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INHIBITORS OF HIV-1 REVERSE TRANSCRIPTASE AND FIDELITY OF *IN VITRO* DNA REPLICATION

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Mechanisms of the effects of the dTTP analogues 3′-azido-3′-deoxythymidine 5′-triphosphate (AZTTP) and 3′-amino-3′-deoxythymidine 5′-triphosphate (NH_2TTP) upon the HIV-1 reverse transcriptase (RT) are discussed. These compounds block the RT *in vitro* and do so by different kinetic mechanisms. Infidelity of replication is a hallmark of the HIV-1 RT, and replication errors by the enzyme on RNA and DNA templates are discussed. The enzyme's infidelity has ramifications for inhibition: On the one hand, the propensity to produce mutations enhances the ability of the virus to escape inhibitors whereas on the other hand, the infidelity of the reverse transcriptase may allow the development of imaginative inhibitor strategies.

KEY WORDS: HIV-1, reverse transcriptase, enzyme inhibition, DNA replication fidelity, frameshift mutation, processivity.

1. INTRODUCTION

Two biological issues have formed the basis for intense investigation of the reverse transcriptase (RT) of human immunodeficiency virus, type 1 (HIV-1). The first issue lies with the fact that the reverse transcriptase is a target for drug therapy against HIV.[1,2] An approach in this regard has been to identify fundamental characteristics of the enzyme, with the idea that such information may facilitate rational drug design. We describe here work to identify the kinetic features of the HIV reverse transcriptase, including studies with various substrate inhibitors such as AZTTP and the primer analogue $Sd(C)_{28}$.

The second biological issue lies with the observation that HIV shows considerable genetic diversity (for review, see references[3]), and appears to have a dynamic genome in the sense that sequences rapidly mutate after introduction of a clonal isolate of HIV proviral DNA into cells. The implication of this is that HIV can rapidly adapt to escape drugs or antibodies targeted to specific viron components. One wishes to know if the reverse transcriptase could provide a source of this genome diversity or dynamics through error-prone replication. We will describe the work which suggests a mechanism by which the HIV reverse transcriptase could produce errors. This article focuses on work in this laboratory, and is not intended as a comprehensive review.

2. KINETIC CHARACTERISTICS OF HIV-1 REVERSE TRANSCRIPTASE

The availibility of highly purified HIV-1 reverse transcriptase[4] allowed detailed

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mechanistic studies of the enzyme, and initial experiments attempted to elucidate a steady-state kinetic mechanism for polymerization by the HIV reverse transcriptase. A system with poly (rA) as template, oligo (dT), and dTTP as substrate was employed. The steady-state kinetic approach could be readily applied to this enzyme, and substrate initial velocity studies combined with product inhibition studies using pyrophosphate suggested an ordered reaction mechanism with free enzyme binding first to template-primer.[5-7]

DNA synthesis by the HIV-1 RT also was examined for processivity. Products of synthesis were separated by electrophoresis and visualized by autoradiography. We examined chain lengths of labeled DNA products formed during a short incubation period (200 s) at a high ratio of template-primer to enzyme. These conditions were chosen so that each product molecule would represent just one cycle of enzyme binding, synthesis, and termination. Under these conditions, a chain elongation rate at 37°C of approximately 2-4 nucleotide/s/enzyme was determined, which was consistent with data obtained for the steady-state rate from initial rate measurements. We found that HIV reverse transcriptase conducts processive DNA synthesis, but exhibits some probabillity of terminating synthesis after each dTMP additon to the nascent chain. After the third and subsequent dTMP additions, the amount of termination was essentially constant and was equal to a termination probability of ~ 0.01, or one termination for every 100 nascent product molecules at any one chain length. Termination after incorporation of the first dTMP residue was $\sim$ 20-fold higher. This observation of higher termination after the first incorporation event supports the idea that the initiation of synthesis may be kinetically distinct from subsequent elongation.

Further studies revealed that $d(C)_{28}$ is a linear competitive inhibitor of DNA synthesis by the HIV reverse transcriptase, against poly r(A) · oligo d(T) as template-primer, indicating that $d(C)_{28}$ and the template-primer combine with the same form of the enzyme in the reaction scheme, the free enzyme. The phosphorothioate oligodeoxynucleotide $Sd(C)_{28}$ also is a linear competitive inhibitor against template-primer, but the K_i for inhibition (~ 2.8 nM) was $\sim$200-fold lower than the K_i for inhibition by $d(C)_{28}$. Substrate kinetic studies of DNA synthesis by the HIV reverse transcriptase using $Sd(C)_{28}$ as primer and poly r(I) as template revealed that K_m for the phosphorothioate primer was 24 nM.[8]

Annealing the phosphorothioate primer to poly r(I) template yielded the same inhibition of the HIV reverse transcriptase as the inhibition with primer alone, whereas poly r(I) alone caused no inhibition of DNA synthesis on a poly r(A) · oligo d(T) template-primer. Thus, enzyme binds the model primer as tightly as it binds a complex of the same primer annealed to template. The interpretation in the poly r(I) · $Sd(C)_{28}$ system is that the template-primer recognition process is controlled by primer binding to free enzyme. The template clearly directs association of the nucleotide substrate, but free enzyme can recognize the primer about as well as the primer · template complex.[8]

Various phosphorothioate oligonucleotides are being evaluated as potential antiviral agents of acquired immunodeficiency syndrome.[9,10] Antiviral effects of phosphorothioate oligodeoxynucleotides have been found and can be placed in two general categories: oligonucleotide sequence dependent and sequence independent.[10] A proposed mechanism for sequence-dependent antiviral activity involves "antisense" hybridization to viral mRNA. Based on the findings described above with the $Sd(C)_{28}$ primer, a plausible mechanism for the sequence independent activity could be reverse transcriptase-phosphorothioate oligonucleotide interaction. We note that this primer

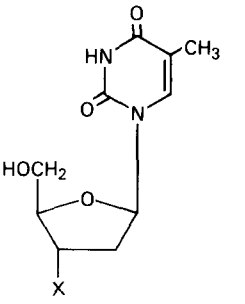

FIGURE 1 Structure of AZT and NH_2T. These thymidine analogues differ only at the position indicated by X. For 3′-azido-3′-deoxythymidine (AZT), X = N_3; for 3′-amino-3′-deoxythymidine (NH_2T), X = NH_2.

analogue ($Sd(C)_{28}$) also inhibited the activity of cellular DNA polymerases α and γ, with K_i values similar to that observed against the HIV reverse transcriptase.[8] Therefore, phosphorothioate oligonucleotides are potentially toxic for the host cell, although such toxicity does not appear to be strong.[10]

Subsequent studies of the HIV reverse transcriptase were carried with dNTP analogues, 3′-azido-dTTP (AZTTP) and 3′-amino-dTTP (NH_2TTP). The nucleoside structures of these compounds are indicated in Figure 1. In the model DNA synthesis system with poly r(A) · oligo d(T) as template-primer, AZTTP was a strong inhibitor of the HIV-1 reverse transcriptase. This has been known for some time and had been shown for a number of HIV-1 RT preparations. Double-reciprocal plots were linear at each fixed concentration of AZTTP in the range 10–60 nM, and the patterns converge to the same point on the ordinate. The replot of slope with each fixed concentration of AZTTP also was linear. These results indicate that AZTTP blocks enzymatic activity through competitive inhibition with the normal substrate (dTTP) for binding to the primer-enzyme complex.[11] The K_i value for AZTTP inhibition, 20 nM, was considerably lower than the K_m for dTTP incorporation, 2–3 μM.[5]

Kinetic studies with AZTTP as substrate revealed a K_m for incorporation of 2.9 μM, and a k_{cat} of 0.27 s^{-1}. That the K_i inhibition (20 nM) is 2 orders of magnitude less than K_m may indicate that binding of AZTTP to enzyme-template complex is a more important factor in interfering with normal dTTP incorporation than actual AZTMP incorporation and chain termination.[11] Nonetheless, Heidenreich *et al.*[7] found that once incorporated into a growing nascent chain, the AZTMP terminated template-primer complex is a potent dead-end inhibitor of DNA synthesis by the HIV-1 RT. Müller *et al.*[12] (1991) postulated three effects by which chain terminators might act to inhibit reverse transcriptases: First, there is direct competitive inhibition with incorporation of the natural nucleotides. This effect could be a consequence of the tight binding of AZTTP to the enzyme-template-primer complex. Second, chain termination leads to removal of potential incorporation sites. Third, the pool of enzyme available for polymerization will be decreased by formation of stable complexes between chain-terminated primer-template species, since these dissociate very slowly; this last mechanism is equivalent to the formation of a dead-end complex.

Other workers have examined inhibition and kinetic constants for AZTTP incorporation. Huang *et al.*[13] analyzed incorporation reactions on a DNA template using sequencing gels and concluded that AZTTP competed with dTTP as substrate for

HIV-1 RT. Reardon and Miller[14] found on a poly (rA) · oligo dT system k_{cat} of $0.06\,s^{-1}$ and K_m of 220 nM for AZTTP incorporation. Heidenreich *et al.*[7] found for incorporation of AZTTP on the same template-primer system a K_m of 3.0 μM and a V_{max} about 50-fold lower than that with dTTP, which would correspond to a k_{cat} of approximately $0.08\,s^{-1}$. Thus, k_{cat} values from several groups are consistent within about a factor of 4, and our results for K_m are consistent with those of Heidenreich *et al.*[7]; we cannot explain the discrepancy in the K_m value reported by Reardon and Miller,[14] other than to point out the possibility of differences between the enzyme preparations.

A kinetic study was also conducted on the inhibition by the analogue NH_2TTP. In this case, a primary double-reciprocal plot revealed that the pattern of inhibition was noncompetitive, since the lines at different fixed concentrations of inhibitor extrapolated to different points on the ordinate. The replots of slopes and intercepts were linear also and indiated linear noncompetitive inhibition with a K_i value of 42 nM. Taken together, these results with AZTTP and NH_2TTP indicate that the two analogues block DNA synthesis by HIV reverse transcriptase by interacting with different forms of the enzyme. This result was rather unexpected since the two analogues are closely related in chemical composition. To confirm this observation, a "mixed inhibition" experiment was conducted. The presence of 10 nM NH_2TTP increased the slope for AZTTP inhibition, indicating again that the two substrates act on different enzyme forms.[11]

Examination of products of DNA synthesis in the presence of each inhibitor also showed differential effects. As AZTTP concentration increased, the size distribution of products shifted toward smaller lengths, such that the average length of product molecules decreased. With increasing concentrations of NH_2TTP, the overall size distribution of products did not change, but the number of product molecules at each length decreased. These results are consistent with different modes of inhibition by the two analogues. Studies of NH_2TTP as substrate showed a K_m of 2.3 μM, similar to that of AZTTP, but the steady-state rate of incorporation of AZTTP was about 4-fold higher than the rate with NH_2TTP.[11]

In the absence of data on the metabolic fate of AZT, including conversion to NH_2T, the therapeutic implications of our findings are not clear. This is especially important to consider since Larder *et al.*[15] recently reported HIV clinical isolates resistant to AZT but still sensitive to other reverse transcriptase inhibitors, including presumed chain terminators. Although *in vitro* mutagenesis studies have produced reverse transcriptases that were more resistant to AZTTP than wild type,[16] purified RT from these AZT-resistant HIV isolates surprisingly failed to show a significant difference in AZTTP sensitivity.[15,17] Such results would be consistent with a situation where an AZT "metabolite" (not AZTTP), acting by a different mechanism from AZTTP, was important for the inhibition of viral replication in cells. A clue to further experimental approaches toward elucidating this metabolite recently came from the work of Tan *et al.*[18] These investigators found that AZTMP, a metabolite of AZT in cells, is a potent inhibitor of the RNase H activity of HIV-1 RT. This activity, like the polymerase activity, is required for virion replication competence.

These results from clinical experiments, and biochemical findings with the various dNTP analogues, provide at least a suggestion that combined therapy with agents that act upon different forms of the HIV reverse transcriptase occurring during its reaction pathway may be useful, and AZTTP and NH_2TTP may be considered prototypes for such an approach. Unfortunately, NH_2TTP may not seem suitable as a practical

therapeutic candidate since the triphosphate also inhibits cellular DNA polymerase α, and NH_2T is strongly toxic to cells in culture (for discussion, see reference[11]).

3. FIDELITY OF HIV-1 REVERSE TRANSCRIPTASE

3.1. *Infidelity During RNA Template-Directed DNA Synthesis*

In addition to kinetic characteristics of the HIV-1 RT on the poly rA · oligo dT system with correct dNTP substrate, we also investigated misincorporation by the enzyme in this system. We found that misincorporation could be induced with the reverse transcriptases of HIV and avian myeloblastosis virus (AMV) by omitting the correct substrate dTTP, and adding high concentrations (1 mM) of individual incorrect substrates to the reaction mixture. In the presence of dTTP, both reverse transcriptases show highly processive DNA synthesis, and both enzymes failed to extend primer when dATP was the only triphosphate substrate. When 1 mM dCTP was substrate, the enzymes extended the primer, but processivity was markedly reduced; products represented 10 nucleotide additions or fewer.[19]

When dGTP was the substrate, both reverse transcriptases showed activity, suggesting that they tolerate G:A mispairs and incorporate dGMP. With AMV RT, the modal distribution of products corresponded to only about 8–10 nucleotide additions. With the HIV enzyme, however, product molecules were extended by 100 nucleotides or greater, approaching the size of products formed with the normal substrate, dTTP. Analysis of the product molecules following nuclease digestion indicated that dGMP had been incorporated; any contamination by dTMP represented less than 2 percent of incorporated product. We do not see incorporation of dGTP in the absence of template, indicating that HIV-1 RT does not show characteristics of terminal deoxynucleotidyltransferase. The most abundant single product size with dGTP as substrate was primer + 1, indicating that the phenomenon of abortive inititation, or higher probability of terminating synthesis after incorporation of the first nucleotide, occurs with either dTTP or dGTP as substrate. High dGMP misincorporation by HIV-1 RT is still seen even when dTTP is added as substrate; with both triphosphates present, misincorporation of dGMP represents more than 0.5 percent of normal dTMP incorporation by the reverse transcriptase.[19]

High frequency misincorporation *in vitro* by reverse tanscriptases from avian and murine type C retroviruses is well known,[20,21] and it was not unexpected to observe that the HIV reverse trancriptase shares this property. Nonetheless, the rate and processivity of dGMP misincorporation by the HIV enzyme on poly (rA) are surprising, and the HIV enzyme's processivity under these conditions is much greater than that of AMV RT. Takeuchi *et al.*[22] examined misincorporation of dCMP on a poly (rA) template. These workers found that HIV reverse transcriptase showed an error rate of 1/20,000 to 1/30,000 with these substrates, in contrast to the dGMP error rate we observed; nonetheless, this error rate was 3–5 times higher than those seen with other reverse transcriptases.

HIV RT tolerance of the G:A mispair with poly (rA) as template is such that several of the detailed enzymatic features of dGMP incorporation are similar to those of dTMP incorporations. Thus, with higher dGTP concentrations, poly (dG) synthesis was highly processive, and the synthesis product is formed at about the same maximum velocity as that of dTMP incorporation. The similarity in k_{cat} implies, but does not prove, that the rate-limiting step is the same in the two systems. HIV reverse

TABLE I
Kinetic and thermodynamic parameters of deoxynucleotide incorporation by HIV reverse transctriptase.[a]

Nucleotide Incorporated	K_m dNTP (μM)	k_{cat} (sec^{-1})	k_{cat}/K_m (M^{-1} sec^{-1})	ΔG^* (kcal mol^{-1})	ΔH^* (kcal mol^{-1})	ΔS^* (cal K^{-1} mol^{-1})
dTMP (A:T pair)	2	4	2×10^6	17.33	20.3	9.6
dGMP (A:G mispair)	2000	3.6	1.8×10^3	17.38	6.2	−36.1

[a] K_m and k_{cat} for the A:T pair have previously been reported.[5] Other values are taken from reference[19]. ΔG^* was calculated from the values for k_{cat}. ΔH^* was obtained as described in the text. ΔS^* was calculated from the relationship $\Delta G^* = \Delta H^* - T\Delta S^*$ at 37°C.

transcriptase demonstrates a much higher frequency of termination after insertion of the first nucleotide with dGTP than with dTTP as substrate, suggesting that the incorrect primer terminus may provide a means of more precise kinetic analysis of this early step in precessive DNA synthesis. The infidelity of the HIV RT may also have implications for drug therapy; it may be possible to design substrates for the enzyme to insert during replication of the RNA genome, which would then block the second round of synthesis with DNA as template.

We made use of this strong poly (dG) synthesis reaction to examine thermodynamic parameters for misincorporation by the HIV RT. The results are indicated in Table I. The K_m for dGTP is about 2 mM, whereas the K_m for dTTP is about 2 μM. The k_{cat}/K_m term is an apparent "efficiency constant", or second order rate constant, relating the reaction rate to the concentrations of dNTP and enzyme, $v = [E][S]k_{cat}/K_m$. We note that the k_{cat}/K_m value for dGMP misinsertion is 3 orders of magnitude lower than the comparable value for dTMP insertion. Other workers employing a variety of systems have similarly found large decreases in k_{cat}/K_m for mispairing by DNA Pol 1,[23] Pol I large fragment,[24,25] and DNA polymerase α.[26]

ΔH^* values were obtained from Arrhenius plots (log activity versus 1/T) for dTMP and dGMP incorporation. The entropy of activation, ΔS^*, is then calculated from a standard equation. The difference in ΔG^* values is a straightforward result of the slight difference in k_{cat}. The constituents of ΔG^* are significantly different for these two cases, however. Correct base pairing requires a higher enthalpy of activation, which is counteracted by an increase in entropy for the transition state complex; for incorrect base incorporation, activation enthalpy is smaller, and a decrease in entropy occurs with the transition complex. Thus, poly d(G) synthesis involves less enthalpy of activation and less entropy of activation than poly d(T) synthesis. The mechanistic basis for this may be related to solvation and active site contact effects.

The only DNA polymerase for which a detailed kinetic scheme is available is Pol I large fragment. The rate-limiting step for this enzyme is a part of the nucleotide insertion process where the enzyme-substate complex undergoes a relatively slow step between dNTP binding and the nucleotidyl transferase event.[27,28] The HIV RT shares several key properties with Pol I lf, including similar processivity, similar ordered sequential mechanism and similar k_{cat}. By analogy, the rate-limiting complex responsible for the thermodynamic parameters obtained here may also be part of the insertion process.

Fersht[29] has already suggested that the transition state with complementary base

pairs is accompanied by an entropy increase due to displacement (and dispersion) of hydrogen-bonded water molecules in the active site, and Petruska *et al.*[30] have postulated a polymerase active site that excludes water from binding to correct base pairs. This explanation is consistent with the generally held view that positive ΔS^* values observed in polymer formation reactions are due to solvation effects.[31,32] Thus, the entropy values in Table I may reflect different hydration conditions for the two transition state complexes. The implication is that the HIV RT can accomodate a more hydrated structure involved in A : G mispairing within the microenvironment of the binding site, whereas other polymerases, such as Pol I for example, cannot. If water exclusion secondary to charge neutralization, for example, is an enthalpy consuming process, the finding of a lower ΔH^* value for misincorporation may also be related to solvation effects. The difference in ΔH^* could thus reflect a situation where RT would hold dGTP more loosely in the active site, compared with dTTP, in the presence of the poly r(A) template.

Other interpretations of the results reported here may be reasonable also. The dNMP incorporation reaction undoubtedly reflects many individual microscopic steps inherent in the DNA synthesis process by HIV-1 RT in our system. The similarity in k_{cat} and processivity implies that the rate-limiting step and translocation step, respectively, are similar in the two cases. However, measurement of the temperature dependence of each microscopic rate constant would be required to assign the differences found here to individual steps in the reaction, such as incoming nucleotide base-pairing to the template or translocation. Such an approach may be possible in the future for single turnover reactions where dGMP is misincorporated opposite a template dT.[19]

We note also that HIV RT has shown a tendency in other systems to produce mismatch errors involving dGMP. In an M13 mutagenesis system, the enzyme produces (template) dT : dGMP errors about 5-fold more frequently, and (template) dG : dTMP errors about 20-fold more frequently, than AMV reverse transcriptase.[33] Working with a ϕX174 mutagenesis system, another group found that the HIV RT formed (template) dG : dAMP mispairs 5 times more frequently, and (template) dG:dTMP mipairs 50 times more frequently, than reverse transcriptase fropm Moloney murine leukemia virus (MLV)[34]; the HIV reverse transcriptase also exhibited a greater tendency than the MLV enzyme to elongate primer termini mispaired with a template dG.[34]

In addition, Williams *et al.*[35] have reported an analysis of HIV genomic sequences indicating that the variability of oligo (rA) tracts within the viral *env* gene is higher than for all other comparable repetitive sequences in all regions of the viral genome. They note that one explanation for this higher variability could be an inherently lower fidelity of the HIV reverse transcriptase in copying (rA) tracts *in vivo*. Our observations of the enzyme's infidelity *in vitro* on a poly (rA) template are consistent with this idea.

3.2. *Infidelity During DNA Template-Directed DNA Synthesis*

In initial experiments to examine the misinsertion propensity of HIV reverse transcriptase on a DNA template, we used a method termed DNA "bypass" synthesis applied by Beattie and colleagues.[36–38] With this method, template-directed DNA synthesis is carried out with individual dNTPs omitted from the reaction mixture. Displaying reaction products by gel electrophoresis reveals a strong termination before the first position at which the omitted nucleotide is normally inserted. Depending on the

TABLE II
Bypass frequency of DNA synthesizing enzymes[a]

Omitted substrate	Bypass frequency in percent for: Pol I lf	HIV RT	AMV RT
dATP	2.4	58	68
dCTP	1.6	33	75
dGTP	< 1.0	0	0
dTTP	5.6	68	48

[a]DNA synthesis reactions were carried out with ^{32}P-labeled primer hybridized to ϕX plus strand DNA; individual dNTPs were omitted as indicated, and the other three dNTPs were present at 50 μM. Products of reaction were separated by electrophoresis on a 12% polyacrylamide, 7 M urea gel, and were visualized by autoradiography. Product bands were traced by densitometry, and areas under peaks were determined by cutting and weighing. Bypass frequency was determined by the relative amount of DNA synthesis products extended beyond the first "minus dNTP" site for a given substrate, compared to products representing termination at the first "minus" site. The bypass frequencies for the minus dGTP condition reflect detectable but small bypass for Pol I lf, and no detectable bypass synthesis by the reverse transcriptases. Taken from reference.[19]

ability of a given polymerase to misinsert at such sites, product molecules of longer size are also seen.

We compared bypass synthesis by the reverse transcriptases of HIV and AMV, and *E. coli* Pol I large fragment (Pol I lf). A ^{32}P-labeled primer was hybridized to ϕX174 single stranded DNA upstream of a region where the template is lacking in secondary structure, based on previous enzymatic studies and computer projections.[39] In normal reactions with all four dNTPs, processive DNA synthesis was observed with both reverse transcriptases. These enzymes also showed preferential sites for termination of processive synthesis; their termination patterns are similar, but not identical (for a general discussion of termination, see reference[40]).

When an individual dNTP was omitted from the reaction mixture, Pol I lf showed a strong termination band at the first position where the omitted substrate would have been inserted. Weak bands also were seen at downstram positions, suggesting that there is some bypass synthesis due to misinsertion. The incoming substrate for the first two template residues is dGTP, and very little synthesis can be seen when dGTP is omitted, however, longer exposure of the gel revealed faint bands at positions corresponding to sites where dGTP is the incoming substrate. The reverse transcriptases, in contrast, showed no detectable synthesis when dGTP was omitted. For the reactions where dATP, dCTP, and dTTP were omitted, the reverse transcriptases showed much more bypass than Pol I lf. Thus, the bypass patterns for the reverse transciptases are similar to each other and distinctly different from Pol I lf.[41]

The tendency of a given polymerase to bypass each "minus dNTP" site was quantified from band intensities by comparing the relative proportion of product molecules at the first "minus substrate" site to all product molecules of greater length. One can then calculate a bypass frequency, reflecting the probability that an enzyme will synthesize beyond the first "minus dNTP" site rather than terminate synthesis. Table II displays the bypass frequencies for the threee enzymes examined. One sees for Pol I lf low levels of bypass sythesis for each minus nucleotide reaction. For the -dATP, -dCTP, and -dTTP reactions, the reverse transcriptases exhibit bypass

frequencies that are at least an order of magnitude larger. In several cases, the bypass frequency of HIV reverse transcriptase approaches or exceeds 50 percent, indicating that the enzyme readily continues synthesis in the absence of the correct nucleotide. Only in the minus dGTP siutation is bypass not seen with the reverse transcriptase; Pol I lf shows a small but detectable amount of bypass synthesis in this case.

These experiments indicate that the purified HIV reverse transcriptase conducts error-prone replication on a DNA template. Based on observations for bypass at a single position on the ϕX DNA template, the fidelity of HIV reverse transcriptase appears similar to that of AMV reverse transcriptase, and considerably worse than Pol I lf (Table II). One exception is the minus dGTP reaction, where both viral enzymes showed bypass frequencies less than that of Pol I lf. This is consistent with an earlier observation by Hillebrand and Beattie[37] that there was no striking difference between AMV reverse transcriptase and Pol I if in bypass synthesis on an M13 template in the absence of dGTP, although these workers cautioned that relative fidelity of polymerases may change with template position.

Another group has also reported extensive bypass synthesis by HIV-1 reverse transcriptase. When these workers examined base substitution fidelity by measuring reversion of a ϕX174 *amber* phenotype, they determined an error rate for HIV reverse transcriptase of 1 per 4000 bases incorporated, compared to 1/9000 for AMV reverse transcriptase.[42] Other workers examining base substitutions by reversion of a nonsense codon on an M13 template, found an error rate of 1/18,000 for HIV RT, compared to 1/24,000 for AMV RT.[43] These same groups have also found that the HIV reverse transcriptase can efficiently elongate primers with mispaired 3′ ends.[43,44] Thus, the HIV reverse transcriptase could provide a source of genetic diversity through error-prone replication.

The observation that the HIV reverse transcriptase terminates DNA synthesis in a non-random manner,[40,41] and the availability of a system which can measure the mutation spectrum of any DNA-polymerizing enzyme, allowed a collaboration to examine the influence of termination on mutations.[33] That the tendency of a DNA polymerase to dissociate from the template-primer during synthesis might affect fidelity was suggested by several observations. Kinetic studies with DNA polymerase α,[45] the Pol I large fragment,[46] and HIV-1 reverse transcriptase[5] indicate that initiation and processive synthesis have distinctly different enzymatic properties; it would be of interest to determine if the kinetic differences are reflected in differences in error rate. The model for energy relay proofreading predicts that incorporation of the first nucleotide during DNA synthesis should be more error-prone that subsequent incorporations.[47] In addition, previous work with eucaryotic DNA polymerases in the M13mp2 mutation assay indicated that more processive enzymes are more accurate, particularly for one-base frameshifts.

With the M13mp2 mutation system, it was possible to examine both the mutation spectrum of HIV-1 reverse transcriptase, as well as the termination pattern characteristic of the enzyme.[33] If insertion of the first nucleotide were inherently error-prone, one would expect to observe mutation hot spots at positions immediately following strong termination sites. A comparison of termination and mutation sites suggested that reinitiation is not a strong determinant of base substitution errors for either the HIV-1 reverse transcriptase of the AMV reverse transcriptase.[33,49] Thus Hopfield's model[47] or other kinetic models predicting error-prone incorporation of the first nucleotide, were not corroborated by the these results. This conclusion is consistent with earlier observations at single loci with mammalian DNA polymerases.[50,51]

With HIV-1 reverse transcriptase, however, there was a correlation between termination in runs of three or more identical nucleotides and frameshift mutations in such runs. The HIV reverse transcriptase shows an overall mutation frequency that is an order of magnitude greater than those of other reverse transcriptases,[43] and the dominant features of the HIV enzyme's mutation spectrum are "hot spots" for one-base frameshifts.[33] Each major frameshift site lies within a homopolymer sequence of 3 to 5 residues, and a distinguishing feature of the major frameshift is the presence of a strong termination site. The observation of frameshifts within runs of identical residues is consistent with a model proposed by Streisinger and colleagues[52] for mutations caused by slippage between template and primer, and termination within a run could facilitate such a mechanism. The implication is that the HIV reverse transcriptase is able to tolerate primer-template misalignment during synthesis, or may cause such misalignment when it reinitiates synthesis.[33]

On the M13 template, mutation was still a low-probability event, compared to termination. For example, at a position where the enzyme terminates synthesis about half the time, the error rate for plus-one errors was much less than one percent. In addition, the correlation between termination probability and one-base frameshifts was not quantitative. The highest error rates did not necessarily occur at sites exhibiting the highest termination probability. Such quantitative differences are not inconsistent with the concept that processivity is important for fidelity, since a number of parameters are expected to influence the error rate, including the length and base composition of the run, the influence of neighboring bases, and the structure of the polymerase and its contacts with the template-primer.[33]

These results indicate a correlation between frameshift error rates and major termination sites in runs of identical nucleotides. To establish a causal relationship between termination and errors will require further experiments to modulate termination frequency at specific sites and observe the effect on error rates. The correlation nonetheless suggests that termination may facilitate slippage-dependent frameshift mutations.[33] With the AMV reverse transcriptase, frameshifts within runs are seen at a much lower frequency,[49] and strong termination sites are generally absent from runs.[33] This suggests that enzymes which have similar mechanisms for DNA synthesis might produce errors during synthesis by different mechanisms.

CONCLUSION

We have described kinetic and fidelity characteristics of the HIV-1 reverse transcriptase. The identification of fundamental properties of the reverse transcriptase may facilitate rational inhibitor design, and thereby assist in the development of drug therapy against the HIV virus. The results with AZTTP and NH_2TTP indicate that a small change in chemical structure can alter the mechanism of inhibition. The existence of alternative mechansims of inhibition may also offer a strategy to overcome viral resistance of individual inhibitors.

The findings on fidelity also have ramifications for inhibitor design. On the one hand, the propensity of the virus to rapidly mutate enhances its ability to evade inhibitors whereas on the other hand, the infidelity of the HIV reverse transcriptase may allow the development of imaginative therapies. The enzyme's promisucity may allow the design of inhibitors with specificity for the HIV RT, and with little effect on cellular enzymes. In addition, the tendency of the HIV RT to misincorporate on RNA

templates may allow the design of inhibitors which the enzyme would insert during synthesis directed by the RNA genome, and which would then block DNA replcation during the next round of DNA template-directed replication.

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