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RNA Probes of Steric Effects in Active Sites: High Flexibility of HIV-1 Reverse Transcriptase

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As part of the replication of the HIV-1 virus, the HIV reverse transcriptase enzyme (HIV-RT) makes a DNA copy of the existing HIV-1 RNA single-stranded genome. The enzyme has been widely studied because of its biomedical importance as a target for drugs that treat AIDS.[1] It is a highly mutagenic polymerase, a property that leads to rapid development of drug resistance during treatment of the disease.[2] It has been proposed that one could make use of this property by employing nucleotide analogues that increase the mutagenicity beyond which the virus can tolerate.[3] Study of the origins of mutagenicity in this enzyme could contribute new information on strategies for treatment of this disease, as well as basic understanding of the mechanisms of replication and evolution.

Recent studies have suggested that polymerase enzymes can use steric mechanisms to enforce high fidelity of DNA base pairing during DNA synthesis.[4] In such mechanisms, polymerases tightly surround the incipient base pair, using rigid structure to enforce correct base pair size and shape. Conversely, enzymes that process DNA with low selectivity might present a sterically more flexible active site.[5,6] Although recent studies have begun to assess the rigidity of DNA polymerases,[6] no such studies have been directed to RNA-templated DNA synthesis as carried out by reverse transcriptases. Here we describe the development of a set of ribonucleoside analogues as tools for probing RNA steric effects in sub-angstrom increments and their application in systematic studies of the active site flexibility of HIV-RT. We report that this enzyme is markedly more flexible than other nucleic-acid-synthesizing enzymes studied to date.

The variably sized RNA probe nucleosides (Figure 1) were designed to be shaped similarly to uridine but to have increasing size over a small 0.8 Å range. The analogues rH and rF were reported previously,[7,8] and we prepared rL and rB to complete the variable set (see Supporting Information (SI) for experimental details). We attempted to prepare an analogous diiodo-substituted rI nucleoside from the dibromo case using a procedure analogous to that used in preparation of a related diiodo deoxynucleoside analogue.[9] However, varied attempts to use a copper-catalyzed halogen-exchange reaction[10] resulted in conversion of a single aromatic bromine to iodine, while the other bromine was unreactive. This led us to limit the RNA probe substituent variation to the series H < F < Cl < Br. The 5′-OH groups of the nucleoside analogues were protected using dimethoxytrityl chloride, and the 2′-OH groups were protected using TOM or TBDMS groups. Subsequently, 3′-*O*-phosphoramidite derivatives of the series were prepared for use in the automated solid-phase synthesis of RNAs. Mass spectrometry data confirmed intact incorporation of the new nucleoside analogues into RNA oligomers.

To test the functional flexibility of the HIV-1 reverse transcriptase, we prepared 28mer RNA template strands containing the set of analogues rH, rF, rL, and rB and measured their ability to be reverse transcribed into DNA strands. Steady-state single nucleotide incorporation and single nucleotide extension experiments were

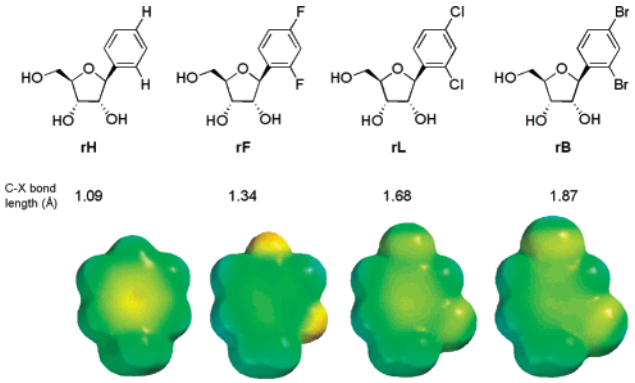

Figure 1. Structures of nonpolar uridine analogues having gradually increased size. Space-filling models of bases (with methyl in place of ribose) are shown below, with electrostatic potentials mapped on the surfaces (PM3 calculations, Spartan, Wavefunction Inc.).

performed with a 28mer/23mer or 28mer/24mer template-primer duplexes, respectively (see SI). For incorporation experiments, the unnatural base was positioned in the template to pair with an incoming natural dNTP. Radiolabeled products of single nucleotide addition were resolved from unreacted primer by denaturing polyacrylamide gel electrophoresis, and the amounts of incorporation were quantified by autoradiography. The kinetics data are given in detail in Tables S1 and S2 (SI) and are shown graphically in Figures 2 and 3. For comparison, we show data with a related DNA steric series for a previously studied DNA polymerase;[6a] the current study is the first with an RNA-dependent enzyme, and it is of fundamental interest to compare the RNA-dependent response with the available literature data for other classes of polymerases having varied flexibility and varied fidelity.

The experiments involving HIV-RT with RNA templates showed much lower size dependence than previous experiments with DNA polymerases. All of the uracil analogues selectively directed preferential incorporation of dATP over the other nucleoside triphosphates. As for the effect of size, the smallest analogue, rH, was less well tolerated (by a factor of 35) than other analogues in the template (Figure 2A), but there was otherwise little or no difference in dATP incorporation efficiencies opposite analogues ranging from the difluoro, dichloro, to dibromo. This is in marked contrast to high-fidelity DNA polymerases.[6] For example, in previous studies, the Klenow fragment of *E. coli* DNA Pol I (Kf) displayed a clear preference for a dichloro-substituted analogue of thymine (Figure 2B) and showed a much larger 182-fold drop in efficiency for decreasing size to the dihydrogen case.[6a] Similarly, while HIV-RT here exhibits no drop in efficiency on increasing size beyond the difluoro or dichloro cases, Kf showed a 14-fold decrease from dichloro to dibromo.

Remarkably, the sensitivity to size increases for HIV-RT measured here is even less than that of a low-fidelity repair polymerase, Dpo4.[5] The latter enzyme showed a 6-fold drop in

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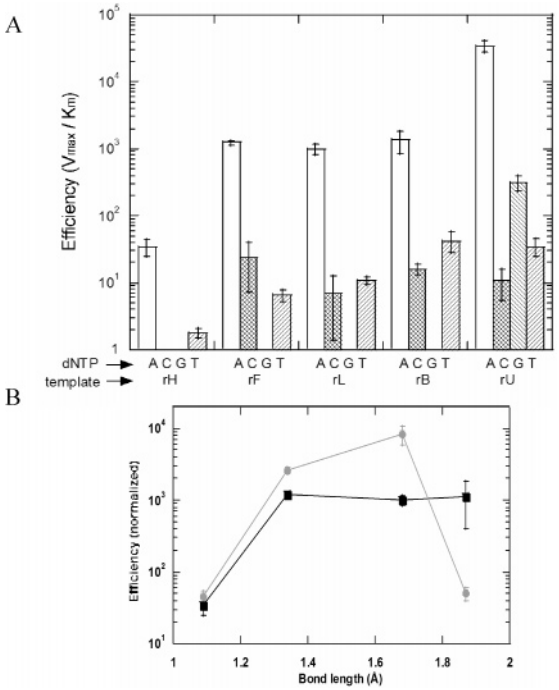

Figure 2. Flexibility of HIV-RT, as shown by effects of varying RNA template base size on nucleotide incorporation. Data are steady-state efficiencies for insertion of natural dNTPs opposite unnatural bases in the template, with natural U data shown for comparison. Note log scales for efficiency; empty columns indicate no observable incorporation. (A) Plot showing incorporation efficiencies of the four nucleoside triphosphates opposite the size-varied RNA series. (B) Comparison of varying base size data for HIV-RT (black) and the Klenow fragment of DNA Pol I (Kf; gray).[6a] The latter is a higher efficiency enzyme; for ease of comparison, we divided its efficiencies by 10^3.

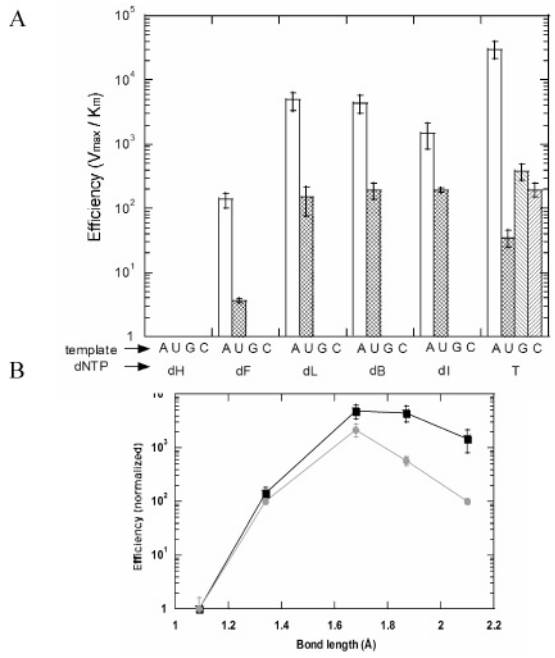

Figure 3. Effects of incoming nucleotide base size on base pair synthesis by HIV-RT. Data are steady-state efficiencies for insertion of size-varied dNTP analogues opposite natural RNA bases. (A) Incorporation efficiencies of the size-varied nucleoside triphosphates. (B) Comparison of the effect of varying dNTP base size on efficiencies of HIV-RT (black) and the Klenow fragment of DNA Pol I (gray).[6a]

efficiency on increasing size from dichlorotoluene to a dibromo analogue, whereas HIV-RT shows no change within experimental error. Thus the data are consistent with the HIV-RT active site for incipient pair formation being sterically quite flexible as compared with other polymerase enzymes. We suggest that this flexibility is likely to be a strong contributor to the mutagenic properties of this enzyme (and thus of the HIV virus as a whole).

To test whether this flexibility is observed on both sides of a base pair during RNA-to-DNA reverse transcription, we carried out similar experiments with a set of variably sized incoming deoxynucleoside triphosphates, using natural RNA bases in the template RNA. The dNTP series contains bases (dH, dF, dL, dB, dI) analogous to the RNA base analogues described here.[6a,9] Steady-state kinetics data (see SI) are graphed in Figure 3. The results again showed remarkable tolerance to increasing base size. For example, increasing size from dichloro to diiodo gives a drop of only 3.3-fold in efficiency (Figure 3), whereas the Kf DNA polymerase gave a 22-fold drop for the same size increase. Moreover, fidelity of HIV-RT pairing of U(T) analogues with A (relative to mispairings with U,C,G) was relatively low for the analogue series (see Figures 2A and 3), whereas it is an order of magnitude higher with the Kf enzyme.[6a] Our results suggest that the mutagenicity and flexibility of the enzyme are directly related.

In summary, we report the development of a set of variably sized uridine analogues, and we have used them to show that the HIV reverse transcriptase is an unusually flexible polymerase enzyme. Interestingly, the RNA template-dependent synthesis by this enzyme is known to proceed with lower fidelity than the DNA-dependent activity;[11] thus it would be of interest in the future to compare steric responses of these two distinct activities that proceed in the same active site. In general, the development of a set of systematically varied nucleotide analogues that retain biological activity may be broadly useful in probing steric effects in varied subfields of RNA biology.

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Supporting Information Available: Details of nucleoside synthesis, RNA synthesis, and enzyme kinetics. This material is available free of charge via the Internet at http://pubs.acs.org.

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