistance to a level similar to the highly resistant virus HIVRTMC, which is derived from a molecular clone containing the four previously identified mutations (7, 8). Also, Leu-41 in the HIVRTMC/F background caused an increase in resistance similar to the level of the highly resistant virus HIVRTMCY, which has the same genotype as HIVRTMC except at codon 215 where a tyrosine replaces the phenylalanine. This con-

firmed the Met-41 → Leu change as a fifth amino acid substitution contributing to zidovudine resistance.

**Detection of Met-41 → Leu by Selective PCR.** Selective PCR has been used to study the appearance of zidovudine resistance-conferring mutations in sequential isolates from a cohort of initially asymptomatic individuals (2, 17). We extended this technique to survey 16 individuals from this cohort for the presence of methionine or leucine at codon 41 (Fig. 4). The amino acid substitution Met → Leu can be conferred by one of two single nucleotide changes, ATG(methionine) to TTG(leucine) or CTG(leucine). Both of these codons could be detected in the cohort studied by using two different diagnostic mutant oligonucleotides [5M to detect TTG, 5M(C) to detect CTG]. After 24–32 weeks of treatment, 3 of 16 samples consisted of a mixed or mutant population at codon 41. However, by 72 weeks of treatment, 12 of 16 isolates were mutant. Four isolates remained wild type at codon 41 after 96 weeks of treatment. To support these findings, nucleotide sequence analysis of isolates from two patients at early and late times during drug therapy gave results consistent with the PCR analysis (data not shown).

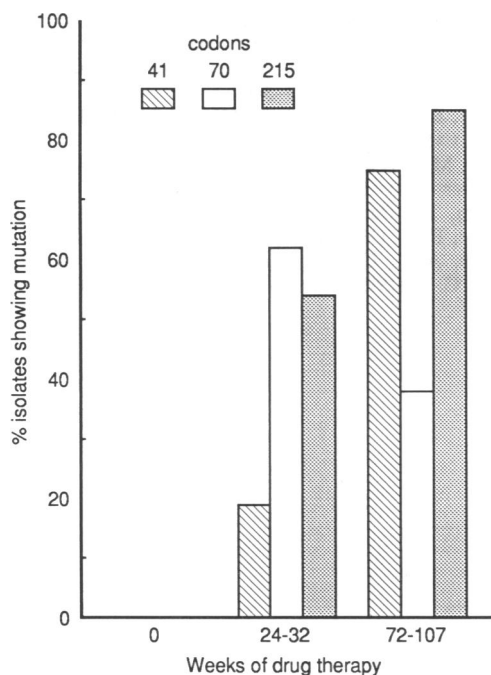


FIG. 4. Frequency of isolates having evidence of mutations at codons 41, 70, and 215 of the RT gene either as a homogeneous population or as mixture of mutant and wild-type variants.

**DISCUSSION**

The observation that some HIV isolates highly resistant to zidovudine only had three of the four identified resistance-conferring mutations raised the possibility that these isolates contained an additional mutation (7, 8). The identification of this mutation has become of increasing importance with the advent of intensive investigation into the clinical significance of zidovudine resistance. Clinical studies thus far have analyzed the genotype of PBL virus populations at the four identified mutations to assess the degree of resistance (2, 17, 18). However, for a complete assessment of zidovudine resistance by genotypic analysis, all commonly occurring mutations must be monitored.

Using the powerful technique of marker rescue, we have localized and identified the fifth mutation as a methionine to leucine change at codon 41 of the HIV-1 RT coding region. The presence of Leu-41 in certain combinations with other zidovudine resistance-conferring mutations caused further decreases in drug sensitivity (Table 2). When present with Asn-67, Arg-70, and Tyr-215, the combination resulted in a 179-fold increase in resistance relative to wild type. Therefore, this mutation must account for the similar increase in resistance seen in the clinical isolates studied. A retrospective survey of the RT sequence previously obtained from clinical isolates revealed that Leu-41 was present in two highly resistant viruses, A018C (7, 8) and patient 105 (130-week sample) (17) that were also mutant at the other four codons. Since the level of resistance of these isolates is

similar to other highly resistant isolates studied, the significance of Leu-41 in this context remains unclear at present.

In the light of the sequence and selective PCR data, it is interesting to speculate about the role of the codon 41 mutation. As reported elsewhere, the mutation Arg-70 → Lys is commonly detected first during zidovudine treatment. This mutation then seems to disappear from the viral population, being replaced by the mutation at codon 215 (17). Our PCR analysis showed that the mutation at codon 41 only appeared after the change at 215 (Fig. 4). This genotype has been shown to confer an increase in resistance of ≈4-fold over a virus containing solely Tyr-215. This is likely to cause a selective advantage for the virus containing both Leu-41 and Tyr-215 in the presence of zidovudine. The PCR analysis also showed that the appearance of Leu-41 in the viral population precedes the reappearance of arginine at codon 70. Therefore, Leu-41 may also serve as a compensatory mutation, allowing the acquisition of Arg-70 in the presence of Tyr-215. The level of resistance of this combination of mutations is as yet unknown. However, it is likely to exhibit only partial resistance, with the subsequent appearance of Asn-67 conferring higher level resistance. The creation of further mutant proviral clones is being used to address the role of Leu-41 in the development of zidovudine resistance.

It is interesting to note that this new mutation is in fairly close proximity to amino acids 67 and 70, which are involved in zidovudine resistance, and to the more recently identified ddI resistance-conferring mutation at codon 74 (6). In the small number of examples studied where zidovudine-resistant isolates were initially present, the appearance of a mutant codon 74 was accompanied by the reversion of Leu-41 to wild-type Met-41. Whether this was true reversion or the outgrowth of a different virus population under ddI selection is unclear. However, from the data available, it would appear that Leu-41 might not be compatible with Val-74, in contrast to the other zidovudine resistance-conferring mutation Tyr-215, which persists in ddI-resistant isolates.

There has been a recent report claiming that different novel resistance mutations can occur. However, this was based solely on the nucleotide sequence determination of the RT coding sequence from a single isolate (18). Such analysis cannot distinguish specific mutations conferring resistance from the abundant natural amino acid variation that occurs in the RT of different HIV strains, and more rigorous genetic analysis will be required to establish whether such mutations are indeed relevant to the development of resistance.

In conclusion, we have identified a fifth mutation, Met-41 → Leu in HIV RT, causing an increase in resistance to zidovudine when present in combination with other specific changes. Although we cannot rule out the existence of other mutations conferring zidovudine resistance, we believe we

have now identified the five most common amino acid substitutions. Clearly, any mutations suspected of conferring resistance must be introduced into a defined genetic background by site-directed mutagenesis, and the resulting virus must be analyzed by standard sensitivity assays to determine their contribution to the resistance phenotype. Finally, in clinical studies designed to assess zidovudine resistance, analysis of the five codons described here should give a clear indication of the level of resistance HIV has acquired.

We thank Eithne O'Sullivan and Susan Hartman for technical assistance, P. F. Ertl for help in preparing the manuscript, and Dr. G. Darby for his continued support. We also thank Dr. Lee Ratner for the clone HXB2, Dr. Jean-Francois Nicolas for the cell line HT4LacZ-1, and Dr. Jan Willem Mulder and Dr. Joep Lange for clinical samples.

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