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Implications of Active Site Constraints on Varied DNA Polymerase Selectivity

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Transfer of genetic information to the offspring relies essentially on the selectivity of DNA polymerases. How these enzymes distinguish faithfully between nucleotide substrates according to the Watson−Crick rules is still not fully understood.[1] Selectivity during nucleotide insertion is believed to be achieved mainly by editing nucleotide shape and size within a tight nucleotide binding pocket.[1] Yet, DNA polymerase selectivity often varies significantly depending on the DNA polymerase.[1f,2,3] The origin of this varying error propensity is elusive. It is assumed that DNA polymerases form nucleotide binding pockets that differ in properties such as shape and tightness.[1f,2a] Thus, high fidelity DNA polymerases are believed to form more rigid binding pockets tolerating less geometric deviation, while low fidelity enzymes exhibit more flexibility leading to decreased fidelity. However, this concept of active site tightness remains to be tested experimentally.

Recently, we have introduced new functional means to investigate the effect of mainly steric constraints on the mechanism of DNA polymerase selectivity.[4] Increasing the bulk of nucleoside triphosphates by substitution of the 4′-hydrogen position of the sugar with alkyl groups that gradually increase in steric demand (see Figure 1A) led to a marked increase in nucleotide insertion selectivity catalyzed by the Klenow fragment (Kf) of *Escherichia coli* DNA polymerase I.[4] These results support the model that steric constraints are at least one crucial determinant of DNA polymerase selectivity.[1] If varied active site tightness is indeed a crucial determinant of varied DNA polymerase selectivity, steric probes such as **T^{R}TP** should reflect this in differential action on various DNA polymerases. Here we report on functional investigations of human immunodeficiency virus type 1 reverse transcriptase (HIV-1 RT), an enzyme known for its error propensity.[5] To gain new insight into the mechanism of selectivity of this polymerase, we combined chemical and genetic means. The results presented differ significantly from those obtained for Kf and give new valuable insight into enzyme properties responsible for variations of DNA polymerase fidelity.

We tested the effect of thymidines **T^{R}TP** on wild-type HIV-1 RT as well as the prominent M184V mutant of the enzyme. Through M184V mutation, a β-methyl side chain present in valine is introduced that is believed to contact the sugar ring of the incoming triphosphate.[6] Interestingly, this mutation has been shown to result in increased nucleotide insertion selectivity which is attributed to increased steric constraints within the active site.[7] Thus, such studies should further elucidate whether differential steric constraints introduced by mutations are involved in processes that cause varying HIV-1 RT selectivity.

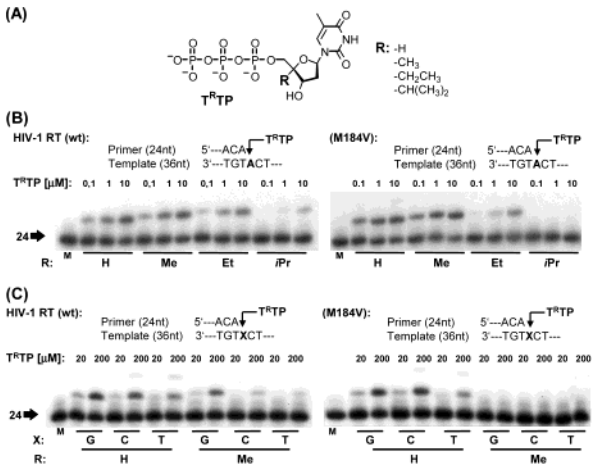

Figure 1. (A) Steric probes used in this study. (B) Nucleotide insertion.[8] (C) Nucleotide misinsertion.[8] Nucleotide concentrations applied are indicated in the figure. M: marker.

First, we qualitatively evaluated the effect of **T^{R}TP** on HIV-1 RTs in canonical base pair formation using a gel-based single nucleotide insertion assay (Figure 1B).[8] The results show that both enzymes are able to insert the steric probes in the nascent DNA strand albeit to a different extent. Both wild-type and M184V HIV-1 RT insert **T^{H}TP** and **T^{Me}TP** with similar efficiency, while insertion of bulkier probes is somewhat hampered. Interestingly, as is already apparent from the qualitative results, **T^{Et}TP** and **T^{iPr}TP** are more efficiently inserted by the wild-type enzyme as compared to the M184V mutant. To verify these observations, we performed steady-state kinetic measurements under single completed hit conditions as described recently.[9] The data obtained suggest little difference in insertion efficiency of unmodified **T^{H}TP** as compared to **T^{Me}TP** by both RTs (Table 1). However, the bulkier probes **T^{Et}TP** and **T^{iPr}TP** are less efficiently inserted by both enzymes, and this effect is more pronounced in the case of the M184V mutant. These observations suggest differential steric constraints within the nucleotide binding pocket, causing a more pronounced steric clash in the mutated enzyme.

Next, we investigated whether size expansion by 4′-alkylation has an impact on the fidelity of nucleotide insertion. Because **T^{H}TP** and **T^{Me}TP** are incorporated by both HIV-1 RTs with comparable efficiency, we focused on the investigation of these two analogues. If steric constraints are a crucial determinant for the mechanism of HIV-1 RT fidelity, one would expect reduced nucleotide insertion efficiency opposite noncanonical nucleotides caused by bulkier sugar residues. Furthermore, these effects should be more pronounced in the case of M184V if active site size reduction is indeed involved in the increase of selectivity observed due to the mutation.

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Table 1. Steady-State Kinetic Analyses of Nucleotide Insertion Opposite Template A and Misinsertion Opposite G, C, and T

	wt	M184V
T^RTP	V_{max}/K_M [M^{-1} min^{-1}][a]	V_{max}/K_M [M^{-1} min^{-1}][a]
opposite A:		
H	1 020 000	830 000
Me	1 500 000	1 500 000
Et	120 000	36 000
^{i}Pr	2000	270
opposite G:		
H	3500	706
Me	2030	38
opposite C:		
H	340	378
Me	77	8
opposite T:		
H	290	310
Me	43	11

[a] Data shown represent averages derived from multiple experiments.[8]

Qualitative experiments already suggested varying effects of the nucleotide modification on RT selectivity depending on the enzyme variant and the mismatch formed (Figure 1C). The steady-state kinetic analysis (Table 1) suggests that 4′-methylation of thymidine triphosphate has little effect on mismatch formation opposite template G when the wild-type enzyme is used. Interestingly, $\mathbf{T^{Me}TP}$ misinsertion opposite C or T occurs approximately 4−7-fold less efficiently as compared to natural $\mathbf{T^HTP}$. The M184V mutation effects mainly $\mathbf{T^HTP}$ misinsertion opposite template G, while insertion of $\mathbf{T^HTP}$ opposite C and T is comparable to the wild-type enzyme. Remarkably, the M184V mutation causes a marked decrease in misinsertion efficiency of $\mathbf{T^{Me}TP}$ opposite all noncanonical template nucleobases as compared to the unmodified nucleotide. For example, incorporation of $\mathbf{T^{Me}TP}$ opposite G is 19 times less likely than incorporation of $\mathbf{T^HTP}$. In the case of misinsertion opposite C and T, the effect is even more pronounced (Table 1).

The results presented are highly interesting in many respects. First, the M184V mutant incorporates the 4′-methylated thymidine $\mathbf{T^{Me}TP}$ with significantly higher fidelity than the wild-type enzyme. This property can be attributed to the additional β-side chain present in valine and absent in methionine, which is believed to point toward the sugar moiety of the incoming triphosphates. Thus, the additional β-methyl group should reduce the size of the nucleotide binding pocket. This size-augmentation is monitored by the increased bulk of the steric probe $\mathbf{T^{Me}TP}$ resulting in significantly lower misinsertion efficiency by the M184V mutant as compared to the natural substrate. Noteworthy, a similar mechanism based on steric hindrance is suggested to be responsible for the observed 3TC resistance of HIV-1 RT caused by M184V mutation.[6] Second, our study shows that most significant effects of 4′-methylation are observed when thymidines are inserted opposite pyrimidines C or T, which cannot be easily explained with a pure steric model.[1] As suggested by Kool, this might be due to water molecules that bind to pyrimidines and render them too large to fit opposite another pyrimidine in the template. Because no new hydrogen bonding can be formed that compensates for their loss, solvation persists and makes pyrimidine pairs effectively too large to be accommodated efficiently by the enzyme.[1d,f] Taken together, these observations further support the steric model for DNA polymerase selectivity.

If varied active site tightness is indeed essential for differential nucleotide insertion selectivity, one would propose HIV-1 RT to process the bulkier thymidines more efficiently than the more selective enzyme Kf. As published, we observed Kf being capable to insert $\mathbf{T^{Me}TP}$ and $\mathbf{T^{Et}TP}$ with high efficiency opposite a canonical template base, while misinsertion opposite noncanonical bases is approximately 100-fold less efficient as compared to the natural substrate $\mathbf{T^HTP}$.[4] Concerning "correct" insertion of the different $\mathbf{T^RTP}$ used here, there is little difference between HIV-1 RT and Kf. However, analyzing misinsertion, the two enzymes behave differently. While 4′-methylation has little effect on the selectivity of HIV-1 RT, significant effects are observed for Kf. Thus, on the basis of the concept of active site tightness, our results suggest that both enzymes most significantly differ when promoting misinsertion rather than insertion opposite canonical template bases. This might be the result of differential active site conformations causing different steric constraints while promoting "incorrect" nucleotide insertion.

In conclusion, we gained new valuable insight into the mechanism of DNA polymerase fidelity. The presented study provides experimental evidence that variations of steric constraints within the nucleotide binding pocket of at least two DNA polymerases cause differences in nucleotide incorporation selectivity. Thus, our results support the concept of active site tightness as a causative of differential fidelity among DNA polymerases.

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Supporting Information Available: DNA sequences, assay conditions, details of kinetic analyses (PDF). This material is available free of charge via the Internet at http://pubs.acs.org.

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