Pre-steady-state methods were used to study the fidelity of human immunodeficiency virus reverse transcriptase. Fidelity of DNA-directed DNA synthesis can be attributed to a 1-2 order of magnitude reduction in affinity for noncomplementary dNTPs, and a 1-4 order of magnitude reduction in the rate of the conformational change that limits the rate of nucleotide addition. Affinities of reverse transcriptase for paired or mispaired primer termini are similar. Discrimination against a mispaired primer is due to reduction in affinity for the next dNTP and reduction in rate of extension. Extension of mispaired termini proceeds 20-700-fold faster than the rate of dissociation of reverse transcriptase from the primer-template and is 2-3 orders of magnitude more frequent than nucleotide misincorporation. The rate-limiting step for extension of a mispaired terminus occurs at the conformational change or chemical step, depending on the nature of the mispair. Presence of a mismatch at the 3' penultimate position reduces pyrophosphorolysis of the primer by a factor of 10^6, indicating that mismatches 5' to the site of chemistry can also affect catalysis.

Genomic variability of human immunodeficiency virus (HIV) is predicated, in large part, on the low fidelity of its DNA biosynthetic enzyme. HIV reverse transcriptase (RT) has lower fidelity than other DNA polymerases (1,2), including other reverse transcriptases, which lack HIV RT, do not possess a proofreading exonuclease (3). Several laboratories (1,2,4-13) have demonstrated that HIV RT can generate substitution errors by direct misincorporation and subsequent extension. Individual misincorporation events measured by a forward reaction assay range from 2 x 10^{-4} to 2 x 10^{-3} for dGMP incorporation across a template U, to <3 x 10^{-5} for dTMP incorporation across a template T (14). Steady-state parameters for misincorporation and extension of a mispaired primer by the enzyme have been described by a number of laboratories (1,6,8-12). In addition to direct misincorporation, slippage of primer-template has been shown to be a major source of replication errors, including one-base frame-shift and substitution errors (5,15,16). Reports conflict as to whether RNA-dependent or DNA-dependent polymerization is more precise (14,17,18).

Pre-steady-state analysis of several DNA polymerases (19-23) has provided insight into the mechanisms of polymerase fidelity and has provided an intellectual framework for this study of HIV RT. A comprehensive analysis of RT DNA polymerase has shown that an induced-fit conformational change provides a major selection against incorrect dNTPs and mispaired primer termini (22). In contrast, Escherichia coli DNA polymerase I discriminates against incorrect dNTPs primarily at the chemical step (20,23). Work on these two enzymes has demonstrated the utility of pre-steady-state methods for identification of those steps that are important in substrate discrimination.

We (24) and others (25-27) have described a detailed kinetic mechanism for DNA-directed nucleotide incorporation by the HIV enzyme. A key feature of the HIV RT mechanism is a rate-limiting conformational change that occurs subsequent to dNTP binding (24), which effectively renders nucleotide addition kinetically irreversible. In this report, we show that this conformational change plays a key role in the selectivity of nucleotide addition, while nucleoside triphosphate binding provides an additional contribution. We also demonstrate these steps are also involved in the discrimination against extension of mispaired termini.

EXPERIMENTAL PROCEDURES

Protein and Nucleotides—T4 polynucleotide kinase was from United States Biochemical Corp. (Cleveland, OH). Ribonucleoside-5'-triphosphates, deoxyribonucleoside-5'-triphosphates, and their phosphorothioate analogues were from Pharmacia LKB Biotechnology Inc. Radioisotopes of nucleotides were from DuPont-NEN. HIV RT was prepared as described (24), with 80% of the heterodimers being catalytically proficient.

Oligodeoxyribonucleotides—DNA primers, ranging from 22 to 24 nucleotides in length, and a 32-mer DNA template (see Table I for nomenclature) were synthesized on an Applied Biosystems 381A DNA synthesizer. Concentration determination, purification, 5'-end labeling, and primer-template annealing were performed as described (24). Duplex [3'-32P]22N/32-mer with a specific mispair at the penultimate position of the primer terminus was prepared by incubating 1 pm 22N/32-mer (where N is A, G, or T), HIV RT (500 nm), [α-32P]dATP (1.7 μM, 3000 Ci/mmol) in buffer A (6 mM MgCl_2, 40 mM KCl, and 50 mM Tris-HCl, pH 8.0) at 37 °C for 20 min. Greater than 95% of the 22N/32-mer was extended. The product was purified as described (24).

Single Nucleotide Addition—Experiments were performed at 37 °C using a KinTek chemical quench apparatus (KinTek Instruments) for time sampling in the range of 10 ms to 4 s. RT [5'-32P]primer-template complex (40 μl) in buffer A was mixed with 40 μl of a solution of the specified dNTP in buffer A. Reactions were quenched by mixing with 170 μl of 0.2 mM EDTA, pH 8.0. Time samples for incubation periods exceeding 4 s were obtained manually. In this case, an equal volume of RT DNA complex (50 μl) was mixed with dNTP as indicated, or 0.1-6 mM dNTP (50 μl). Samples (5 μl) were withdrawn and quenched into 20 μl of 0.2 mM EDTA, pH 8.0. Products were separated on 12% polyacrylamide gels containing 8% urea and quantitated using a PhosphorImager (Molecular Dynamics).

Pulse-Chase Analysis—Complexes of RT (4 μl) and 22N/32-mer (2 μl) in buffer A were mixed with an equal volume of 1 mm [α-32P]dATP (2.9 Ci/mmol) in buffer A at 37 °C for the times indicated. Reactions (5 μl) were directly quenched with 5 μl of 0.5 mM EDTA, pH 8.0, or chased with 5 μl of 10 mM dATP in buffer A for 300 s and then quenched with 10 μl of 0.5 mM EDTA, pH 8.0. Products were quantitated as described for nucleotide addition reactions. To minimize experimental error, the...
amount of (α-32P)dAMP incorporated for each time sample was normalized using [5'-32P]22T/32-mer as the internal standard.

Pyrophosphorolysis—Pyrophosphorolysis of primer-templates containing a mismatched primer terminus was complicated by precipitation of MgPPi at (PPi) > 2 mM, with co-precipitation of DNA becoming significant (>50%) at times greater than 10 min. This problem, which was not significant at shorter reaction times (24), precluded accurate determination of the dissociation constant for PPi, and rate of pyrophosphorolysis for mispaired primer termini. However, under conditions of short reaction times or low PPi concentrations or both, it was possible to compare relative pyrophosphorolysis rates of various mispaired primer termini.

The 5'-end labeled primer-template containing a mispaired primer terminus was incubated with HIV RT in the presence of Mg-PPi, and the reduction in length of primer was monitored. A solution (10 μl) of either 20 mM or 5 mM PPi in buffer B (50 mM Tris-HCl, pH 8.0, and 40 mM KCl) was added to 96 μl of pre-equilibrated HIV RT (500 nM) and [α-32P]PPi/32-mer (100 nM) in buffer B containing 8 mM or 6.5 mM MgCl2, respectively, such that a final concentration of 6 mM free Mg2+ was present in the solution. At indicated times, samples (5 μl) were removed and quenched into 20 μl of 97% formamide, 170 mM EDTA, pH 8.0. Products were quantitated as described above for nucleotide addition reactions.

Pyrophosphorolysis of (3'-32P)primer-template complexes containing a penultimate mispair was examined by monitoring the release of [α-32P]dTPP as described (24). Products were resolved by thin layer chromatography and quantitated as described (24), except that polyethyleneimine-cellulose plates (EM Separations) were developed in 0.3 M acetic acid containing 1 M LiCl.

Lifetimes of the Complex of RT and Mispaired Primer-Template—Dissociation of enzyme from a mispaired primer-template was monitored by virtue of the ability of released enzyme to extend a distinct 32-mer (100 nM) in buffer containing 1 mM MgCl2, 8 mM or 6.5 mM MgCl2, respectively, and the indicated primer-template (800 nM) was mixed in a chemical equilibrium dissociation constant for the incorrect dNTP.

The dramatic reduction in kobs for misincorporation, relative to that for correct incorporation, might be accounted for by rate-limiting dNTP association at low dNTP concentration. To distinguish between rate-limiting dNTP association and rapid equilibrium binding models, the partition of E-DNA-dNTP complex between incorporation and dissociation was examined by pulse-chase analysis (Fig. 2, lower panel). Equilibrated RT-DNA complex was pulsed with (α-32P)dATP for various periods of time and quenched with EDTA or chased with a 10-fold excess of unlabeled dATP, followed by EDTA quench. The amount of RT-DNA-bound (α-32P)dATP incorporated as opposed to that undergoing dissociation will depend on koff (koff + k2) and [RT-DNA-dATP]. Computer simulation (not shown) to the conditions and mechanism in the lower panel of Fig. 2 using the experimentally determined koff value and a set of k2 values ranging from 0.1 koff to 100 koff (k2 calculated from the relationship koff = k2 (Kp) was employed to evaluate possible outcomes of pulse-chase analysis. Significant differences at early times between the pulse-chase and pulse-quench reactions are predicted if koff < 4 k2. In contrast, a rapid equilibrium dNTP binding model predicts no difference between the pulse-quench and pulse-chase reaction profiles because koff >> k2.

The chase did not produce additional labeled product (Fig. 2, lower panel), consistent with the rapid equilibrium model. The possibility of slow dNTP association was further evaluated by simulation. Assuming rate-limiting dNTP association and given the pseudo-first-order conditions maintained experimentally, a second-order rate constant k2 of 20 M−1 s−1 can be derived from the initial rates of the reactions in the inset of Fig. 2. If nucleotide misincorporation is governed by rate-limiting association, a lag in product formation (Fig. 2, lower panel, broken lines) is predicted by computer simulation to the conditions and mechanism described in the legend to Fig. 2. However, no lag was observed experimentally, and none of these simulated curves fit the observed data. These results further argue against rate-limiting dNTP association. We have therefore concluded that the binding of dNTP is in rapid equilibrium.

Similar kinetic profiles like that shown in Fig. 2 were observed for other incorrect dNTPs opposite a template dA, dC, or dG, except that dCMP incorporation opposite template dC was not detected. These results are summarized in Table II. The variation in dissociation constants (Kp) and incorporation rates (koff) with different incorrect dNTPs indicates two stages of discrimination against incorrect dNTPs by HIV RT. The first stage of selection is dNTP binding by the E-D complex, as exemplified by an increased Kp for incorrect dNTP binding.
relative to that for the correct dNTP (25-fold for dTTP opposite a template dG, to 200-fold for dTTP opposite a template dC or dCTP opposite template dA). The second stage of discrimination is due to a reduction in rate of incorporation by a factor ranging from 18 to 20 for dTMP incorporation opposite template dG to $1.7 \times 10^4$ for dGMP incorporation opposite template dA, relative to that for addition of a correct nucleotide.

As shown above, both $k_{pol}$ and $K_a$ are substrate selectivity parameters. We define specificity as $k_{pol}/K_a$, which is not equivalent to the conventional steady-state parameter $k_{pol}/K_a$. This parameter is useful for comparison of relative propensities for misincorporation, defined as $(k_{pol}/K_a)_{correct}/(k_{pol}/K_a)_{incorrect}$. Misincorporation propensities of the dNTPs tested range from $10^{-4}$ to $10^{-8}$, with the exception of dTMP incorporation opposite a template dG ($2 \times 10^{-5}$), and dCMP opposite a template dC (undetectable). This indicates that dTTP/dG is a common misincorporation event, consistent with other reports (5, 12), while dCTP/dC a rare one. While reduced binding of incorrect dNTPs significantly affects the misincorporation events tested, discrimination results primarily from a dramatic reduction in $k_{pol}$.

As shown above, this rate is determined by a conformational change following dNTP binding.

Due to possible competition by rNTPs in vivo, incorporation of rUMP, rAMP, rCMP, and rGMP opposite template dA and rGMP opposite template dC was tested. Complementary rNMPs are incorporated with a propensity of approximately $10^{-5}$, with discrimination evident at both binding and incorporation steps (Table II). Misincorporation of noncomplementary ribonucleotides opposite template dA was limited to a propensity of less than $1 \times 10^{-10}$ (data not shown). A complementary rNTP is therefore no more likely to be incorporated by HIV RT than is an incorrect dNTP, while noncomplementary rNTPs are not utilized to a detectable degree with a DNA template.

**Elemental Effects on Misincorporation.—**Formation of a conformationally distinct intermediate subsequent to dNTP binding but prior to chemistry is rate-limiting for incorporation of a correct dNTP by HIV RT (24). To determine whether chemistry is rate-limiting for misincorporation, we have tested the reaction for phosphorothioate elemental effects. Direct comparison of misincorporation kinetics at saturating dNTP or dNTPoS concentrations yielded only small elemental effects (Table II). Although the full magnitude of a phosphorothioate elemental effect on HIV RT-catalyzed nucleotidyl transfer chemistry is unknown, we presume it to be greater than 18, since we have observed an elemental effect of this magnitude for extension of a mispaired primer terminus (see below). Based on the small elemental effects observed with incorrect dNTPoS, we have concluded chemistry is not rate-limiting for misincorporation.

We therefore assign the rate-limiting step for addition of an incorrect nucleotide to a conformational change after dNTP binding. This conclusion is supported by three lines of evidence: (i) the absence of burst kinetics for misincorporation places the rate-limiting step at or prior to chemistry; (ii) the lack of a substantial phosphorothioate elemental effect rules out the chemical step; and (iii) as shown above, binding of an incorrect dNTP is in rapid equilibrium relative to incorporation, thus placing the rate-limiting step between dNTP binding and chemistry.

**Absence of Proofreading Pathways.—**The absence of editing exonuclease activity in HIV RT prompted us to test for other possible proofreading pathways that might contribute to fidelity. Since pyrophosphorolysis has been suggested as a possible fidelity mechanism for DNA polymerases (30, 31), we tested for a role of this activity in excision of primer terminal mispaired residues. As shown in Fig. 3, the estimated rates of pyrophosphorolysis of 3'-C/A and 3'-A/A primer termini at 2 mM PP, were $9 \times 10^{-5}$ s$^{-1}$ and $1 \times 10^{-5}$ s$^{-1}$, respectively. Pyrophosphorolysis of G/A was not detectable within the time scale of Fig. 3 (not shown). In contrast, a very fast, albeit small, burst accounting for the chemical step occurring at $>160$ s$^{-1}$ has been previously observed for pyrophosphorolysis of a correctly paired terminus (24). The absence of a corresponding burst with mispaired termini indicates that pyrophosphorolysis of such termini is limited by the rate of the chemical step, or by preceding steps. Furthermore, the extremely slow nature of this reaction indicates pyrophosphorolysis of mispaired termini does not contribute significantly to the fidelity of RT. In fact, pyrophosphorolysis of correctly paired primer is at least $10^{6}$-fold faster than of a mispaired terminus.

Kinetic proofreading via template directed hydrolysis of a bound incorrect dNTP has been proposed as a method for increasing fidelity of a polymerase (32). Such a mechanism is

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**Table I**

<table>
<thead>
<tr>
<th>Name</th>
<th>Used as</th>
<th>Sequence</th>
<th>$k_{pol}$ μM$^{-1}$ cm$^{-1}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>22 nucleotides in length</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>22-mer Correct primer</td>
<td>5'-d(GGGAGTCCCCTGTTGGGCCGCA)</td>
<td>223</td>
<td></td>
</tr>
<tr>
<td>23 nucleotides in length</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>23-mer Correct primer</td>
<td>5'-d(GGGAGTCCCCTGTTGGGCCGCA)</td>
<td>230</td>
<td></td>
</tr>
<tr>
<td>22A-mer Mispaired primer$^a$</td>
<td>5'-d(GGGAGTCCCCTGTTGGGCCGCA)</td>
<td>238</td>
<td></td>
</tr>
<tr>
<td>22G-mer Mispaired primer$^a$</td>
<td>5'-d(GGGAGTCCCCTGTTGGGCCGCA)</td>
<td>235</td>
<td></td>
</tr>
<tr>
<td>22T-mer Mispaired primer$^a$</td>
<td>5'-d(GGGAGTCCCCTGTTGGGCCGCA)</td>
<td>231</td>
<td></td>
</tr>
<tr>
<td>24 nucleotides in length</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>22A-mer Mispaired primer$^a$</td>
<td>5'-d(GGGAGTCCCCTGTTGGGCCGCA)</td>
<td>246</td>
<td></td>
</tr>
<tr>
<td>22C-mer Mispaired primer$^a$</td>
<td>5'-d(GGGAGTCCCCTGTTGGGCCGCA)</td>
<td>237</td>
<td></td>
</tr>
<tr>
<td>22G-mer Mispaired primer$^a$</td>
<td>5'-d(GGGAGTCCCCTGTTGGGCCGCA)</td>
<td>242</td>
<td></td>
</tr>
<tr>
<td>22T-mer Mispaired primer$^a$</td>
<td>5'-d(GGGAGTCCCCTGTTGGGCCGCA)</td>
<td>239</td>
<td></td>
</tr>
<tr>
<td>32 nucleotides in length</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>32-mer Correct primer</td>
<td>3'-d(CCCTCAGGGACAGCCCGCGGTTGAAGAGGG)</td>
<td>348</td>
<td></td>
</tr>
</tbody>
</table>

$^a$ These oligomers were used in mispair extension experiments and are identical to 23-mer except for the 3'-end terminal residues (underscores) which form mispairs opposite a template guanine.

$^b$ These oligomers were used in pyrophosphorolysis of mispaired termini and are identical to 24-mer except the 3'-end residues (underscores) which form mispairs opposite a template adenine.

$^c$ These oligomers were used in pyrophosphorolysis of correct termini next to a penultimate mispair and are identical to 24-mer except the 3'-end penultimate residues (underscores) which form mispairs opposite a template guanine.

$^d$ Sequence is shown from 3'- to 5'-end.
Fidelity of Reverse Transcriptase

Table II
Parameters for correct and incorrect nucleotide addition onto 23/32-mer and 22/32-mer

| dNTP | $K_D$ | $k_{ps}$ | $k_{ps}/K_D$ | Propensity* | Elemental effect
<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>22/32-mer</td>
<td>5'—A↓</td>
<td>3'—TGAC—</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>dCTP</td>
<td>6.71</td>
<td>11.8</td>
<td>1</td>
<td>ND+</td>
<td></td>
</tr>
<tr>
<td>dTTP</td>
<td>150</td>
<td>4.0</td>
<td>2.7 x 10^{-2}</td>
<td>2 x 10^{-3}</td>
<td>ND</td>
</tr>
<tr>
<td>dATP</td>
<td>2500</td>
<td>0.12</td>
<td>4.8 x 10^{-6}</td>
<td>4 x 10^{-6}</td>
<td>ND</td>
</tr>
<tr>
<td>rUTP</td>
<td>190</td>
<td>0.04</td>
<td>2.1 x 10^{-4}</td>
<td>2 x 10^{-5}</td>
<td>ND</td>
</tr>
<tr>
<td>23/32-mer</td>
<td>5'—AC↓</td>
<td>3'—TGAC—</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>dCTP</td>
<td>18</td>
<td>83</td>
<td>4.6</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>dATP</td>
<td>3700</td>
<td>2</td>
<td>5.4 x 10^{-4}</td>
<td>1 x 10^{-4}</td>
<td>1</td>
</tr>
<tr>
<td>dATP</td>
<td>770</td>
<td>0.03</td>
<td>4 x 10^{-6}</td>
<td>9 x 10^{-6}</td>
<td>3</td>
</tr>
<tr>
<td>dGTP</td>
<td>500</td>
<td>0.005</td>
<td>1 x 10^{-6}</td>
<td>2 x 10^{-6}</td>
<td>3</td>
</tr>
<tr>
<td>rUTP</td>
<td>1000</td>
<td>0.02</td>
<td>2 x 10^{-6}</td>
<td>4 x 10^{-6}</td>
<td>ND</td>
</tr>
<tr>
<td>24/32-mer</td>
<td>5'—ACT↓</td>
<td>3'—TGAC—</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>dGTP</td>
<td>16</td>
<td>23</td>
<td>1.4</td>
<td>1</td>
<td>ND</td>
</tr>
<tr>
<td>dATP</td>
<td>2000</td>
<td>0.06</td>
<td>3 x 10^{-6}</td>
<td>2 x 10^{-6}</td>
<td>ND</td>
</tr>
<tr>
<td>dGTP</td>
<td>3800</td>
<td>0.03</td>
<td>8 x 10^{-6}</td>
<td>6 x 10^{-6}</td>
<td>ND</td>
</tr>
<tr>
<td>rGTP</td>
<td>6300</td>
<td>0.08</td>
<td>1 x 10^{-5}</td>
<td>9 x 10^{-6}</td>
<td>ND</td>
</tr>
</tbody>
</table>

* Relative propensity = ($k_{ps}/K_D$)correct / ($k_{ps}/K_D$)incorrect.
+ Calculated as dNMP addition rate / dNMPaS addition rate.
+ Not determined.

* All parameters for correct dTMP addition are from Hsieh et al. (1993).
* No dCMP addition was observed within 20 min for dCTP concentration up to 3 mM.

Fig. 3. Single turnover pyrophosphorylation of mispaired primer termini. RT (450 nM) equilibrated with [5'-32P]23A/32-mer (90 nM) (●, ○) or [5'-32P]23C/32-mer (90 nM) (△, ▲) was mixed with PPi (final concentration: 2 mM (filled symbols), or 0.5 mM (open symbols)) and reaction quenched at indicated times. Data were fit to the first-order equation described in Fig. 2 for reactions utilizing [5'-32P]23C/32-mer. Rates were derived from the slope of tangents to the curves through the origin. For reactions utilizing [5'-32P]23A/32-mer, fits to the first-order equation proved erroneous due to scatter in the data near the origin. Since less than 2% of the substrate was utilized over the time course, a linear fit accurately approximates this very early portion of a first-order curve.

distinct from the proofreading exonuclease activities well characterized for DNA polymerases, where incorporation is a prerequisite to hydrolysis. The possibility that HIV RT might catalyze preinsertion hydrolysis of incorrect dNTPs was tested by incubating [α-32P]dATP, [α-32P]dGTP, or [α-32P]dUTP with RT-22/32-mer complexes. Production of [α-32P]dNMP was not observed, for the three incorrect pairings opposite a template dA, for reaction times ranging from 20 s to 2 h. Thus, no evidence was found to suggest HIV RT hydrolyzes incorrect dNTPs to increase fidelity in DNA-directed DNA synthesis.

Extension of 3'-Terminal Mispaired Primer—The absence of an effective proofreading pathway provides misincorporated nucleotides with the opportunity to be permanently fixed in the genome, if mispaired primer termini can be efficiently extended. The ability of HIV RT to extend a mispaired primer terminus was examined by monitoring the addition of the next correct nucleotide (dTMP) onto 22T/32-mer (Table I) containing a 3'-T opposite a template G. Under conditions where more than 95% of DNA was complexed with enzyme (see below), extension of the mispaired primer displayed biphasic kinetics (Fig. 4, upper panel, inset). The rapid phase, with a maximal burst amplitude corresponding to 60% of the primer-template, was complete within the first 100 ms. Rates ($k_{ps}$) governing the
yielded an equilibrium dissociation constant of 270 nM for the 32P122T/32-mer extension was observed after prolonged incubation (60 min), demonstrating that primer-template not participating in the burst phase of the reaction was a substrate for extension.

An alternate expression for the substoichiometric burst would be fast dissociation of enzyme from the ternary complex of E-D-dNTP following incorrect dNTP binding (e.g. a dissociation rate (k_d) half as fast as the misincorporation rate). Partitioning between incorporation and dissociation would reduce the amount of E-D-dNTP involved in covalent chemistry, and therefore decrease the burst amplitude by k_d/(k_{pol} + k_d) (26). In such a mechanism, the slow postburst phase would then correspond to the reassocation of free enzyme with the primer-template and dNTP. If this were the case, the postburst phase would be abolished by trapping the dissociated form of the enzyme with a large excess of unlabeled primer-template.

However, when extension of 22T/32-mer by HIV RT was monitored after mixing preformed RT-22T/32-mer complex with a dTTP solution containing a 50-fold excess of unlabeled 23/32-mer, the amplitude of the postburst phase was 60% of that observed in the absence of trap, thus ruling out this possibility.

A third explanation for the biphasic kinetic curve of mispaired terminus invokes a rate-limiting step (e.g. k_4, Fig. 1) after the chemical step (k_2) with an equilibrium constant of two (k_2/k_{d1} = 2). However, such a mechanism is not supported by the parameters for the reverse reaction of mispaired terminus extension (see below). Taken together, these results indicate that bimodal kinetics of extension of the 22T/32-mer are due to an inherent property of the RT-22T/32-mer complex. We interpret these results as indicating the existence of the RT-22T/32-mer complex in two states at a roughly 2:1 ratio, with the minor form incapable of efficient extension of a mispaired terminus.

Similar biphasic kinetics (not shown) were observed with two other mispaired primers (22G and 22A) annealed to the same template, forming either a 3-G/G or an 3-A/A mispaired 3'-end. Compared with extension of the 22T/32-mer (Fig. 4, upper panel, inset), the burst amplitudes are reduced (not shown), corresponding to 50% of 22G/32-mer and 20% of 22A/32-mer. The decreased burst amplitudes observed with these substrates indicate that the fraction of mispaired primer-template enzyme complex in a nonproductive form is dependent on the nature of the terminal mismatch. As in the case of the 22T/32-mer (Fig. 4, upper panel), pre-steady-state rates for extension 22G/32-mer and 22A/32-mer displayed hyperbolic dependence on dTTP concentration (not shown), allowing determination of the maximal pre-steady-state extension rates (h_{max}) and the K_D values for dTTP binding (Table III). A comparison of the kinetic parameters governing mismatch extension with those for correct primer extension (Table III) indicates that a mismatched primer terminus decreases the affinity of the E-DNA complex for the next dTTP by roughly an

As described in the text, biphasic kinetics of mismatch extension under single turnover conditions can be accounted for by placing a rate-limiting step (k_d) after the chemical step (k_2). In order to account for a maximal burst amplitude of 60% that expected, this mechanism requires the chemical step (k_2) to be in a rapid equilibrium with an equilibrium constant of approximately two (i.e. k_2/k_d = 2). The pre-steady-state rate (42 s^{-1}) of 22T/32-mer extension represents the rate-limiting conformational change (i.e. k_3 = 42 s^{-1}) prior to the chemical reaction. The rate of the chemical step (k_2) is unknown but is at least 42 s^{-1} because it is not a rate-limiting step, thus placing a lower limit of 21 s^{-1} for the chemical step of reverse reaction (i.e. k_4 > 21 s^{-1}), assuming that its rate is of the same order of magnitude as the measured pyrophosphorolysis rate of 0.09 s^{-1}.

Consequently, the placement of a rate-limiting step after the chemical step in the forward reaction of mismatched primer extension is not consistent with the kinetic parameters for the reverse reaction.
Effects on extension rates were examined. Elemental effects of 3' nucleoside triphosphates. Comparison of the propensities of extension of either a correct or an incorrect nucleotide onto a correct primer is a conformational change prior to the chemical step. In order to test for rate-limiting chemistry for the misincorporation of a nucleotide, the rates of extension of either a correct or an incorrect nucleotide onto a correct primer-templates (Table III) indicates that discrimination against mispaired primer-templates is less than that against incorrect nucleoside triphosphates, but the two act in a synergistic manner in overall fidelity.

Interestingly, the hierarchy of rates for extension of the mispaired primer-templates tested correlates with that of the burst amplitudes. For example, 70% of 22T/32-mer was extended at a burst rate of 42 s⁻¹ and 50% of 22G/32-mer at 15 s⁻¹ while only 20% of 22A/32-mer was extended at a burst rate of 1.4 s⁻¹, although the significance of this correlation is not clear.

Rate-limiting Step in Extension of Mispaired Primer Terminus—As discussed above, the rate-limiting step for addition of either a correct or an incorrect nucleotide onto a correctly paired primer is a conformational change prior to the chemical step. In order to test for rate-limiting chemistry for extension of mispaired primer, phosphorothioate elemental effects on extension rates were examined. Elemental effects of 3' and 18 were observed for extension of 22T/32-mer and 22A/32-mer, respectively, at dNTPoS concentrations at 1.5 times the respective \( K_D \) values. The small elemental effect with 22T/32-mer indicates chemistry is not rate-limiting for extension of this mispaired terminus. In contrast, the large elemental effect with 22A/32-mer clearly indicates that chemistry is at least partially rate-limiting for extension of this mispaired terminus. It is noteworthy that 22A/32-mer was also extended at the lowest rate.

Dissociation of HIV RT from Mispaired Primer-Templates—Dissociation of enzyme from a mispaired primer-template was examined by an approach previously employed with DNA polymerase I (29). Pre-equilibrated RT-DNA was mixed with a 10-fold excess of 22/32-mer with its next correct nucleotide triphosphate ([α-32P]dCTP) and addition of a single [α-32P]dCMP onto 22/32-mer (Table I) was monitored. As observed previously (24), a biphasic curve for a single dCMP addition onto 22/32-mer by free RT was observed (Fig. 5), indicating the binding of free enzyme to 22/32-mer and subsequent nucleotide addition occur very rapidly (estimated pre-steady-state rate = 30 s⁻¹). However, when RT was initially complexed with 22G/32-mer or 22T/32-mer dissociation is prerequisite to 22/32-mer extension. In these cases, the pre-steady-state burst of nucleotide addition onto 22/32-mer was not observed (Fig. 5), indicating that dissociation of enzyme from the mispaired primer-template is rate-limiting. A nearly identical profile was observed with RT pre-equilibrated with 23/32-mer (Table I), indicating similar rates of RT dissociation from a paired or mispaired primer-template. This is further supported by computer simulation of the data in Fig. 5 to the mechanism in Scheme I:

\[
RT-DNA \xrightarrow{k_{pr}} RT + DNA \xrightarrow{k_{pc}} RT + dCMP \xrightarrow{k_m} RT + 23/32-mer
\]

where \( k_{pr} \) and \( k_{pc} \) are the dissociation rates of RT-DNA and RT-22/32-mer, respectively, and \( k_m \) is the rate of [α-32P]dCMP addition onto 22/32-mer. An \( k_{pc} \) value of 30 s⁻¹ was estimated by simulating the data for dCMP addition by free enzyme to above mechanism. Based on this \( k_{pc} \) value, simulated fitting constraints the dissociation rate (\( k_{pc} \)) of enzyme from paired and mispaired primer termini to a value between 0.2 s⁻¹ and 0.3 s⁻¹. The sensitivity of this method in constraining \( k_{pc} \) is illustrated by the hypothetical curves (Fig. 5, broken lines) generated with \( k_{pc} \) values of 5 s⁻¹, 1 s⁻¹, or 0.01 s⁻¹. These results indicate similar lifetimes among the complexes of RT and primer-template having a paired or mispaired primer terminus.

Pyrophosphorolysis of Extended Mispaired Primer-Templates—As discussed above, the substoichiometric burst in extension of mispaired terminus could be due to a rapid equilibration at the chemical step, followed by a rate-limiting step. To test this possibility, the parameters for the HIV RT-catalyzed pyrophosphorolysis of the extended form of a mispaired primer-template was examined by employing [3',5'-32P]22/32-mer which contain a mismatch at the 3' penultimate position of the primer opposite a template dG. First-order kinetics were observed in single-turnover pyrophosphorolysis of 22T/32-mer (Fig. 6, inset). These rates display a hyperbolic dependence on PPi concentration, yielding a maximal rate (\( k_{pyn} \)) of 0.09 s⁻¹ and

![Figure 5](image-url)

**Fig. 5.** Dissociation of HIV RT from primer/template. RT (100 nM) pre-equilibrated with 23/32-mer (●), 22G/32-mer (○), 22T/32-mer (□), or none (■) was mixed with unlabeled 22/32-mer (4 μM) and [α-32P]dCTP (10 μM, 5 Ci/mmol) as described. The formation of [α-32P]dCMP was monitored as a function of time. The solid lines are computer simulations of the data to Scheme I with \( k_{pc} \) = 30 s⁻¹, \( k_m \) = 0.3 s⁻¹, and \( k_{pc} \) = 0.2 s⁻¹. The broken lines are generated by simulation using the model in Scheme I with above \( k_{pc} \) and \( k_m \) values, and a \( k_m \) value of 5 s⁻¹, 1 s⁻¹, or 0.01 s⁻¹.

Table III: Parameters for dTMP addition onto mismatched primer/template

<table>
<thead>
<tr>
<th>Primer-template</th>
<th>( K_D )</th>
<th>( k_{pr} )</th>
<th>( k_{pc} )</th>
<th>( k_{pc}/K_D )</th>
<th>Relative propensity</th>
<th>Elemental effect</th>
</tr>
</thead>
<tbody>
<tr>
<td>5'-AC</td>
<td>1.4</td>
<td>3.6</td>
<td>1.1</td>
<td>1.1</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>3'-TG</td>
<td>2.7</td>
<td>1.2</td>
<td>1.1</td>
<td>1.1</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>5'-AC</td>
<td>270</td>
<td>42</td>
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<td>1.1</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>3'-TG</td>
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<td>15</td>
<td>1.1</td>
<td>1.1</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>5'-AA</td>
<td>690</td>
<td>1.4</td>
<td>1.1</td>
<td>1.1</td>
<td>1</td>
<td>1</td>
</tr>
</tbody>
</table>

a See Table II.

b Calculated as dTMPoS addition rate.

c All parameters for dTMP addition on correct 23/32-mer are from Hsieh et al. (1993).

d Not determined.
otide addition reaction: the dNTP binding step and a subsequent conformational change that precedes the covalent chem- 

tation. A 500-500,000-fold discrimination against incorrect 
dNTP selection by a factor of 25-240, corresponding to a free 
nucleus, further clarifying mechanisms utilized by this enzyme to 
excision of mispaired termini (Tables II and III) indicates HIV RT 
discriminates against incorrect dNTPs more vigorously than 
against mispaired primer termini. This has been observed with 
avian myeloblastosis virus reverse transcriptase (35) but not 
with Drosophila melanogaster DNA polymerase α (35), suggesting 
that differential discrimination may be a characteristic of 
retroviral DNA polymerases.

Previous steady-state studies (11, 12) have indicated that 
discrimination against an incorrect dNTP by the HIV enzyme is 
due mainly to a dramatic increase in $K_\alpha$ with a slight decrease in $V_{\text{max}}$. These steady-state parameters are not equivalent to 
the pre-steady-state parameters $K_\alpha$ and $k_{\text{cat}}$ reported here. By 
identifying the steps governed by $K_\alpha$ and $k_{\text{cat}}$ for correct and 
incorrect nucleotide addition, we are able to directly compare these 
pre-steady-state parameters to assess nucleotide selectivity. Such an assessment based on steady-state parameters is 
problematic due to the difficulty in relating steady-state parameters to fundamental steps of mechanism. The steady-state 
$V_{\text{max}}$ for addition of a correct nucleotide is limited by dissociation 
of DNA (24, 26, 27); however, $V_{\text{max}}$ for misincorporation may be limited by a different step, as illustrated by our finding that 
certain dNTPs misincorporate more slowly than dissociation 
of DNA, while others misincorporate faster. Thus, it is 
generally not appropriate to compare the steady-state rates of 
correct and incorrect nucleotide incorporation as the rate-limiting 
steps for the two kinds of reaction may differ. In addition, since the $K_\alpha$ parameter for a multistep reaction is in general 
a complex function of rate constants, this parameter cannot be 
directly related to steps in the mechanism of nucleotide addition.

Our results predict frequent dTTP/dG misincorporation by 
HIV RT and are consistent with the report that G → A transi-
tion is the most frequent base substitution error observed (5,
Furthermore, our results indicate misinsertion of dCMP opposite a template dC is a rare event, consistent with the failure to detect C → G mutation in a gapped DNA synthesis assay that can score 219 different single-base substitution errors at 113 template positions (5). The propensity for other misincorporation events reported here are in general agreement with the average base substitution error rate of 6 × 10⁻⁵ estimated for HIV RT from in vitro DNA synthesis reactions (5). With the exception of dTMP incorporation opposite a template dC, the nucleotide selectivity of HIV RT is comparable to estimated for HIV RT from ways such as primer-template slippage.

REFERENCES