HIV RT strand transfer and nontemplated base addition are mechanistically independent

Joanna Luczkowiak*, Tania Matamoros & Luis Menéndez-Arias
Centro de Biología Molecular "Severo Ochoa" (CSIC-UAM), Madrid, Spain
jluczkowiak@cbm.csic.es

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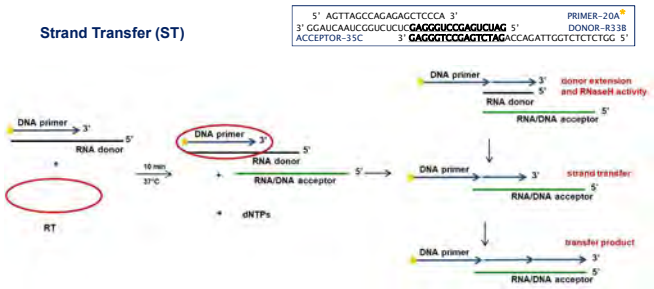
Background

Reverse transcription is an essential step in the life cycle of retroviruses. This complex process is catalyzed by the retroviral reverse transcriptase (RT), possessing RNA- and DNA-dependent DNA polymerase and RNase H activities. The combination between these two activities during reverse transcription process produces strand transfers (ST). ST events occur twice during HIV reverse transcription, the first during minus-strand DNA synthesis and the second, when the plus-strand DNA is polymerized. Both events rely on RNase H cleavage and template homology. Previous studies have shown that template switching is favored by short unpaired 3´-end tails in the DNA, suggesting that RT's nontemplated nucleotide addition (NTA) activity could promote ST. We used *in vitro* ST and NTA assays and a panel of HIV-1 RTs to determine whether increased ST associates with high NTA activity. Moreover, we have studied the binding affinity constants (K_d) for all RTs and its possible correlation with ST and NTA.

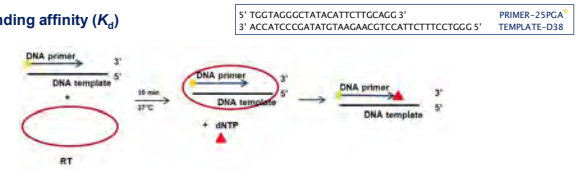
Methods

Tested enzymes were the wild-type (WT) HIV-1_{BH10} RT and mutant L92P; and the WT HIV-1_{ESP49} (group O) RT and ten mutants containing the connection subdomain substitutions K358R/A359G/S360A (3M) alone, or in combination with F61A, K65R/E478Q, L92P, L92P/D443N, L92P/E478Q, V148I, T355A/Q357M, D443N or E478Q. RTs were expressed and purified by ionic exchange followed by affinity chromatography. ST activity was assessed *in vitro* with three synthetic oligonucleotides, including a radiolabeled DNA primer, a RNA donor, and partially homologous DNA or RNA acceptors. Nucleic acid binding affinity constants (K_d) were obtained with ^{32}P-labeled DNA/DNA template-primer (T/P). NTA activity was measured with ^{32}P-labeled blunt-ended DNA/DNA T/P. ^{32}P-labelling was indicated in the scheme with yellow asterisks.

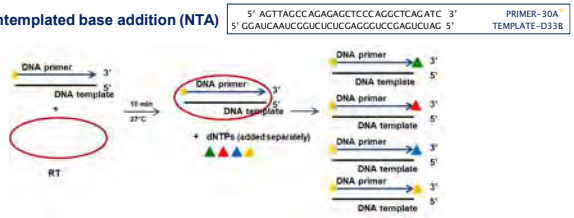
Strand Transfer (ST)



Binding affinity (K_d)



Nontemplated base addition (NTA)



Results

RTs containing RNase H-inactivating mutations (e.g. all those having the substitutions D443N or E478Q) were devoid of ST activity, while mutants containing F61A or L92P had very low ST activity (<7.5%). RTs with the highest ST efficiencies were 3M_T355A/Q357M (34.9%), 3M_V148I (29.3%), 3M (28.1%), WT HIV-1_{ESP49} (13.9%) and WT HIV-1_{BH10} (8.5%) (Table 1, Fig. 1).

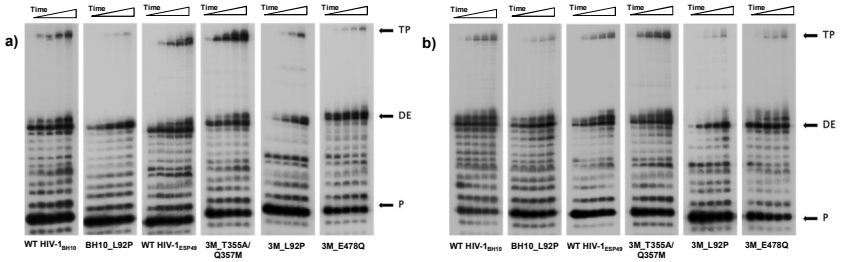


Figure 1. ST activity of HIV-1 RT mutants. Tested enzymes shown in the gel were: WT HIV-1_{BH10} RT, HIV-1_{BH10} RT mutant L92P, WT HIV-1_{ESP49} (group O) RT, and its mutants 3M_T355A/Q357M, 3M_L92P and 3M_E478Q. ST activity was determined with the 5' ^{32}P-radiolabeled DNA primer 20A, a RNA donor 33B, and partially homologous DNA and RNA acceptors (35C and 35D, respectively). Aliquots were taken at 1, 5, 15, 30 and 60 min and visualized on a 20% denaturing polyacrylamide electrophoresis gel. **a)** ST activity assays carried out with a DNA acceptor (35C); **b)** ST activity assays carried out with a RNA acceptor (35D). Arrows indicate the positions of the transfer product (TP); the donor extension product (DE) and the unextended primer (P).

All RTs except those having L92P showed NTA activity, and their best substrate was dATP, followed by dGTP, dCTP and dTTP (Table 1). RTs with the highest NTA activity were those of WT HIV-1_{BH10} (k_{obs}= 0.227 min^{-1}), WT HIV-1_{ESP49} (k_{obs}= 0.216 min^{-1}), and mutants 3M (k_{obs}= 0.198 min^{-1}), 3M_T355A/Q357M (k_{obs}= 0.214 min^{-1}), 3M_D443N (k_{obs}= 0.130 min^{-1}) and 3M_E478Q (k_{obs}= 0.203 min^{-1}).

RT	ST $_{60\ min}$ (%)	*Kd* (nM)	k_{obs} min^{-1} dATP	dGTP	dCTP	dTTP	ATP
WT HIV-1_{BH10}	8.5 ± 0.9	6.2 ± 2.3	0.227 ± 0.1	(1.8 ± 0.1) x 10^{-2}	(8.5 ± 3.0) x 10^{-4}	(3.7 ± 0.1) x 10^{-3}	0
BH10_L92P	1.4 ± 0.3	18.6 ± 6.7	(5.0 ± 2.0) x 10^{-4}	0	0	0	0
WT HIV-1_{ESP49}	13.9 ± 1.4	4.6 ± 0.5	0.216 ± 0.1	(6.1 ± 0.4) x 10^{-3}	(1.2 ± 0.2) x 10^{-3}	(1.4 ± 0.1) x 10^{-3}	(4.3 ± 0.6) x 10^{-4}
3M	28.1 ± 2.6	1.5 ± 0.6	0.198 ± 0.1	(1.7 ± 0.1) x 10^{-2}	(4.0 ± 0.3) x 10^{-3}	(3.7 ± 0.2) x 10^{-3}	(4.4 ± 0.6) x 10^{-4}
3M_T355A/Q357M	34.9 ± 2.1	2.0 ± 0.4	0.214 ± 0.1	(1.9 ± 0.1) x 10^{-2}	(1.6 ± 0.1) x 10^{-2}	(5.0 ± 0.1) x 10^{-3}	(1.3 ± 0.1) x 10^{-3}
3M_L92P	7.5 ± 1.2	4.6 ± 1.5	(3.2 ± 0.3) x 10^{-3}	0	0	0	0
3M_D443N	1.2 ± 0.2	1.6 ± 0.2	0.130 ± 0.1	(9.0 ± 0.3) x 10^{-3}	(8.5 ± 0.2) x 10^{-3}	(2.7 ± 0.1) x 10^{-3}	(1.0 ± 0.1) x 10^{-3}
3M_L92P/D443N	0	5.3 ± 2.6	(1.0 ± 0.1) x 10^{-3}	0	0	0	0
3M_E478Q	4.2 ± 0.7	1.6 ± 0.7	0.203 ± 0.18	(1.2 ± 0.1) x 10^{-2}	(1.0 ± 0.1) x 10^{-2}	(4.0 ± 0.1) x 10^{-3}	(1.8 ± 0.1) x 10^{-3}
3M_L92P/E478Q	1.5 ± 0.2	6.0 ± 2.1	0	0	0	0	0
3M_K65R/E478Q	2.2 ± 0.3	7.2 ± 1.9	(1.2 ± 0.1) x 10^{-2}	(9.4 ± 1.1) x 10^{-4}	0	(9.5 ± 2.4) x 10^{-5}	0
3M_F61A	3.8 ± 0.7	8.6 ± 1.2	(3.3 ± 0.1) x 10^{-2}	(5.1 ± 0.2) x 10^{-3}	(1.2 ± 0.3) x 10^{-4}	(1.7 ± 0.1) x 10^{-3}	0
3M_V148I	29.3 ± 2.7	3.5 ± 0.8	(1.3 ± 0.1) x 10^{-2}	(2.1 ± 0.1) x 10^{-3}	(4.7 ± 0.6) x 10^{-4}	(1.1 ± 0.2) x 10^{-3}	0

Table 1. Summary of ST activities, K_d values and NTA for all tested HIV-1 RTs. ST activity (%) for all RTs at 60 min was assessed with 20A*/R33B and a DNA acceptor 35C. K_d values for RT mutants were determined with 25PGA*/D38 and calculated by fitting the data to a quadratic equation (Menéndez-Arias, 1998). NTA was assessed by using a blunt-ended 30A*/D33B T/P. The velocity of the NTA incorporation (k_{obs}) for dATP was determined by fitting the data to one-phase exponential association equation. dGTP, dCTP and dTTP were too slow to be calculated by this equation, therefore in these cases the data were fit using a linear regression model. ST and NTA activity values are given as a mean value ± SEM. K_d values are presented as a mean value ± SD. All statistical analysis were performed using GraphPad Prism v6.

The ST efficiency of RTs showing a functional RNase H activity correlated negatively with their T/P binding affinity (p = 0.039*) (Fig. 2).

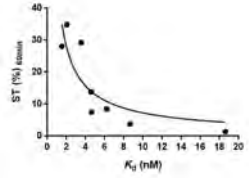


Figure 2. Correlation between overall ST activity and K_d (Table 1) for WT and mutant HIV-1 RTs. RTs containing RNase H-inactivating mutations (all those having D443N or E478Q) were devoid of ST activity and were therefore excluded from the analysis. ST efficiencies were measured at 60 min incubation time. Correlation between ST activity and K_d were calculated by Pearson r test using GraphPad Prism v6.

The 3M_V148I RT had high ST activity but low NTA activity (k_{obs}= 1.3 x 10^{-2} min^{-1}). In contrast, WT HIV-1_{BH10} RT incorporated dATP efficiently on blunt-ended T/P (k_{obs}= 0.227 min^{-1}) (Table 1, Fig. 3), but had relatively low ST activity. Overall, we found no correlation between ST and NTA activities of tested RTs (as determined with a Pearson r test).

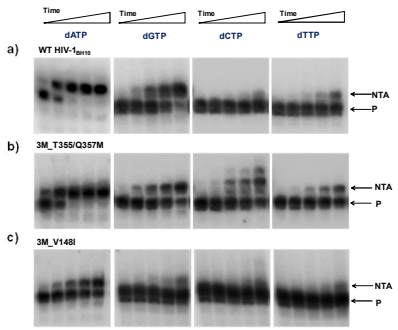


Figure 3. NTA of dATP, dGTP, dCTP and dTTP by HIV-1 RT mutants. **a)** WT HIV-1_{BH10} RT; **b)** 3M_T355A/Q357M RT; and **c)** 3M_V148I RT. NTA activity was obtained by using a blunt-ended 30A*/D33B T/P. NTA activity for RT mutants was measured at 1, 5, 15, 30, 60 min and visualized on 20% denaturing polyacrylamide gel electrophoresis. NTA, nontemplated base addition; and P, unextended primer are indicated with arrows.

Conclusions

Our results demonstrate that ST is efficiently promoted by the high affinity of RTs for T/P binding. We found that ST and NTA are mechanistically independent, meaning that for a specific RT, high NTA activity is not necessarily associated with increased template switching. These data are expected to be helpful to understand HIV variability at putative recombination sites, while providing further insight towards the characterization of ST as a distinct target for antiretroviral intervention.

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