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Human immunodeficiency virus type 1 reverse transcriptase: role of Tyr115 in deoxynucleotide binding and misinsertion fidelity of DNA synthesis

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Tyr115 is located in the vicinity of the polymerase catalytic site of human immunodeficiency virus type 1 (HIV-1) reverse transcriptase. Site-directed mutagenesis was used to generate variant enzymes having Phe, Trp, Ala, Ser, Asp or Lys instead of Tyr115. The substitution of Tyr115 by Phe renders a fully active polymerase, displaying similar kinetic parameters, processivity and misinsertion fidelity of DNA synthesis as the wild-type enzyme. In contrast, the replacement of Tyr by Asp or Lys produced enzymes with a very low polymerase activity. The activity of the variant enzymes having Trp, Ala or Ser instead of Tyr115 was reduced significantly, particularly when poly(rA)$_{484}$ was used as template. This effect was caused by a dramatic increase in the K_m value for dTTP, and was detected using a DNA template mimicking a proviral HIV-1 *gag* sequence. Misinsertion fidelity assays revealed that mutants Y115W, Y115A and Y115S had a higher misinsertion efficiency than the wild-type reverse transcriptase. The low fidelity of these mutants appears to be related to nucleotide recognition rather than altered DNA–DNA template–primer interactions. The effects observed on the steady state kinetic constants, processivity and fidelity were mediated by the 66 kDa subunit, as demonstrated using chimeric heterodimers with the Y115A substitution in either p66 or p51.

Keywords: AIDS/fidelity/polymerase/quasispecies/retrovirus

Introduction

Human immunodeficiency virus (HIV) reverse transcriptase (RT) converts the viral genomic RNA to a double-stranded DNA intermediate which integrates into host cell DNA. HIV-1 RT is composed of two asymmetric units of 66 and 51 kDa (termed p66 and p51, respectively). Crystal structures of RT in its native form (Rodgers *et al.*, 1995) and complexed with double-stranded DNA (Jacobo-Molina *et al.*, 1993) or non-nucleoside inhibitors (Kohlstaedt *et al.*, 1992; Ding *et al.*, 1995; Esnouf *et al.*, 1995; Ren *et al.*, 1995) have revealed different folding for each subunit. Both subunits contain four common subdomains. In the p66 subunit they form a nucleic acid binding cleft, which is absent in p51. Aspartic acid residues at positions 110, 185 and 186 define the polymerase active site in the palm of p66, which also contains an RNase H domain located at its C-terminus.

HIV-1 RT is an attractive target for chemotherapeutic intervention in the control of acquired immunodeficiency syndrome (AIDS). However, the selection of HIV-1 variants resistant to antiretroviral agents has become an important obstacle in the control of AIDS (Richman, 1993; Larder, 1994; Nájera *et al.*, 1995; and references therein). The rapid emergence of drug-resistant viruses results from a high intrinsic mutation rate of HIV, which leads to viral genetic heterogeneity because of the low fidelity exhibited by the RT (Preston *et al.*, 1988; Roberts *et al.*, 1988; Weber and Grosse, 1989; reviewed in Bebenek and Kunkel, 1993). Single-base substitution errors could arise directly either by the insertion of a noncomplementary nucleotide or by a transient primer template slippage mechanism (Perrino *et al.*, 1989; Ricchetti and Buc, 1990; Yu and Goodman, 1992; Boyer *et al.*, 1992a). Site-directed mutagenesis studies have revealed that the substitution of Gly262 or Trp266 by Ala led to RT mutants with a low frameshift fidelity because of template–primer slippage errors (Bebenek *et al.*, 1995). Gly262 and Trp266 are located on α-helix H and are involved in interactions with the primer strand of the template–primer (Beard *et al.*, 1994). The simultaneous replacement of Cys38 and Cys280 by Ser rendered HIV-1 RTs with an increased efficiency of mispair extension (Bakhanashvili and Hizi, 1992), and a similar effect has been reported for mutants having Ala instead of Met184 (Pandey *et al.*, 1996). In both cases, the mechanism leading to a decreased fidelity remains unclear. In other polymerases, residues involved in misinsertion fidelity usually affect nucleotide binding. Examples are Tyr766 in the fingers domain of *Escherichia coli* DNA polymerase I (Carroll *et al.*, 1991) or Tyr865 of human DNA polymerase α (Dong *et al.*, 1993). The Tyr865 of human DNA polymerase α is conserved in many polymerases, including HIV-1 RT (Poch *et al.*, 1989; Delarue *et al.*, 1990; Blanco *et al.*, 1991), where the equivalent residue (Tyr115) could also participate in dNTP binding as suggested by modelling studies based on the crystal structure of RT complexed with DNA (Tantillo *et al.*, 1994; Patel *et al.*, 1995). Here we describe a series of mutants of Tyr115 of HIV-1 RT to show a role for this amino acid in dNTP binding and misinsertion fidelity of DNA synthesis.

Results

Expression and purification of mutant and wild-type RTs

Purification of the mutant and wild-type RTs was performed after independent expression of their subunits. The 51 kDa subunit was obtained with an extension of six histidines located at its N-terminal end. The poly-histidine

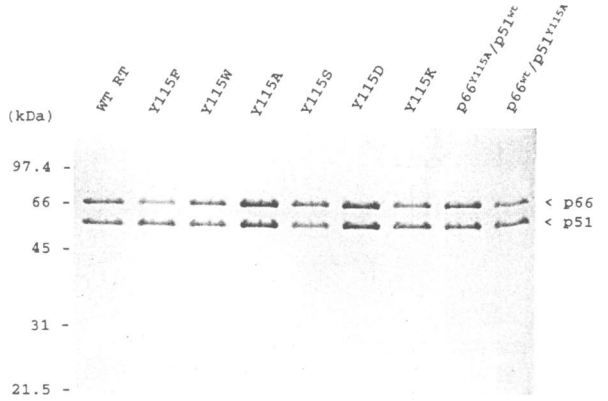

Fig. 1. SDS–PAGE analysis of mutated HIV-1 RTs. Electrophoretic mobility of p66 and p51 is shown for the wild-type RT and the mutated enzymes. Molecular weight markers and their masses were: phosphorylase b (97.4 kDa), bovine serum albumin (66 kDa), ovalbumin (45 kDa), carbonic anhydrase (31 kDa) and soybean trypsin inhibitor (21.5 kDa). Details on the purification are given in Materials and methods.

tract was introduced to facilitate its purification by metal chelate affinity chromatography (LeGrice *et al.*, 1991). The 66 kDa subunit was constitutively expressed by cultures harbouring plasmid p66(RT) (Hizi *et al.*, 1988), and contained two extra amino acids at the N-terminal end (Met–Val–) which were not found in the viral RT obtained from purified HIV (Di Marzo Veronese *et al.*, 1986). Site-directed mutagenesis was used to generate a series of mutants where Tyr115 was replaced by Phe, Trp, Ala, Ser, Asp or Lys. The purification of heterodimeric wild-type and mutant RTs was made after combining bacterial extracts containing p66 and p51. The formation of heterodimers was facilitated by changing the ionic strength and temperature conditions while processing the bacterial extracts to favour the association of the RT subunits (Lebowitz *et al.*, 1994). After purification of the heterodimeric RT, enzymes were found to be >90% pure, as judged by SDS–PAGE (Figure 1). The overall yield of the process was ~0.3–0.5 mg protein/l bacterial culture. In this study, the amino acid substitutions were introduced in both subunits of the RT. However, this purification procedure was also used to produce chimeric heterodimers containing the mutation in either p66 or p51 ($p66^{wt}/p51^{Y115A}$ and $p66^{Y115A}/p51^{wt}$).

RT activity and steady state kinetic constants

The effects of different substitutions on the polymerase activity of the heterodimeric RT are shown in Figure 2. The replacement of Tyr115 by Phe produced a variant RT with a higher specific activity than the wild-type enzyme. In contrast, the replacement of Tyr by Trp, Ala or Ser rendered enzymes which were less active, particularly when poly(rA)·oligo$(dT)_{12-18}$ was used as the template–primer. The polymerase activity of the Y115K and Y115D mutants was almost negligible with such a substrate. Interestingly, Y115D or Y115K showed a measurable activity when assayed in the presence of poly(dC)·oligo-$(dG)_{12-18}$ and dGTP. The specific activity of the heterodimeric wild-type RT was similar to that obtained with commercial preparations of HIV-1 RT lacking the polyhistidine tract at the N-terminal end of p51 (e.g. Boerhinger-Mannheim HIV-1 RT; Müller *et al.*, 1989).

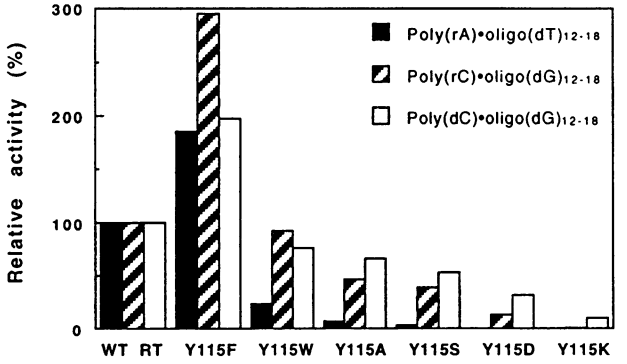

Fig. 2. Effect of substitutions at Tyr115 of HIV-1 RT on polymerase activity. Activities were determined with the different homopolymeric template–primers [poly(rA)·oligo$(dT)_{12-18}$, poly(rC)·oligo$(dG)_{12-18}$ and poly(dC)·oligo$(dG)_{12-18}$], and normalized to the value obtained with the wild-type RT and the corresponding template–primer. The specific activity of the wild-type enzyme was 1700 U/mg with poly(rA)·oligo$(dT)_{12-18}$, 500 U/mg with poly(rC)·oligo$(dG)_{12-18}$ and 1000 U/mg with poly(dC)·oligo$(dG)_{12-18}$. One activity unit is the amount of enzyme that incorporates 1 nmol [^{3}H]TMP into acid-insoluble products in 10 min at 37°C under the conditions described in Materials and methods. Deviations between triplicates were always <20%.

Table I. Kinetic parameters for dTTP binding of wild-type and mutant RTs[a]

Enzymes	k_{cat} (s^{-1})	K_m (μM)	k_{cat}/K_m ($mM^{-1}s^{-1}$)
WT	0.47 ± 0.16	6.7 ± 1.7	70.1 ± 10.8
Y115F	0.23 ± 0.10	3.0 ± 1.0	76.7 ± 17.8
Y115W	0.51 ± 0.02	44.8 ± 7.7	11.4 ± 2.0
Y115A	0.50 ± 0.05	156.7 ± 19.9	3.2 ± 0.9
Y115S	0.65 ± 0.05	235.2 ± 26.4	2.8 ± 0.4
$p66^{Y115A}/p51^{wt}$	0.64 ± 0.05	154.4 ± 26.0	4.1 ± 0.7
$p66^{wt}/p51^{Y115A}$	0.49 ± 0.04	5.6 ± 2.8	87.5 ± 17.1

[a]Poly$(rA)_{484}$·oligo$(dT)_{20}$ was used as substrate. The template/primer nucleotide ratio was 10:1 (approximate molar ratio 1:2.5). The template–primer concentration, which was fixed at 1 μM (expressed as 3′-hydroxyl primer termini), was saturating for all enzymes tested. The source of each polypeptide in a heterodimer is shown by a superscript.

The effects of substituting Tyr115 were also observed when kinetic parameters were determined using poly(rA)·oligo$(dT)_{20}$ as a template–primer (Table I). While Y115F and the wild-type RT showed similar and low K_m values for dTTP incorporation (<7 μM), other enzymes, such as Y115A or Y115S, showed K_m values >150 μM. Differences in the k_{cat} values were less significant. In addition, we observed that the effects on the kinetic parameters were also caused by substitutions in the 66 kDa subunit. Thus, chimeric heterodimers with a mutated p51 showed a wild-type phenotype, while enzymes with a mutated p66 were similar to Y115A. These data indicate that Tyr115 on the p66 subunit of HIV-1 RT plays a major role in dNTP recognition.

Effect of amino acid substitutions on template–primer interactions

The dissociation equilibrium constants (K_d) were determined with poly$(rA)_{484}$·oligo$(dT)_{20}$ and with the heteropolymeric DNA–DNA template–primer complex shown in Figure 3. As shown in Table II, the estimated K_d values for RNA–DNA binding were not strongly affected by mutations at Tyr115. For example, the substitution of Tyr

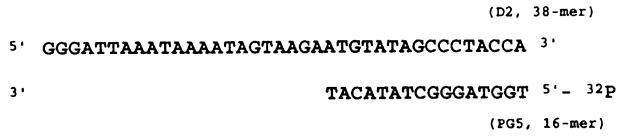

Fig. 3. Nucleotide sequence of the 38/16mer DNA complex used. Nucleotide sequences of template (D2) and primer (PG5) were taken from Ricchetti and Buc (1993). The template strand mimics the HIV-1 *gag* sequence including nucleotides 1137 (5′ end)–1174 (3′ end), according to the numbering given by Ratner *et al.* (1985).

Table II. Estimated K_d values for wild-type and mutant RTs

Enzymes	Estimated K_d (nM) (RNA–DNA)[a]	K_d (nM) (DNA–DNA)[b]
WT	7.3 ± 1.8	3.9 ± 0.6
Y115F	1.8 ± 0.6	1.7 ± 0.5
Y115W	7.7 ± 1.8	2.1 ± 0.5
Y115A	16.6 ± 2.6	1.8 ± 0.2
Y115S	18.7 ± 4.2	5.9 ± 0.9
Y115D	ND[c]	2.0 ± 0.5
Y115K	ND[c]	2.1 ± 0.7
$p66^{Y115A}/p51^{wt}$	33.0 ± 4.5	2.7 ± 0.8
$p66^{wt}/p51^{Y115A}$	4.7 ± 2.0	1.8 ± 0.5

[a]The estimated K_d values were obtained as described by Reardon *et al.* (1991), using poly(rA)$_{484}$·oligo(dT)$_{20}$ as the template–primer complex.
[b]The dissociation constant was determined by a gel mobility shift assay, using the heteropolymeric template–primer complex shown in Figure 3.
[c]Not determined.

by Ala or Ser resulted in a mere 2- to 3-fold increase in the K_d value. These differences were even less significant when the 38/16mer complex was used as the template–primer. Interestingly, poorly active enzymes, such as Y115D or Y115K, showed a K_d for DNA–DNA binding comparable with the wild-type RT, suggesting that Tyr115 does not exert a major influence on DNA binding.

Processivity

Qualitative measurements of the processivity of the wild-type RT and the mutant derivatives were carried out by the DNA trap method using poly(rA)$_{484}$·oligo(dT)$_{16}$ and the 38/16mer template–primer complex. When poly(rA)$_{484}$·oligo(dT)$_{16}$ was used (Figure 4A), primers were extended for >60 nucleotides, in agreement with previous data reported for the wild-type RT (Huber *et al.*, 1989; Beard and Wilson, 1993). Polymerization by wild-type RT, Y115F and $p66^{wt}/p51^{Y115A}$ proceeded at a somewhat higher catalytic rate than with the other mutants, while the lowest elongation rate was observed with Y115W. Elongation by this enzyme may be slower under the buffer conditions used in this assay than under the conditions used in the assays reported in Table I. All the enzymes tested were able to complete long processive extensions (over 60 bases). When the 38/16mer template–primer complex was used, differences became more significant (Figure 4B). Although full extension of the primer was obtained with most of the enzymes, stops were observed at positions +1, +2, +3, +6 and +14 in all cases. For Y115W, the incorporation of the last nucleotide appears to be slower than for the other enzymes. The amount of fully extended primer was low for Y115W and Y115A, and almost undetectable for the chimeric heterodimer $p66^{Y115A}/p51^{wt}$ and for Y115S. The dissociation rate constant during processive polymerization (k_{off}) was determined with poly(rA)$_{484}$ as a template and 5′-phosphorylated (dT)$_{20}$ as primer (Table III). Its value was found to be lower for Y115F and higher for Y115S, ranging from 0.13 to 0.89 min^{-1}, and correlates quite well with the K_d values for poly(rA)$_{484}$·oligo(dT)$_{20}$ binding given in Table II. The k_{off} value for the wild-type RT (0.23 min^{-1}) was similar to that reported by other authors (Beard and Wilson, 1993; Sarafianos *et al.*, 1995a). The k_{off} values suggest that in the assay conditions, interactions between the RT and the template–primer complex are not strongly affected by the replacement of Tyr115 by another amino acid. The k_{cat} values obtained in the absence of trap were somewhat higher than those reported in Table I. The observed differences could be attributed to the higher template/primer molar ratio used in the processivity assays.

Misinsertion fidelity

Steady state parameters for the incorporation of the first nucleotide at the 3′ end of a 16mer template–primer were determined using the 38/16mer complex (Figure 3). The misinsertion efficiency (f) of the wild-type RT was similar to that observed for Y115F and $p66^{wt}/p51^{Y115A}$ (Table IV). In contrast, the f values obtained with other variant RTs, such as Y115W, Y115A or Y115S, were around two orders of magnitude higher than those determined for wild-type RT. The K_m values for the incorporation of the correct nucleotide (dTMP) were largely affected by nonconservative substitutions of Tyr115. As observed with poly(rA)$_{484}$·oligo(dT)$_{20}$, the replacement of Tyr by Trp, Ala or Ser rendered enzymes with a lower affinity for dTTP. These changes were not observed with the V_{max} values, which show little variation. The V_{max} and K_m values for the incorporation of the incorrect nucleotide (dGMP) did not show the same trend. Both parameters were lower for the mutant RTs than for the wild-type enzyme or Y115F. However, unlike the case of dTTP, the V_{max}/K_m ratio for the incorporation of dGTP was similar for all the studied RTs. Taken together, these data suggest that the lower misinsertion fidelity of the Tyr115 mutants is caused by a loss of affinity for the correct nucleotide (dTTP). In addition, the poly-histidine tract did not have an influence on fidelity because heterodimeric RT lacking those residues showed similar kinetic properties to our wild-type RT in misinsertion fidelity assays (data not shown). All the fidelity data obtained with the chimeric RTs confirmed the dominant role of p66 in the catalysis.

Discussion

Several residues in HIV-1 RT have been proposed as potential dNTP binding sites, i.e. residues 65–75 (Cheng *et al.*, 1993; Wu *et al.*, 1993), Gln151 (Sarafianos *et al.*, 1995b) and Lys263 (Basu *et al.*, 1989). The region comprising residues 65–75 includes sites involved in resistance to several nucleoside-analogue inhibitors (Larder, 1994; Arts and Wainberg, 1996; and references therein). However, recent evidence suggests that resistance could also be influenced by the positioning and conformation of the template–primer (Martin *et al.*, 1993b; Boyer *et al.*, 1994b; Tantillo *et al.*, 1994). In the case of Lys263, Martin *et al.* (1993a) have shown that highly purified

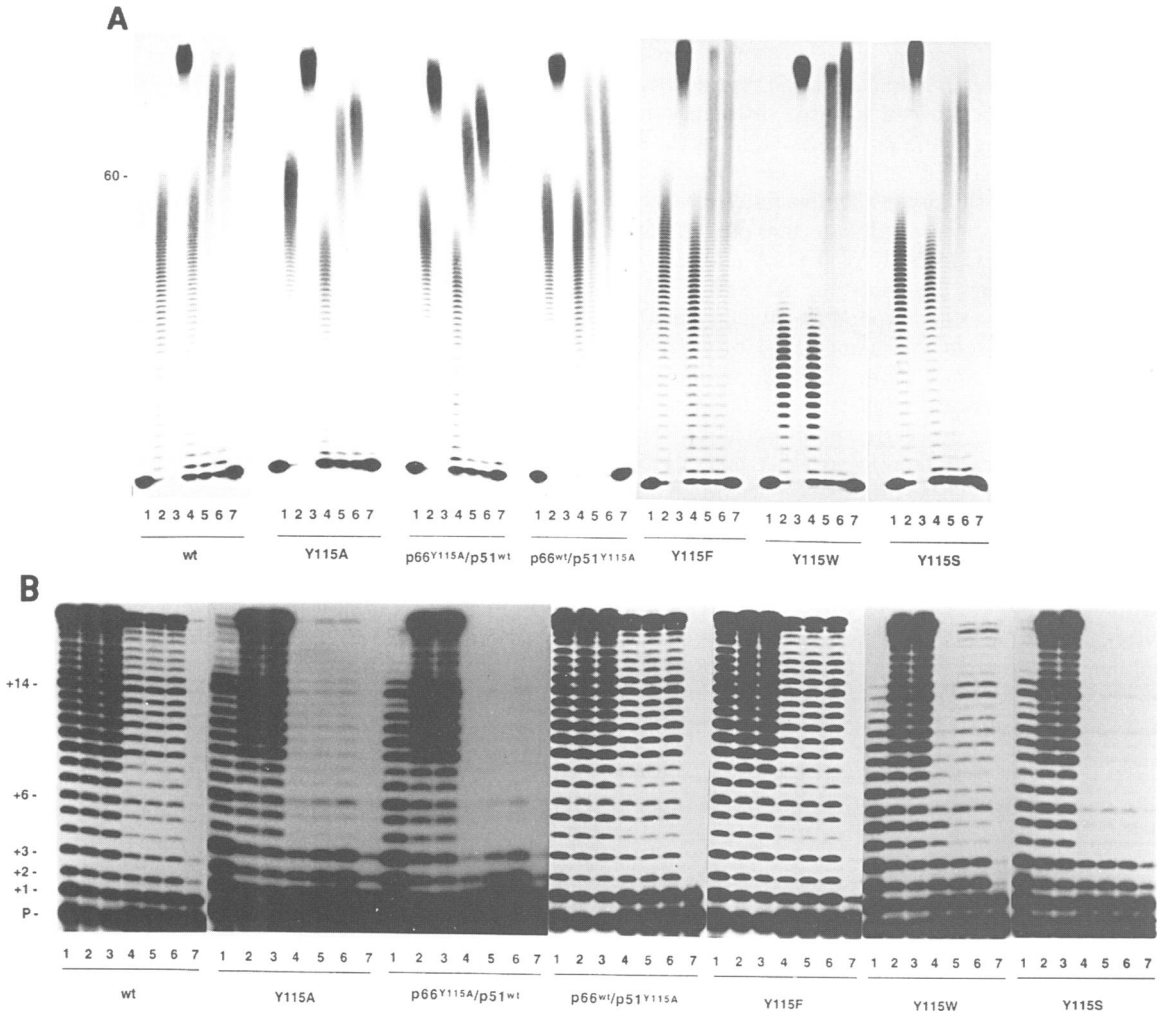

Fig. 4. Processivity of the wild-type and mutant HIV-1 RTs. The results of processivity assays performed with poly(rA)$_{484}$·oligo(dT)$_{16}$ as the template–primer are shown in (**A**), while the results obtained with the 38/16mer complex are shown in (**B**). In (A), lanes 1 are controls with no enzyme, dTTP or DNA trap; lanes 2 and 3 correspond to reaction products with no added DNA trap after 1 and 10 min incubation; lanes 4–6 correspond to 1, 10 and 20 min reactions where dTTP and DNA traps were added simultaneously; lanes 7 show the reaction products in assays where the enzyme was added after dTTP and DNA trap (10 min reactions are shown). In (B), lanes 1–3 show the reaction products after 45 s, 10 min and 20 min in the absence of DNA trap; lanes 4–6 correspond to 45 s, 10 and 20 min reactions where dNTPs and DNA traps were added simultaneously; lanes 7 show the reaction products in assays were the enzyme was added after the DNA trap (10 min reactions are shown). P stands for primer.

mutant RT with Ser instead of Lys263 bound natural dNTP and primed polynucleic acid substrates with equal affinity when compared with the wild-type RT. Furthermore, in the crystal structure of RT complexed with double-stranded DNA (Jacobo-Molina *et al.*, 1993), Lys263 is located near the phosphates of the primer strand, away from the potential dNTP binding site (Beard *et al.*, 1994; Tantillo *et al.*, 1994). Modelling studies based on such a 3-D structure suggested that regions involved in dNTP binding included part of β6 and the loop connecting β6 and αC (residues Asp110–Phe116), the β9–β10 hairpin (residues Tyr183–Asp186) and portions of the β8–αE loop (Gln151), αE (Phe160 and Gln161) and β11b (Lys219 and His221) (Tantillo *et al.*, 1994). It has been suggested that Tyr115 could be involved in interactions with the base of the incoming nucleotide (Patel *et al.*, 1995). Here we demonstrate the role of Tyr115 in dNTP binding and misinsertion fidelity. The replacement of Tyr115 with Phe by site-directed mutagenesis did not affect the polymerase activity of the enzyme, in agreement with results reported by other authors (Larder *et al.*, 1989; Lowe *et al.*, 1991; Boyer *et al.*, 1992b). However, the introduction of nonconservative substitutions, as in Y115A or Y115S, produced a significant decrease in the specific activity of the mutated RTs. Charged amino acids like Asp or Lys were detrimental for the activity when introduced in this position. In our study, the specific activity of the mutants was found to be correlated with the hydrophobicity of the side chain of the introduced residue. Thus, Y115F was the most active RT, followed by the wild-type enzyme, Y115W, Y115A and Y115S. This trend is also observed in data obtained by other groups with different mutants. It has been shown that wild-type RT, Y115F and Y115V were fully active *in vitro* (Larder *et al.*, 1989; Lowe *et al.*, 1991; Boyer *et al.*, 1992b, 1994a), while Y115N and Y115H showed <15% of the wild-type RT activity and

were resistant to 3′-azido-3′-deoxythymidine triphosphate and phosphonoformate (Larder *et al.*, 1989). In our series of mutated RTs, their kinetic characterization revealed that the correlation could be ascribed to large differences in the K_m values for dNTP, while k_{cat} values were affected only slightly. The observed effects were caused by mutations in the 66 kDa subunit, as demonstrated by using chimeric heterodimers. The results of the processivity studies revealed that mutants Y115W, Y115A and Y115S were less processive than the wild-type RT and Y115F. These differences were more pronounced when heteropolymeric DNA was used as a template. However, all mutants have a similar equilibrium dissociation constant for DNA binding, and the differences in the dissociation rate constant (k_{off}) during processive polymerization were only three or four times higher for Y115A and Y115S than for the wild-type RT. These differences were relatively small when compared with those reported for mutants involved in template binding interactions, such as G262A or W266A, whose k_{off} value is >200 times higher than for the wild-type enzyme (Beard *et al.*, 1994). The low processivity of Y115W, Y115A or Y115S could be caused by a combined effect of low k_{cat} and high K_m for dNTP during processive polymerization.

Our data demonstrate that Tyr115 plays an important role in dNTP binding. As expected, this residue is strictly conserved in all isolates of HIV-1 and HIV-2 (Myers *et al.*, 1995), although Phe has been reported to appear at this position after passage of the virus in the presence of the RT inhibitor 1592U89 (Mellors *et al.*, 1995). Tyr or Phe are the only amino acids found at the equivalent position of other retroviral RTs (Poch *et al.*, 1989). In addition, Tyr is also conserved in other RNA-dependent polymerases and in DNA polymerases α (Delarue *et al.*, 1990; Blanco *et al.*, 1991). In this group of enzymes, this residue has also been involved in dNTP binding, as demonstrated for Tyr254 of ϕ29 DNA polymerase (Blasco *et al.*, 1992) and Tyr865 of human DNA polymerase α (Dong *et al.*, 1993).

The low fidelity of HIV-1 RT reflects the lack of proofreading activity, the relative ease of mispaired extension, the high frequency of T·G error formation and elevated rates of other error-forming pathways, such as template–primer slippage. Error frequencies appear to depend in a complex manner on the type of mispair formed and the sequence context (Ricchetti and Buc, 1990). However, base hydrogen bonding, base stacking, geometry of the base pair and interactions of specific amino acids of the polymerase with single-stranded template and double-stranded primer nucleotides are likely to be important for fidelity (Perrino *et al.*, 1989; Boyer *et al.*, 1992a; Yu and Goodman, 1992; Bebenek *et al.*, 1993; Zinnen *et al.*, 1994). Mutations at the nucleotide binding site of the RT are expected to have an influence on misinsertion fidelity. The replacement of Tyr115 by Trp, Ala or Ser resulted in enzymes with a higher misinsertion efficiency than the wild-type RT. Interestingly, substitution of the equivalent residue in human DNA polymerase α (Tyr865) by Ser rendered an enzyme with a 10-fold higher misinsertion efficiency than the wild-type enzyme in Mg^{2+}-catalysed reactions (Dong *et al.*, 1993). Under these conditions the differences in misinsertion fidelity between mutant Y865F and wild-type human DNA polymerase α were insignificant, as observed in our study with the equivalent mutant Y115F and the wild-type HIV-1 RT. Again, these data point to the side chain of Tyr115 as an important determinant of fidelity in HIV-1 RT. Previously, it was shown that the substitutions of Gly262 or Trp266 by Ala in the α-helix H of HIV-1 RT lead to enzymes showing a frameshift mutator phenotype (Beard *et al.*, 1994; Bebenek *et al.*, 1995). In that case, errors would arise from alterations in the template–primer binding properties of the polymerase. While this paper was being

Table III. Results of quantitative processivity studies with wild-type and mutant RTs[a]

Enzymes	k_{off} (min^{-1})	$(N/E)_f$	k_{cat} (s^{-1})
WT	0.23 ± 0.04	231 ± 96	0.87 ± 0.15
Y115F	0.13 ± 0.02	152 ± 43	0.33 ± 0.03
Y115W	0.31 ± 0.04	142 ± 35	0.73 ± 0.08
Y115A	0.71 ± 0.06	90 ± 16	1.05 ± 0.08
Y115S	0.89 ± 0.08	47 ± 11	0.83 ± 0.06
$p66^{Y115A}/p51^{wt}$	0.62 ± 0.08	189 ± 51	1.02 ± 0.21
$p66^{wt}/p51^{Y115A}$	0.26 ± 0.04	137 ± 37	0.61 ± 0.04

[a]These assays were performed with poly(rA)$_{484}$·oligo(dT)$_{20}$ as substrate. The template/primer molar ratio was 1:1. Experimental data were fitted to the equation.
$N/E = (k_{cat}/k_{off})\ (1 - e^{-k_{off}t})$, where N/E is the number of dTMP incorporated per dimer of RT, k_{cat} is the steady state rate constant observed in the absence of trapping agent (heparin in these experiments), and k_{off} is the dissociation rate constant for the RT/template–primer complex during processive polymerization. In the presence of trap, a plateau is achieved after a sufficiently long time period ($t >> 1/k_{cat}$). This plateau is described by the relationship: $(N/E)_f = k_{cat}/k_{off}$, where $(N/E)_f$ is the limiting value for the incorporation of dTTP into polymer (Bryant *et al.*, 1983).

Table IV. Misinsertion fidelity of wild-type and mutant RTs[a]

Enzymes	dTTP (correct)			dGTP (incorrect)			f[b]
	K_m (μM)	V_{max} (nM/min)	V_{max}/K_m (min^{-1})	K_m (μM)	V_{max} (nM/min)	V_{max}/K_m (min^{-1})	
WT	0.180 ± 0.010	17.8 ± 2.0	0.104	1498 ± 173	2.4 ± 0.4	1.6×10^{-6}	1.54×10^{-5}
Y115F	0.052 ± 0.001	13.8 ± 0.4	0.266	1121 ± 189	4.6 ± 0.6	4.1×10^{-6}	1.53×10^{-5}
Y115W	9.7 ± 1.6	12.2 ± 1.0	1.3×10^{-3}	32 ± 25	0.04 ± 0.01	1.4×10^{-6}	1.0×10^{-3}
Y115A	29.3 ± 4.6	21.0 ± 8.3	7.2×10^{-4}	135 ± 30	0.29 ± 0.08	2.1×10^{-6}	2.9×10^{-3}
Y115S	66.7 ± 9.0	16.1 ± 3.4	2.4×10^{-4}	48 ± 18	0.10 ± 0.02	2.2×10^{-6}	9.1×10^{-3}
$p66^{Y115A}/p51^{wt}$	90.3 ± 9.5	17.8 ± 1.0	2.0×10^{-4}	39 ± 30	0.04 ± 0.01	1.0×10^{-6}	5.0×10^{-3}
$p66^{wt}/p51^{Y115A}$	0.074 ± 0.007	18.0 ± 0.4	0.245	1727 ± 235	4.6 ± 0.2	2.7×10^{-6}	1.04×10^{-5}

[a]The template–primer complex used as a substrate in this experiment is given in Figure 3. The insertion position is at the 3′ end of the primer. Data are the mean value ± standard deviation of at least three independent experiments.
[b]$f = [V_{max}\ (incorrect)/K_m\ (incorrect)]/[V_{max}\ (correct)/K_m\ (correct)]$.

revised, Wainberg *et al.* (1996) and Pandey *et al.* (1996) reported that the replacement of Met184 by Val, which arises after monotherapy with (–)2′,3′-dideoxy-3′-thiacytidine (3TC), renders a variant RT with increased misinsertion fidelity. The substitution of Met184 by Val leads to an enzyme which is catalytically as efficient as the wild-type enzyme, but it is not clear whether the observed effects on the fidelity of DNA synthesis are mediated by a possible interaction with the sugar moiety of the primer terminus or with the dNTP substrate (Pandey *et al.*, 1996). In contrast, the misinsertion mutator phenotype of Tyr115-substituted HIV-1 RTs is apparently caused by an altered dNTP binding function.

The identification of mutator phenotypes in HIV-1 RT has significant implications for AIDS therapy because it opens the possibility of designing compounds targeting the fidelity properties of HIV-1 RT. According to the Eigen's theory of quasispecies (Eigen and Schuster, 1979), it could be theoretically possible to drive RNA populations into error catastrophe by decreasing the fidelity of RNA synthesis. Although viruses carrying the mutation Y115N have been shown to be poorly infectious (Larder *et al.*, 1989), it will be interesting to test whether other mutants such as those described here are viable, and to analyse quasispecies evolution upon introducing misinsertion fidelity mutations in proviral HIV clones.

Materials and methods

Plasmid constructions and site-directed mutagenesis

For the expression of the RT subunits we used two plasmids: (i) p66(RT) (Hizi *et al.*, 1988), which contains the coding region of the 66 kDa subunit; and (ii) pT51H, which was used for the expression of the 51 kDa subunit with a poly-histidine extension at its N-terminal end. Plasmid p66(RT) was kindly provided by S.H.Hughes and P.Boyer (Frederick Cancer Research and Development Center, Frederick, MD). Plasmid pT51H was obtained after cloning the coding region of p51 in the expression vector pTrcHisB (Invitrogen Corporation). For such a purpose, a DNA fragment containing the nucleotide sequence of p51 was synthesized by PCR using p66(RT) as a template. Two oligodeoxynucleotides were used as primers: (i) 5′-CGCGGGATCCGCTAGCCCCATTAGCCCTATTGAG-3′, which contains the nucleotide sequence of the 5′ end of the RT gene and two restriction sites (for *Bam*HI and *Nhe*I); and (ii) 5′-CCTGCAGAATTCAGAAGGTTTCTGCTCCTAC-3′, which contains the 3′ end of p51 and an *Eco*RI site. PCR was carried out in a Perkin-Elmer 9600 thermal cycler for 30 cycles. After 2 min incubation at 95°C, the initial five cycles were 94°C for 30 s, 45°C for 30 s and 72°C for 2 min, followed by 25 cycles in which annealing was performed at 55°C. The programme was terminated with a 10 min incubation at 72°C. The amplified DNA was analysed on an agarose gel, purified and cleaved with *Nhe*I and *Eco*RI. Then it was cloned into the plasmid pTrcHisB (Invitrogen Corporartion), digested previously with both enzymes. Site-directed mutagenesis was performed with the Altered Sites *in vitro* mutagenesis system kit from Promega, following the manufacturer's instructions (Menéndez-Arias *et al.*, 1995). Previously, the p66-coding region was amplified by PCR and cloned into the *Bam*HI and *Eco*RI sites of pALTER-1 (Promega). Plasmid p66(RT) was used as a template and two oligodeoxynucleotides were used as primers: (i) 5′-CGCGGGATCCATGGTACCCATTAGCCCTATTGAG-3′, which contains the nucleotide sequence of the 5′ end of the RT gene and two restriction sites (for *Bam*HI and *Nco*I); and (ii) 5′-GCTGCAGAATTCATAGTATTTTCCTGATTCC-3′, which contains the 3′ end of RT and an *Eco*RI site. All the synthetic oligonucleotides were obtained from Isogen Bioscience (Maarssen, The Netherlands). The RT mutations and oligodeoxynucleotides used in the mutagenesis reaction are shown in Table V. The introduced mutations were confirmed by digestion with *Nsi*I and by DNA sequencing. The pALTER-derived plasmids containing the mutated RTs were digested with *Nco*I and *Eco*RV to isolate a 429 bp fragment, which was then cloned at the corresponding sites in p66(RT). In a similar way, 1207 bp fragments derived from pALTER and containing the introduced mutations were obtained by digestion with *Asp*718 and *Msc*I, and cloned at the appropriate sites of pT51H.

Table V. Synthetic oligonucleotides used in the mutagenesis of HIV-1 RT

Mutation[a]	Oligodeoxynucleotide[b]
Y115F	GGGAACTGAAAAA**A**ATGCATCACCCAC
Y115W	GGGAACTGAAAA**CC**ATGCATCACCCACATC
Y115A	GGGAACTGAAAAA**GC**TGCATCACCCACATC
Y115S	GGGAACTGAAAAA**G**ATGCATCACCCAC
Y115D	GGGAACTGAAAAAT**C**TGCATCACCCAC
Y115K	GGGAACTGAAAA**TTT**TGCATCACCCACATC

[a]Mutations are identified by amino acid and position number in HIV-1 RT, followed by the substituted amino acid. Amino acids are denoted by the single-letter code.
[b]Bold nucleotides correspond to mutations introduced in the RT coding region. The introduced mutations lead to the loss of an *Nsi*I restriction site in Y115A, Y115D and Y115K.

Expression and protein purification

Escherichia coli DH5α cultures containing the plasmid p66(RT) constitutively express the 66 kDa subunit of the HIV-1 RT. Therefore they were grown overnight at 37°C in 2–3 l Luria broth medium containing 50 μg/ml ampicillin. Expression of the 51 kDa subunit by the pT51H constructs was induced with isopropyl-β-D-thiogalactopyranoside (Promega). In this case, freshly prepared *E.coli* DH5α cultures containing the pT51H construct were grown at 37°C in 200 ml Luria broth medium containing 50 μg/ml ampicillin, to an A_{600} of 0.8–1.0. After induction with 0.4 mM isopropyl-β-D-thiogalactopyranoside for 60 min, these cultures expressing p51 were mixed with those grown overnight and expressing p66. Then, cells were harvested by centrifugation at 4000 *g* for 15 min at 4°C. After removal of the supernatant, cells were resuspended in 80 ml 50 mM sodium phosphate, pH 7.8, containing 0.3 M NaCl and 1 mM phenylmethylsulfonyl fluoride, and treated at 4°C for 5 min with lysozyme (0.2 mg/ml). The salt concentration of the solution was then increased by adding 30 ml 50 mM sodium phosphate, pH 7.8, containing 4 M NaCl, and samples were sonicated. The extract obtained was centrifuged at 12 000 r.p.m. for 10 min at 4°C using a Sorvall SS-34 rotor. The pooled supernatants were incubated for 30 min at 37°C and the salt concentration was then lowered to facilitate heterodimer formation by adding 250 ml of a cold solution containing 50 mM sodium phosphate, pH 7.8, and 0.3 M NaCl. The sample was left on ice for 15 min, and then filtered and loaded on a 3 ml Ni^{2+}-nitrilotriacetic acid–agarose column (Invitrogen Corporation). The column was washed successively with 60 ml 50 mM sodium phosphate, pH 7.8, containing 0.3 M NaCl; 50 ml 50 mM sodium phosphate, pH 6.0, containing 0.5 M NaCl; and 50 ml 50 mM sodium phosphate, pH 6.0, containing 0.5 M NaCl and 50 mM imidazole. The RT was eluted with a 40 ml gradient from 50 to 500 mM imidazole in 50 mM sodium phosphate, pH 6.0, containing 0.5 M NaCl. Fractions of 2 ml were collected and analysed for RT content by SDS–PAGE (10% acrylamide). The fractions containing p66 and p51 were pooled and dialysed against 50 mM Tris–HCl, pH 7.0, containing 25 mM NaCl, 1 mM EDTA, 1 mM dithiothreitol (DTT) and 10% glycerol. The RT-containing dialysate was applied to a 3 ml Q-Sepharose column (Pharmacia) equilibrated in the dialysis buffer. The RT was recovered in the nonbinding fraction, and then applied to a 2.5 ml Bio-Rex 70 column (Bio-Rad) equilibrated in the dialysis buffer. After washing the column with 20 ml of this buffer, the RT was eluted with a 50 ml gradient from 0.0 to 0.6 M NaCl. All material eluting from the salt gradient was analysed by SDS–PAGE, and fractions containing the heterodimer were pooled, dialysed against 50 mM Tris–HCl, pH 7.0, containing 25 mM NaCl, 1 mM EDTA, 1 mM DTT and 10% glycerol, concentrated in Centriprep-30 and Centricon-30 to <0.5 ml, and stored at –20°C. All the chromatographic steps were performed at 4°C, and all buffers were sterilized prior to use. Protein concentrations were determined using the Bio-Rad protein assay. The concentration of active molecules in our preparation was determined by titration with a template–primer duplex (Figure 3), by essentially following a procedure described previously (Reardon and Miller, 1990). These experiments showed that ~40–60% of the p66/p51 heterodimers were catalytically active.

RT activity assays

The polymerase activity of the purified RT was determined in 50 µl of a standard reaction mixture containing 50 mM Tris–HCl, pH 8.0, 20 mM NaCl, 10 mM $MgCl_2$ and 8 mM DTT. Assays were carried out using poly(rA)·oligo(dT)$_{12-18}$ and [^{3}H]dTTP, poly(rC)·oligo(dG)$_{12-18}$ and [^{3}H]dGTP or poly(dC)·oligo(dG)$_{12-18}$ and [^{3}H]dGTP. The template–primer concentration was 1 µM (expressed as 3′-hydroxyl primer termini). The concentration of nucleotide triphosphate was 5 µM and its specific activity was 1 mCi/µmol. Template–primers and nucleotide solutions were purchased from Pharmacia, and [^{3}H]dTTP and [^{3}H]dGTP were obtained from Amersham. Reactions were initiated by the addition of enzyme and were incubated at 37°C for 10–30 min. The amount of polymerized deoxynucleotide triphosphate was determined by acid-insoluble precipitation (Hizi *et al.*, 1988). Steady state kinetics of polymerization were studied using poly(rA)$_{484}$·oligo(dT)$_{20}$ and dTTP as substrates. The K_m and k_{cat} values for dTTP were determined in the presence of 1 µM template–primer (concentration expressed as 3′-hydroxyl primer termini). The template/primer nucleotide ratio in these assays was 10. Template–primer annealing was performed as reported previously (Beard and Wilson, 1993). Velocities for each substrate concentration were fitted to the Michaelis–Menten equation using the UltraFit Macintosh program (version 1.03; Biosoft). Kinetic experiments were performed at least twice, and values were averages of at least duplicate samples.

Determination of K_d

To determine estimated K_d values for poly(rA)$_{484}$·oligo(dT)$_{20}$ binding to HIV-1 RT, we performed RT assays where the concentration of substrate was held fixed at 1/5 its K_m value while varying the template–primer concentration. This method provides apparent K_m values for template–primer binding which approximate K_d, as shown previously by Reardon *et al.* (1991). The K_d for DNA binding to wild-type RT and mutant derivatives was determined by a gel mobility shift assay. We used the 38/16mer template–primer shown in Figure 3. Fast protein liquid chromatography-purified template and primer were purchased from Pharmacia. The primer was labelled at the 5′ end using T4 polynucleotide kinase and [γ-^{32}P]ATP. The 38/16mer duplexes were annealed in 150 mM magnesium aspartate and 150 mM NaCl. For a typical gel-shift assay, the labelled template–primer was diluted to a concentration of 0.75 nM in 50 mM HEPES, pH 7.0, containing 15 mM NaCl, 15 mM magnesium acetate, 130 mM KCH_3COO, 1 mM DTT and 5% polyethylene glycol 6000. The molar ratio of template to primer was 5:1. DNA was incubated for 10 min at 37°C with increasing amounts of enzyme, and loaded onto a 4% polyacrylamide gel (in 12 mM Tris acetate, 1 mM EDTA, pH 7.5) that had been pre-run for 10 min at 4°C. The gel was run at 150 V for 90 min at 4°C, and then dried down, exposed to an imaging plate and quantitated on a BAS 1500 scanner (Fuji Corporation). The fraction of bound DNA was plotted against enzyme concentration, and the K_d value was obtained as the RT concentration at which 50% of the DNA is bound.

Processivity studies

The processivity of the mutant enzymes was qualitatively compared with the wild-type RT using poly(rA)$_{484}$·oligo(dT)$_{16}$, as well as the 38/16mer template–primers. The 16mer complexes were 5′ ^{32}P-labelled and annealed 1:1 to (rA)$_{484}$ in 60 mM magnesium aspartate and 60 mM NaCl. The labelled template–primer (0.05 pmol) was preincubated at 37°C for 10 min with 1.4–3.8 pmol enzyme in a 19 µl reaction mixture containing 50 mM HEPES (pH 7.0), 35 mM NaCl, 15 mM magnesium acetate, 100 mM KCH_3COO, 1 mM DTT, 2 mg/ml bovine serum albumin and 2% glycerol. DNA synthesis was initiated by adding 16 µl preincubation buffer containing 1 mM dTTP, with or without the DNA trap (2 mg/ml herring sperm DNA). The reaction was allowed to continue at 37°C. 10 µl aliquots were taken at the indicated time points, mixed with 5 µl of stop solution (10 mM EDTA in 90% formamide) and frozen until further analysis. The products were analysed on a denaturing 16% polyacrylamide gel containing 8 M urea, followed by autoradiography. For the assays performed using the 38/16mer template–primers, pre-incubations were carried out with 0.6 pmol template–primer and 1–5 pmol enzyme in 17 µl of a reaction mixture containing 50 mM HEPES (pH 7.0), 15 mM NaCl, 15 mM magnesium acetate, 130 mM KCH_3COO, 1 mM DTT and 5% polyethylene glycol 6000. Reactions were started by adding an equal amount of the preincubation buffer containing a 0.4–5.0 mM concentration of dNTPs with or without DNA (1–2 mg/ml). At different times, aliquots were withdrawn and analysed as above in a denaturing 20% polyacrylamide gel containing 8 M urea. Quantitative processivity assays were performed essentially as described previously (Bryant *et al.*, 1983). Briefly, reaction mixtures (90 µl) containing 40–100 nM enzyme, 1.5 µM poly(rA)$_{484}$·oligo(dT)$_{20}$ (annealed at a 1:1 ratio with respect to the 5′ ends), 90 mM NaCl, 5 mM DTT and 75 mM Tris–HCl (pH 8.0) were preincubated at 37°C for 10 min. Then, a 180 µl aliquot of 15 mM $MgCl_2$ and 600 µM [^{3}H]dTTP (10–30 c.p.m./pmol) was added to initiate control reactions. In the challenged reactions, heparin was added to a final concentration of 5 mg/ml. Samples were incubated at 37°C. 25 µl aliquots were removed at different times, mixed with 12 µl 0.5 M EDTA, and processed for liquid scintillation counting.

Fidelity assays

Fidelity assays were performed essentially as described previously (Boosalis *et al.*, 1987; Ricchetti and Buc, 1990) with the modifications introduced by Sala *et al.* (1995), except that the molar ratio of template–primer to enzyme was 5:1 and the incubation time for the reaction was 30 s.

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