

# Gln<sup>84</sup> of moloney murine leukemia virus reverse transcriptase regulates the incorporation rates of ribonucleotides and deoxyribonucleotides

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**Abstract** Moloney murine leukemia virus reverse transcriptase (RT) selectively uses deoxyribonucleotides over ribonucleotides (rNTPs) as substrates. Substitution of F155 with valine (F155V) was previously found to increase the enzyme's affinity for rNTPs, though without affecting the  $V_{\max}$  for catalysis, and thereby conferred to the enzyme significant RNA polymerase activity. We have sought new mutations that might increase the RNA polymerase activity of the F155V mutant. We report here that substitution of Q84 with alanine improved RT-F155V's RNA polymerase activity, but also its DNA polymerase activity. Kinetic analysis and gel-retardation assays suggested that the substitution increased the enzyme's general affinity for the template-primer.

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**Keywords:** MMLV RT; DNA polymerase; RNA polymerase

## 1. Introduction

Moloney murine leukemia virus (MMLV) reverse transcriptase (RT) is a monomeric 75 kD protein, composed of a DNA polymerase domain at the N-terminus and a ribonuclease (RNase) H domain at the C-terminus [1]. Through the RNA-dependent and DNA-dependent DNA polymerase activities and the RNase H activity, RT converts the single-stranded viral RNA genome into a double-stranded integration-competent DNA during reverse transcription [2].

X-ray crystallographic structure studies revealed that the polymerase domain of MMLV-RT, like other nucleic acid polymerases, is shaped like a half-opened right hand, comprising of the palm, fingers and thumb subdomains [3,4]. The RNase H domain is connected to the polymerase domain through the "connection" domain and positioned close to the thumb subdomain. The catalytic active site of the polymerase is localized in the palm subdomain and composed of a trio of carboxylic acid residues, Asp150, Asp224 and Asp225, which are thought to directly participate in catalysis through two bound metal ions [3]. Multiple residues in the fingers sub-

domain have been implicated to regulate the processive DNA synthesis through interacting with the template-primer or the incoming deoxyribonucleotide (dNTP) substrate [3,5–8].

As a DNA polymerase, MMLV-RT selectively uses dNTPs over ribonucleotides (rNTPs) as substrates. Based on the structure of MMLV-RT and modeling of the protein with a template-primer complex, F155, a residue located in the active site (Fig. 1), was predicted to play a critical role for the enzyme to prevent the incorporation of rNTPs through an unfavorable interaction between the aromatic ring of F155 and the 2'-OH group of the incoming rNTP [3]. Substitution of F155 with valine dramatically increased the enzyme's affinity for rNTPs and thereby allowed the enzyme to incorporate rNTPs into the product [9,10]. However, the  $V_{\max}$  of the RT-F155V mutant enzyme using rUTP as a substrate was little affected, remaining about 100-fold lower than the  $V_{\max}$  using dTTP as a substrate, and the mutant enzyme could synthesize a stretch of RNA of only seven nucleotides long [9].

We have sought new mutations that might improve the RNA polymerase activity of RT-F155V. Here, we report that substitution of Q84 with alanine or asparagine conferred to RT-F155V higher RNA polymerase activity and allowed the enzyme to synthesize longer RNA. The Q84A or Q84N substitution did not affect the enzyme's selectivity for dNTPs over rNTPs; the mutant enzyme also displayed higher enzymatic activity using dNTPs as substrates.

## 2. Materials and methods

### 2.1. Plasmid construction

The coding sequence of MMLV-RT with six histidines at the C-terminus was PCR amplified from pRT30-His [10] and cloned downstream of the *tac* promoter of plasmid pMAL-c2 (New England Biolabs) to generate pTacRT-His. RT-WT-H and RT-F155V-H have been described previously [9,10]. In this report, pTacRT-His-D524N and pTacRT-His-F155V-D524N express RT-WT-H and RT-F155V-H, respectively. The Q84 mutations in the RT-WT-H or RT-F155V-H backbone were constructed by standard molecular cloning methods. All the constructs were verified by sequencing that only desired mutations were introduced.

### 2.2. Enzyme purification

Recombinant RTs were expressed in *Escherichia coli* BL21. For all the assays, the enzymes were purified to near homogeneity by chromatography on HiTrap chelating HP column (Pharmacia), and MonoS (Pharmacia) fast protein liquid chromatography.

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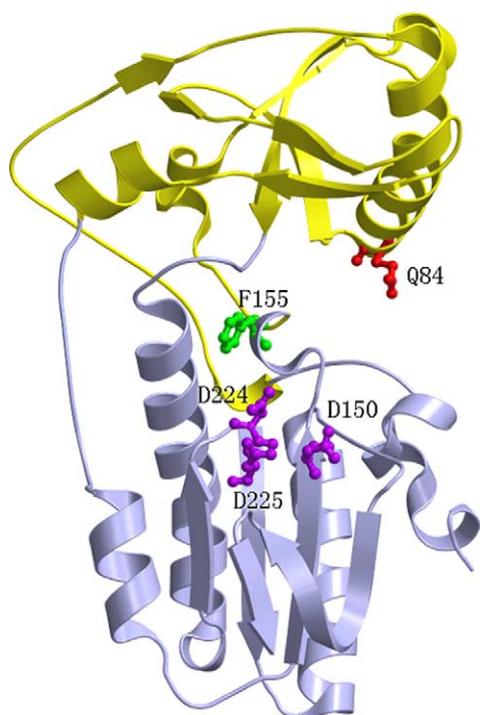


Fig. 1. Structure of the catalytically active fragment of MMLV RT. The fingers and palm subdomains are shown as a ribbon schematic in yellow and gray, respectively. The catalytic aspartate residues (D150, D224 and D225), Q84 and F155 are shown in a ball-and-stick representation in purple, red and green, respectively. The figure was generated from the data with PDB Accession No. 1MML using BOBSCRIPT.

### 2.3. Homopolymer assay

Typical assays were performed at 37 °C using an appropriate amount of RT in 50  $\mu$ l of reaction containing 60 mM Tris-HCl (pH 8.0), 75 mM NaCl, 0.7 mM MnCl<sub>2</sub>, 5 mM DTT, 12  $\mu$ g/ml poly(rA) template, 6  $\mu$ g/ml oligo(dT)<sub>18</sub> primer, 10  $\mu$ Ci/ml  $\alpha$ -<sup>32</sup>P-dTTP or  $\alpha$ -<sup>32</sup>P-UTP (1 Ci = 37 GBq) and 12  $\mu$ M unlabeled dTTP or UTP. At a desired time point, an aliquot of the reaction was removed and spotted on DE81 paper, followed by washing twice with 2 $\times$  SSC and radioactivity quantification. One unit of RT is defined as the amount of the protein that incorporates 1 nmol of <sup>32</sup>P labeled dTMP in 15 min at 37 °C into a form retained on DE81 paper. Kinetic analyses were performed as previously described [9]. The kinetic parameters were determined by double reciprocal plot.

### 2.4. RNA synthesis

Primer oligonucleotide P21 was end-labeled with  $\gamma$ -<sup>32</sup>P-ATP and annealed to template oligonucleotide T36. The primer (0.1  $\mu$ M) was extended with 3  $\mu$ g of RT at 37 °C in a 60  $\mu$ l reaction using 500  $\mu$ M each ultrapure rNTPs (Amersham). The products were resolved by electrophoresis on 23% urea polyacrylamide gels and detected by autoradiography.

### 2.5. Gel-retardation assay

The radiolabeled T36-P21 template-primer was prepared as described above. The gel-retardation assay was performed as previously described [11]. Briefly, 0.3 pmol of the template-primer was incubated with various amounts of RT at 37 °C in a 10- $\mu$ l reaction containing 60 mM Tris-HCl (pH 8.0), 75 mM NaCl, 7.5 mM MgCl<sub>2</sub>, 5 mM DTT. After 15 min of incubation, 1.5  $\mu$ l of 60% (w/v) sucrose was added to the reaction and the samples were loaded on a 5% acrylamide/0.13% bisacrylamide gel and run at 150 V at 4 °C in the running buffer containing 25 mM Tris-HCl and 162 mM glycine (pH 8.4). The intensities of the free and retarded template-primers were measured by Phosphorimager and plotted. The dissociation constant ( $K_d$ ),

defined as the concentration of the enzyme (or the ratio of E/P-T) at which 50% of the template-primer remains unbound, was determined from the data of four independent experiments and presented as means  $\pm$  S.D.

## 3. Results

### 3.1. Substitution of Q84 of RT-F155V-H with alanine or asparagine improved the enzyme's RNA polymerase activity

It has been assumed that the low catalytic rate for a DNA polymerase to synthesize RNA is caused by the enzyme's difficulty to accommodate the RNA product within the active site [12–14]. Based on the analysis of the crystal structure of MMLV-RT ([3,4] and Fig. 1), we inferred that Q84 might be a residue involved in the enzyme's interaction with the template-primer and speculated that substitution of this bulky residue with smaller ones might improve the enzyme's ability to synthesize RNA. Q84 was substituted with alanine or asparagine in the RT-F155V-H backbone, an MMLV-RT double mutant harboring the F155V substitution to allow rNTPs to bind to the enzyme [9], and the D524N substitution, which abolishes the RNase H activity [10], to avoid potential degradation of RNA products by the RNase H activity. The RNA polymerase activity was assayed by the enzyme's ability to incorporate rUTP using poly(rA)-oligo(dT) as template-primer. Indeed, substitution of Q84 with alanine or asparagine conferred to the enzyme higher RNA polymerase activity (Fig. 2, compare RT-Q84A-F155V-H or RT-Q84N-F155V-H with RT-F155V-H). In contrast, substitution of Q84 with arginine failed to do so (Fig. 2, RT-Q84R-F155V-H). It is worth noting that consistent with the role of F155 as a "steric gate" to preclude rNTP binding to the enzyme, the Q84A substitution alone was not sufficient to confer detectable RNA polymerase activity to the wild type RT (Fig. 2, RT-Q84A-H).

RT-Q84A-F155V-H was chosen for kinetic analysis. While RT-Q84A-F155V-H and RT-F155V-H displayed comparable  $K_m$  values for rUTP, the  $V_{max}$  for rUTP of RT-Q84A-F155V-H was four times the level of RT-F155V-H (Table 1).

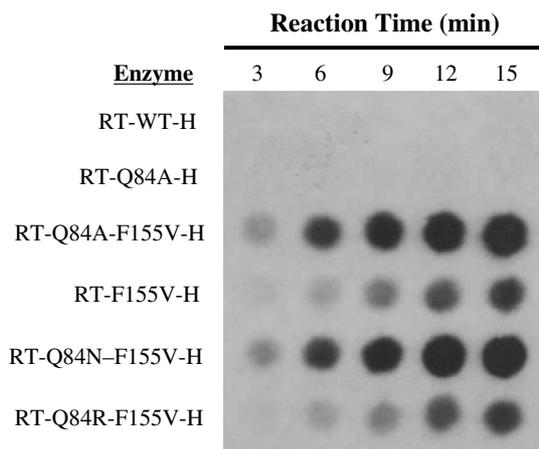


Fig. 2. RNA polymerase activities of the RT enzymes. 800 ng/ml of the indicated enzymes were used to catalyze the incorporation of UMP using poly(rA)-oligo(dT)<sub>18</sub> as template-primer in the presence of  $\alpha$ -<sup>32</sup>P-rUTP. At the indicated time points, an aliquot of the reaction was removed and analyzed by spotting on DE81 paper, washing, and autoradiography.

Table 1  
Kinetic parameters of RTs as RNA polymerases

Enzyme	rUTP	
	$V_{\max}$ (nmol min <sup>-1</sup> g <sup>-1</sup> )	$K_m$ (μM)
RT-F155V-H	2.31 ± 0.30	2.88 ± 0.67
RT-Q84A-F155V-H	9.94 ± 0.90	2.98 ± 0.72

Since the difference in the specific activity between the mutant and wild type enzymes was relatively modest, special care was taken in the preparation of the enzymes. In this report, all the enzymes used for comparison were prepared in parallel and at least three different preparations were analyzed to minimize the possibility that the difference in the enzymatic activities was introduced during the preparations.

### 3.2. RNA synthesis by RT-Q84A-F155V-H

The effect of the Q84A substitution on the enzyme's RNA polymerase activity was further evaluated by analyzing the ability of RT-Q84A-F155V-H to synthesize extended RNA products. Consistent with our previous results [9], RT-F155V-H could synthesize a stretch of RNA of only seven nucleotides even after 120 min reaction (Fig. 3, lane 6). In comparison, RT-Q84A-F155V-H synthesized much longer RNA products after 30 min reaction (Fig. 3, lanes 9–11). Furthermore, the RNA synthesis by RT-Q84A-F155V-H was significantly faster than that by RT-F155V-H (Fig. 3). Compared with the DNA product synthesized using the same template-primer (Fig. 3, lane 12), which is presumably the full-length product, the long RNA product migrated more slowly (Fig. 3). The slower migration of the RNA product could be accounted for by the different migration rates between RNA and DNA products [9], and suggested that the long RNA product is full-length. Nonetheless, due to the lack of appro-

prate molecular weight makers, it is difficult to fully determine the size of the RNA product.

### 3.3. Substitution of Q84 of MMLV-RT with alanine or asparagine improved the enzyme's DNA polymerase activity

The improved RNA polymerase activity of RT-Q84A-F155V-H could be due to either selective improvement of the enzyme's catalytic activity using rNTPs as substrates, or

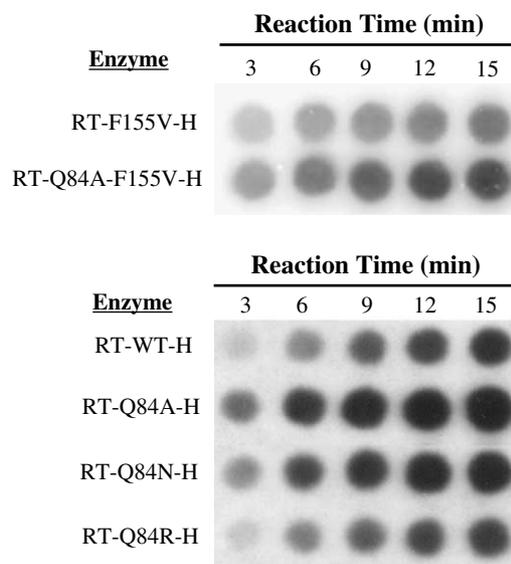


Fig. 4. DNA polymerase activities of the RT enzymes. 200 ng/ml of the indicated enzymes were used to catalyze the incorporation of dTMP using poly(rA)-oligo(dT)<sub>18</sub> as template-primer in the presence of α-<sup>32</sup>P-dTTP. At the indicated time points, an aliquot of the reaction was removed and analyzed by spotting on DE81 paper, washing, and autoradiography.

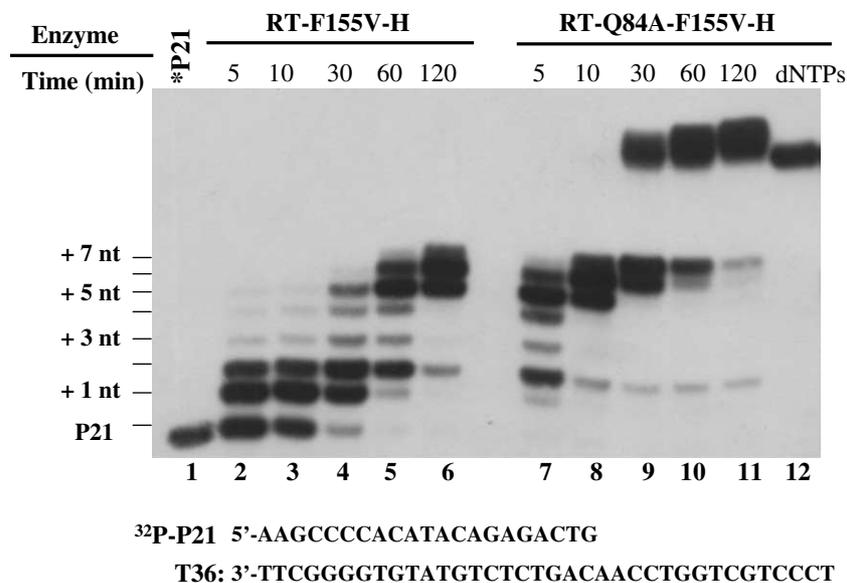


Fig. 3. RNA syntheses by RT-Q84A-F155V-H and RT-F155V-H. The 5' end radiolabeled oligonucleotide P21 was annealed to oligonucleotide T36 to serve as template-primer. Lane 2–11, the P21 primer was extended by RT-Q84A-F155V-H or RT-F155V-H using rNTPs as substrates. At the indicated time points, an aliquot of the reaction was removed and analyzed by gel electrophoresis. Lane 12, the primer was extended by RT-Q84A-F155V-H using dNTPs as substrates.

Table 2  
Kinetic parameters of RTs as DNA polymerases

Enzyme	dTTP		poly(rA)/(dT) <sub>18</sub>	
	$V_{\max}$ ( $\mu\text{mol min}^{-1} \text{mg}^{-1}$ )	$K_m$ ( $\mu\text{M}$ )	$V_{\max}$ ( $\mu\text{mol min}^{-1} \text{mg}^{-1}$ )	$K_m$ (nM)
RT-F155V-H	0.13 $\pm$ 0.04	12.94 $\pm$ 2.08		
RT-Q84A-F155V-H	0.41 $\pm$ 0.04	10.05 $\pm$ 0.72		
RT-WT-H	0.17 $\pm$ 0.02	13.04 $\pm$ 2.71	0.53 $\pm$ 0.10	11.71 $\pm$ 2.31
RT-Q84A-H	0.53 $\pm$ 0.04	11.37 $\pm$ 1.63	0.82 $\pm$ 0.15	5.03 $\pm$ 1.00

generally increased enzymatic activity. To distinguish between these two possibilities, the mutant enzymes were analyzed for their DNA polymerase activities by the homopolymer assay. RT-Q84A-F155V-H did display higher DNA polymerase activity than RT-F155V-H (Fig. 4), suggesting that the Q84A substitution generally improved the enzyme's activity. The substitution of Q84A, Q84N or Q84R was also introduced into RT-WT-H and analyzed for the effect on the enzyme's DNA polymerase activity. RT-Q84A-H and RT-Q84N-H displayed higher DNA polymerase activities than RT-WT-H, but RT-Q84R-H failed to do so (Fig. 4). RT-WT-H and RT-Q84A-H were determined to display 57 and 194 units of DNA polymerase activity per microgram protein, respectively. The specific activity of RT-WT-H is very similar to that reported previously by others [15]. Kinetic analysis revealed that the  $V_{\max}$  of RT-Q84A-H using dTTP as a substrate is about three times that of RT-WT-H, whereas the  $K_m$  values are very similar (Table 2).

### 3.4. The Q84A substitution increased the enzyme's affinity for the template-primer

The position of Q84 in the crystal structure suggested that Q84 might interact with the template. To test whether the

Q84A substitution affected the enzyme's affinity for the template-primer, kinetic analyses were performed to measure the  $K_m$  values of the enzymes for the template-primer using the homopolymer assay. The  $K_m$  of RT-Q84A-H was more than 2-fold lower than RT-WT-H (Table 2), suggesting that the Q84A substitution improved the enzyme's affinity for the template-primer. To substantiate this notion, gel-retardation assays were used to further determine whether RT-Q84A-H and RT-WT-H differ in template-primer binding. As observed by others, two retarded bands were detected (Fig. 5), which were proposed to represent complexes containing one or two RT molecules [11]. The formation of the retarded complexes as a function of the enzyme/template-primer ratio was considered as an indicator of the affinity of the enzyme for the template-primer. At the same ratio of enzyme to template-primer, RT-Q84A-H formed more retarded complexes than RT-WT-H (Fig. 5). The  $K_d$  values were 211  $\pm$  21 nM (or E/P-T: 7.03  $\pm$  0.70) for RT-WT-H and 107  $\pm$  13 nM (or E/P-T: 3.56  $\pm$  0.41) for RT-Q84A-H (see Section 2), further suggesting that the Q84A substitution improved the enzyme's affinity for the template-primer.

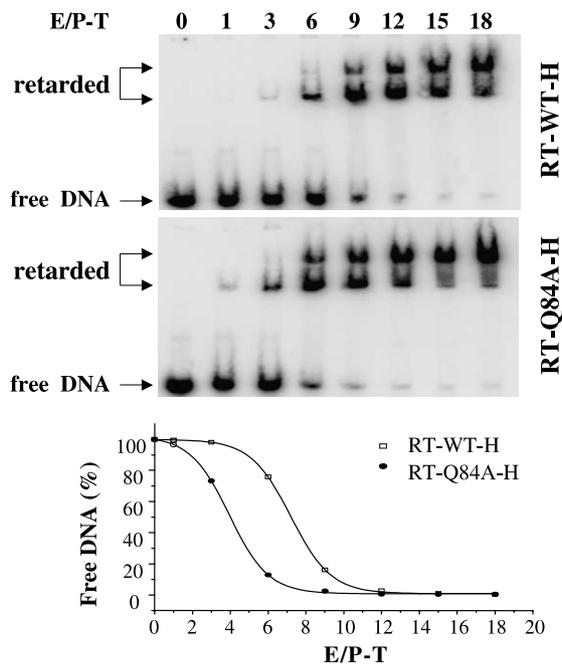


Fig. 5. Gel-retardation assay of complexes formed between RT and template-primer. The radiolabeled template-primer was incubated with the indicated enzymes at various ratios (E/T-P). The resulting complexes were analyzed by electrophoresis on a non-denaturing gel (upper and middle panels). The intensities of free and enzyme-bound template-primers were quantified by Phosphorimager and plotted (lower panel). The data is representative of four independent experiments.

## 4. Discussion

DNA polymerases and RNA polymerases play distinct roles in the life of organisms. It is important for a nucleic acid polymerase to keep its identity as either a DNA polymerase or an RNA polymerase. Substitution of Q84 of MMLV-RT with alanine or asparagine did not affect the enzyme's selectivity for dNTPs over rNTPs. But the substitution conferred to RT-F155V-H higher RNA polymerase activity. In this sense, Q84 of MMLV-RT helps to keep the enzyme's identity as a DNA polymerase.

Analysis of the model of MMLV-RT and the template-primer complex suggests that Q84 might interact with the template. Consistently, our kinetic analysis and gel-retardation assays indicated that the Q84A substitution improved the enzyme's affinity for the template-primer complex. It is conceivable that substitution of Q84 with alanine or asparagines could generate more space for the template-primer complex, resulting in a better fitting of the template-primer in the enzyme's active site. The increased affinity of the enzyme for the template-primer should facilitate processive nucleic acid synthesis.

In HIV-RT, the residue corresponding to Q84 of MMLV-RT is E44. It has been reported that E44D or E44A mutation is associated with HIV-RT drug resistance ([16] and references therein). In the presence of other drug resistance mutations, the E44D mutation affected the enzyme's ability to excise incorporated 3TC-MP [16]. Whether the mutation affects the enzyme's other properties remains to be determined. Nonetheless, since the structure of MMLV-RT is significantly different from the structure of HIV-RT [4,17], it would not be surprising

if the Q84 mutations of MMLV-RT affect the enzyme in a different way than the E44 mutations affect HIV-RT.

It is worth noting that although RT-F155V-Q84A-H synthesized longer RNA products than RT-F155V-H, the pausing sites after addition of 2–7 rNTPs in RNA synthesis by the two enzymes remained similar (Fig. 3). Similar pausing sites were also observed with other DNA polymerase mutants that acquired RNA polymerase activities [9,12,18,19]. One possible explanation is that for the enzyme to accommodate the RNA products containing 2–7 rNTPs, the active site is changed to a position that is not suitable for efficient catalysis. It is also worth noting that once the pausing sites were passed, the primer was extended rapidly to full-length by RT-Q84A-F155V-H, suggesting that the RNA products containing 2–7 rNTPs are the most difficult for the enzyme to accommodate. In addition to conferring to the enzyme higher catalytic activity, the Q84A substitution could help the enzyme to synthesize longer RNA product by accommodating the RNA product better. Further investigation is needed to test this possibility.

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