Effects of Primer-Template Sequence on ATP-dependent Removal of Chain-terminating Nucleotide Analogues by HIV-1 Reverse Transcriptase*

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HIV-1 reverse transcriptase can remove chain terminators from blocked DNA ends through a nucleotide-dependent mechanism. We show that the catalytic efficiency of the removal reaction can vary several hundred-fold in different sequence contexts and is most strongly affected by the nature of the base pair at the 3′-primer terminus and the six base pairs upstream of it. Similar effects of the upstream sequence were observed with primer-templates terminated with 2′,3′-dideoxy-AMP, 2′,3′-dideoxy-CMP, or 2′,3′-dideoxy-GMP. However, the removal of 2′,3′-dideoxy-TMP or 3′-azido-2′,3′-dideoxy-TMP was much less influenced by upstream primer-template sequence, and the rate of excision of these thymidylate analogues was greater than or equal to that of the other chain-terminating residues in each sequence context tested. These results strongly indicate that the primer terminus and adjacent upstream base pairs interact with reverse transcriptase in a sequence-dependent manner that affects the removal reaction. We conclude that primer-template sequence context is a major factor to consider when evaluating the removal of different chain terminators by HIV-1 reverse transcriptase.

Nucleoside analogues are commonly used in treatment of human immunodeficiency virus type 1 (HIV-1)1 infection. After they are phosphorylated to the active triphosphate form, these inhibitors can be incorporated by the viral reverse transcriptase (RT) into nascent viral DNA. Since the drugs lack a 3′-OH group, their incorporation prevents further elongation of the DNA chain, and they must be removed before DNA synthesis can resume. Removal can occur through pyrophosphoryloration catalyzed by RT (1) or through a reaction related to pyrophosphorylation (2), in which the β- and ϒ-phosphates of a nucleoside triphosphate serve as a PPi analogue to attack and excise the 3′-terminal nucleotide of the primer, producing an unblocked primer shortened by one base and a dinucleoside polypophosphate containing the removed nucleotide analogue linked to the NTP acceptor substrate.

Several naturally occurring mutations have been described in RT that increase nucleotide-dependent removal, including mutations associated with thymidine analogue resistance (M41L, D67N, K70R, L210W, T215Y or T215F, and K219Q; also called T-analogue resistance mutations) (3–9) and T69S-XX finger insertion mutations, which are usually accompanied by T-analogue resistance mutations and confer broad resistance to a variety of nucleoside inhibitors (10–13). Mutations have also been described in HIV-1 RT that confer decreased removal activity including A114S (14), W88G (15), and K65R (15, 16). Modeling of ATP into the crystal structure of the HIV-1 RT-DNA primer-template-dNTP complex has suggested that the aromatic ring in ATP can form π-π interactions with the aromatic ring in T215Y or F mutant protein (6, 17). When the π-π interaction was strengthened by providing a nucleotide substrate that favors this interaction, the efficiency of excision by the mutant enzyme was preferentially enhanced (8). When the π-π interaction was disfavored by a nucleotide substrate containing a nonaromatic ring structure, the removal reaction was much less efficient and only slightly enhanced by the T-analogue resistance mutations (8). Studies to identify other interactions between mutant or wild type RT and the excision acceptor substrates may aid in the development of drugs that are refractory to removal. Whereas there is general agreement that the primer-unblocking reaction is an important mechanism for HIV-1 resistance, the preference of RT to remove different chain terminating nucleotides is unclear when results from different laboratories are compared. For example, our laboratory has reported that 2′,3′-dideoxy-AMP (ddAMP) is readily removed from a ddAMP-terminated primer (2, 8, 13), whereas other investigators have reported that it is removed very inefficiently (7). An important difference between these studies is the use of primer-templates (P/Ts) with different sequences. Several activities of HIV-1 RT have been shown to depend on sequence context, including preferential pausing at specific sequences during incorporation (18), misincorporation of nucleotides at hotspots for mutations (19), and discrimination between nucleotide analogues and natural substrates during DNA chain elongation (20). Although effects of sequence context on nucleotide-dependent primer-unblocking activity have been reported for HIV-1 RT (3, 21–23), these effects have not been studied in detail. The rate of pyrophosphoryloration by bacteriophage T7 DNA polymerase has also been reported to be dependent on sequence context (24, 25).

We therefore performed a systematic, quantitative study on
the effects of P/T sequence on the catalytic efficiency ($k_{\text{excis}}/K_d$) of the removal reaction. Removal efficiency varied up to 360-fold depending on sequence context and was most influenced by the nucleotides in the seven base pairs of P/T duplex adjacent to and including the primer terminus. P/Ts terminated with ddAMP, ddCMP, or ddGMP were highly sensitive to sequence context, whereas P/Ts terminated with ddTMP or 3'-azido-2',3'-dideoxy-TMP (AZTMP) were much less affected. In addition, the removal of ddTMP or AZTMP was more efficient than removal of ddAMP, ddCMP, or ddGMP. 

These results suggest that ddTMP or AZTMP at the primer terminus gives a complex that is more active in the unblocking reaction and less influenced by sequence-specific interactions between RT and P/T.

EXPERIMENTAL PROCEDURES

Materials—His-tagged RT containing wild type RT (HIV-1 RTWT), the M41L/T69S-AG/L210W/R211K/L214F/T215Y mutations (HIV-1 RTMDR) or the D67N/K70R/T215Y/K219Q mutations (HIV-1 RTAZT) were expressed and purified as previously described (2, 8, 13). The specific RNA-dependent DNA polymerase activities of HIV-1 RTWT, HIV-1 RTMDR, and HIV-1 RTAZT were 20,000, 27,000, and 7,600 units/mg, respectively, where 1 unit is equal to the amount of enzyme required for incorporation of 1.0 nmol of [3H]dTMP in 10 min when using poly(rA)/oligo(dT) as the substrate (26). These specific activities are consistent with values reported elsewhere in the literature (26–35) and correspond to turnover numbers of 5–9 s$^{-1}$ for RTWT, 7–12 s$^{-1}$ for RTMDR, and 2–3 s$^{-1}$ for RTAZT, assuming that 50–90% of the enzyme molecules are active for each preparation. Poly(rA) and oligo(dT) were from Amersham Biosciences. Other oligonucleotides were obtained from Sigma; M13 DNA was from New England Biolabs. [3H]dTTP was from PerkinElmer, and [α-32P]ddATP and [α-32P]ddNTPs were from Amersham Biosciences. [α-32P]AZTTP was prepared as previously described (3).

Generation of Products Terminated with [32P]ddAMP at Different Positions—M13 single-stranded template was annealed with complementary primer 1 (5'-AAGTTGGGTAACGCCAGGGTTTTCC-AGTCACGAC-3') or primer 2 (5'-ACTCTAGAGGATCCCCGGGTACC-GAGCTCGAATTC-3'), and 5 nM P/T was incubated with 100 nM HIV-1 RTWT, 1 μM [α-32P]ddATP, and 10 μM each of the four dNTPs in reaction buffer (40 mM NaHEPES, pH 7.5, 20 mM MgCl$_2$, 60 mM KCl, 1 mM dithiothreitol, 2.5% glycerol, and 80 μg of bovine serum albumin per ml) for 30 min at 37 °C. The products were separated twice with phenol/chloroform/isooamylalcohol (25:24:1), ethanol-precipitated, and resuspended in DNA buffer (10 mM NaHEPES, pH 7.5, and 40 mM KCl).

ATP-dependent Removal of ddAMP from Products Terminated at Different Positions—Five nM of 3'-[32P]ddAMP-terminated products were incubated with 100 nM HIV-1 RTMDR and 100 nM unlabeled ddATP (to prevent reincorporation of the removed, labeled chain terminator) in reaction buffer containing either no acceptor substrate for removal, 3.2 mM ATP, or 50 μM PP$i$ at 37 °C for 2.5, 5, 10, 20, or 40 min. Reactions were stopped by boiling for 5 min, and the products were separated by electrophoresis through a 20% urea-polyacrylamide gel. The radioactivity in the 3'-[32P]ddAMP-terminated products was quantitated by phosphorimaging, and the amount of ATP- or PP$i$-dependent removal was calculated for each time point. All nucleotides used were...
**Table I**

| Position | ATP-dependent removal | PP_i-dependent removal | Position | ATP-dependent removal | PP_i-dependent removal |
|----------|-----------------------|------------------------|----------|-----------------------|------------------------|
| A1       | −−−−−−               | −−−−−−                | A1       | −−−−−−               | −−−−−−                |
| A2       | ++++++               | ++++++                | A2       | ++++++               | ++++++                |
| A3       | ++                   | ++                    | A3       | ++                   | +                     |
| A4       | ++++                 | ++++                  | A4       | ++++                 | ++++                  |
| A5       | −−−−−−               | −−−−−−                | A5       | −−−−−−               | −−−−−−                |
| A6       | ++                   | +                     | A6       | −                     | +                     |
| A7       | +−                   | −                     | A7       | +−                   | −                     |
| A8       | ++++                 | ++++                  | A8       | ++++                 | ++++                  |
| A9       | −−−−−−               | −−−−−−                | A9       | −−−−−−               | −−−−−−                |
| A10      | −−−−−−               | −−−−−−                | A10      | −−−−−−               | −−−−−−                |
| A11      | ++++                 | ++++                  | A11      | ++++                 | ++++                  |
| A12      | −−−−−−               | −−−−−−                | A12      | −−−−−−               | −−−−−−                |
| A13      | +−                   | −                     | A13      | +−                   | −                     |
| A14      | ++++                 | ++++                  | A14      | ++++                 | ++++                  |
| A15      | ++++                 | ++++                  | A15      | ++++                 | ++++                  |
| A16      | ++++                 | ++++                  | A16      | ++++                 | ++++                  |
| A19      | ++++                 | ++++                  | A19      | ++++                 | ++++                  |
| A20      | ++++                 | ++++                  | A20      | ++++                 | ++++                  |
| A21      | +−                   | −                     | A21      | +−                   | −                     |
| A22      | ++++                 | ++++                  | A22      | ++++                 | ++++                  |
| A23      | −−−−−−               | −−−−−−                | A23      | −−−−−−               | −−−−−−                |
| A24      | ++++                 | ++++                  | A24      | ++++                 | ++++                  |
| A25      | ++++                 | ++++                  | A25      | ++++                 | ++++                  |
| A26      | ++++                 | ++++                  | A26      | ++++                 | ++++                  |
| A27      | ++++                 | ++++                  | A27      | ++++                 | ++++                  |
| A28      | ++++                 | ++++                  | A28      | ++++                 | ++++                  |
| A29      | ++++                 | ++++                  | A29      | ++++                 | ++++                  |
| A30      | ++++                 | ++++                  | A30      | ++++                 | ++++                  |

Removal of ddAMP from Multiple Termination Sites in the M13 Sequence—In order to get an approximate measure of the magnitude of the effect of sequence context on the efficiency of removal by HIV-1 RT, ATP- and PP_i-dependent removal of [32P]ddAMP from multiple termination sites in M13 primer-template was determined (Fig. 1). M13 single-stranded DNA was annealed with either primer 1 or primer 2 (for sequence, see the bottom of Fig. 1) and incubated with HIV-1 RT WT, all four dNTPs, and [α-32P]ddATP to extend the primers and create a mixture of products chain-terminated and 3’-end-labeled at each A incorporation site. The mixture of products was purified and incubated with HIV-1 RT MDR in the absence of an acceptor substrate for removal or in the presence of either 3.2 mM ATP or 50 μM PP_i (Fig. 1). HIV-1 RT MDR was used in most of these experiments because this enzyme supports ATP-dependent removal of ddAMP at a rate that is >30-fold greater than WT RT (13). In the absence of an acceptor substrate (Fig. 1, left lanes), the amount of radioactivity recovered in the chain-terminated products remained constant with incubation time. On the other hand, incubation with either ATP (Fig. 1, middle lanes) or PP_i (Fig. 1, right lanes) led to a time-dependent decrease in radioactivity recovered in most of the chain-terminated products corresponding to removal of the [32P]ddAMP residue through the action of RT.

Removal was very efficient for certain termination products (e.g. A15, A16, A21, and A22), whereas others remained virtually unchanged, even after a 40-min incubation (e.g. A5, A7, and A23). Table 1 (left columns) contains a summary of ATP- and PP_i-dependent removal at each of the first 30 ddA-termination sites (A1–A30) by HIV-1 RT MDR. The rate of removal ranged from very slow (less than 15% removed in 40 min) for A1, A9, and A10 to very fast (more than 50% removed in 2.5 min) for A15, A22, and A24. There was good agreement between susceptibility to ATP- and PP_i-dependent removal, suggesting that the sequence context affects properties of the removal reaction shared by both substrates. Similar experiments were carried out with HIV-1 RT AZT (Table I, right columns), pretreated with thermostable pyrophosphatase (25 μmol of nucleotide was treated with 5 units thermostable pyrophosphatase (Roche Applied Science) in 390 μl of reaction buffer at 75 °C for 20 min. ATP-dependent Removal of Chain Terminators from Synthetic DNA/DNA Oligonucleotide P'Ts—A15P primer (5'-ATGGCTGAGTGGTCAC-TCTAGGATCCCCGGGAT-3') annealed to A15T template (5'-CGAATTCCGAGCTCGGAC-GT-3') or A25P (5'-CGGATCCGAGCTCGGAC-GT-3') annealed to A25T (5'-CACACGAGGAACTCGTTAATCAGTGC-3') or A25T (5'-GCTTAGCTGAGCTCGGAC-GT-3') or A25P (5'-CGGATCCGAGCTCGGAC-GT-3') annealed to A25T (5'-CACACGAGGAACTCGTTAATCAGTGC-3') were 3'-5'-labeled and ddAMP-terminated by incorporation of [32P]ddAMP as previously described (2). Five nm 3'-[32P]ddAMP-terminated P'T was incubated with 200 nm HIV-1 RT MDR, 100 nm unlabeled ddATP, and varying concentrations of ATP for an amount of time allowing a maximum of ~30–35% Ap_ddA formation. Products were separated by electrophoresis through a 20% denaturing polyacrylamide gel. The radioactivity in the products shown in Fig. 1 (and data not shown) was quantitated by phosphorimaging, and the amount of removal was calculated as a function of incubation time and classified as follows: ++++, >50% removal in 2.5 min; ++, >50% removal in 5 min; +++, >50% removal in 10 min; +, >50% removal in 20 min; +−, >50% removal in 40 min; −, 30–50% removal in 40 min; −−, 15–30% removal in 40 min; −−−, <15% removal in 40 min.
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and, although ATP-dependent removal was slower and PP-dependent removal was slightly faster than with HIV-1 RT<sup>MDR</sup>, the relationship between rates of removal and the termination site was similar.

**ATP-dependent Removal of ddAMP from Oligonucleotides Corresponding to a Poor Substrate (A23) and a Good Substrate (A15) for the Removal Reaction**—Two termination sites, A15 and A23, were chosen for further characterization. DNA 34-mer primers and 50-mer templates corresponding to each of these M13 sequences were synthesized, purified, annealed, and termininated with [<sup>32</sup>P]ddAMP. The chain-terminated P/Ts were incubated with HIV-1 RT<sup>MDR</sup> and varying concentrations of ATP. The rates of Ap<sub>4</sub>ddA synthesis (k<sub>obs</sub>) were determined as described in B and plotted versus ATP concentration. B, single turnover experiments were performed for P/T pairs that support different rates of ATP-dependent removal of ddAMP to compare the values of k<sub>obs</sub> determined by analysis of the complete turnover reaction with values estimated from the linear portion of the reaction curve. 5 nM [<sup>32</sup>P]-ddAMP-terminated P/T was incubated with 200 nM HIV-1 RT<sup>MDR</sup>, 100 nM unlabeled ddATP, and 3.2 mM ATP for the indicated times. Radioactivity recovered in primer and Ap<sub>4</sub>ddA were quantitated by phosphorimaging, and the fraction of radioactivity recovered in Ap<sub>4</sub>ddA was determined for each incubation time. Experimental data were fitted to the single exponential, k<sub>obs</sub> = A(1 - e<sup>-t/k<sub>obs</sub></sup>), where A is the amplitude of the reaction, k<sub>obs</sub> is the apparent rate constant for the synthesis of Ap<sub>4</sub>ddA, and t is the time in seconds. The curves represent fits with k<sub>obs</sub> = 2.1 × 10<sup>-3</sup>/s for A23 × 2-11 P/T, k<sub>obs</sub> = 0.50 × 10<sup>-3</sup>/s for A23-4G P/T, and k<sub>obs</sub> = 0.18 × 10<sup>-3</sup>/s for A23 P/T. For comparison, the value of k<sub>obs</sub> was also determined from the fraction of counts in Ap<sub>4</sub>ddA measured at a single time point in the linear phase of the reaction. For A23 × 2-11 P/T, k<sub>obs</sub> was 2.4 × 10<sup>-3</sup>/s (60-s incubation); for A23-4G P/T, k<sub>obs</sub> = 0.59 × 10<sup>-3</sup>/s (360-s incubation); and, for A23 P/T, k<sub>obs</sub> = 0.09 × 10<sup>-3</sup>/s (3600-s incubation). The sequence of A23 P/T is given under “Experimental Procedures.” A23 × 2-11 P/T is described in C. A23-4G P/T is the same as A23 except for a CG to GC substitution at position -4 (see Table II). In many assays, especially at lower ATP concentrations, the fraction of product formed even after prolonged incubation was not sufficient for a reliable fit to the single exponential equation; however, the initial rate could be readily determined. Therefore, reaction rates were derived from the linear portion of the reaction curve. C. ATP-dependent removal of ddAMP from A15 P/T, A23 P/T, and A23-derived P/Ts with segments of the A23 sequence replaced by A15 sequences. k<sub>obs</sub> values were plotted versus ATP concentration as in A and fitted to hyperbole to obtain the maximum excision rate (k<sub>excis</sub>) and the dissociation constant for ATP (K<sub>e</sub>). The catalytic efficiency (k<sub>excis</sub>/K<sub>e</sub>) is shown for each P/T. The filled bars, sequences derived from the A15 P/T; open bars, sequences from the A23 P/T. Catalytic efficiencies were determined in triplicate and are given as the mean ± S.D.

**Effects of Base Substitutions in the A23 P/T on ATP-dependent Removal of ddAMP**—The contribution to ATP-dependent removal of each of the 10 base pairs upstream of the 3′-primer terminus was assessed by measuring the catalytic efficiencies of ATP-dependent removal of ddAMP from 30 A23-derived primer-templates, each containing a different, single nucleotide change at one of the 10 positions in the primer (and corresponding change in the template) (Fig. 3 and Table II).
The nucleotide at each position in the original A23 primer sequence is shaded. Substitution at several positions resulted in large increases in removal; a change from dC to dA or dG at position -4 increased removal by 7- and 5-fold, respectively, whereas a change from dT to dG at position -5 increased removal by 5-fold. Other changes led to more modest increases in removal (dT to dA at position -2, 3-fold increase; da to dT at position -3, 2-fold increase; dT to dA at position -5, 2-fold increase). Although the A23 P/T sequence was an inefficient substrate for removal, sequence changes at some positions decreased removal activity even further. A change from a dG to da at position -6 decreased removal by 6-7-fold, and a change from dG to dC at position -7 decreased it by about 4-fold. Most other substitutions changed the efficiency of removal less than 1.6-fold, including all changes at positions -8 to -11.

Next, we determined the effects of introducing multiple changes into the A23 P/T sequence (Table III). Introducing the three substitutions that had conferred the lowest removal (as determined from Table II), dG at position -3, da at position -6, and dc at position -7, led to a decrease in removal of about 15-fold, whereas introducing the three changes that increased removal the most (da at position -2, da or dg at position -4, and dg at position -5) conferred an increase of about 24-fold in ddAMP removal. Therefore, the effects of upstream sequence substitutions were slightly less than additive.

Comparison of Sequences Preceding Sites in M13 That Are “Poor” Substrates for Removal with Those Preceding Sites That Are “Good” Substrates for ddAMP Removal—To assess whether the effects of upstream sequence we had observed were unique to the A23 P/T sequence, we compared the primer sequences of the 10 bases upstream of six poor removal sites and of 10 efficient removal sites in the M13 sequence (Table IV). Base frequencies at each position are summarized in Table V. Among the poor removal sites, da did not appear at primer position -2, dt did not appear at position -3, and dG did not appear at position -4 or -5, which is in very good agreement with the fact that introducing these bases at positions -2, -3, -4, and -5 in the A23 P/T sequence increased removal activity (Table II). On the other hand, of the 10 termination sites that were efficient substrates for removal, da occurred four times at primer position -2, dT five times at position -3, and dG six times at position -4. Strikingly, none of them had da at position -6.

These results are in good agreement with the effects seen on removal by changes in the A23 P/T sequence (Table II), with some exceptions. The introduction of dt at position -6 in the A23 P/T sequence was associated with little change in the rate of removal, whereas 7 of 10 sites with efficient removal have a dt at this position. In addition, dc at position -7 in A23 P/T conferred a 4-fold decrease in rate of removal, yet 2 of 10 termination sites in the M13 sequence that supported efficient removal had dc at position -7. These exceptions suggest that our understanding of the role of the sequence elements at positions -6 and -7 is still incomplete.

In summary, our data suggest that introduction of specific bases at certain positions in a DNA primer, including da at primer position -11, resulted in significant increases in removal activity. These results are in good agreement with the effects seen on removal by changes in the A23 P/T sequence, with some exceptions. The introduction of dt at position -6 in the A23 P/T sequence was associated with little change in the rate of removal, whereas 7 of 10 sites with efficient removal have a dt at this position. In addition, dc at position -7 in A23 P/T conferred a 4-fold decrease in rate of removal, yet 2 of 10 termination sites in the M13 sequence that supported efficient removal had dc at position -7. These exceptions suggest that our understanding of the role of the sequence elements at positions -6 and -7 is still incomplete.

In summary, our data suggest that introduction of specific bases at certain positions in a DNA primer, including da at

### Table II

| Primer Position | A   | C   | G   | T   |
|-----------------|-----|-----|-----|-----|
| -11             | 0.72 (1.1X) | 0.052 (0.83X) | 0.053 (0.83X) | 0.063 (1.0X) |
| -10             | 0.083 (1.3X) | 0.061 (1.0X) | 0.061 (1.0X) | 0.059 (0.94X) |
| -9              | 0.063 (1.0X) | 0.057 (0.90X) | 0.057 (0.90X) | 0.047 (0.75X) |
| -8              | 0.048 (0.76X) | 0.054 (0.86X) | 0.054 (0.86X) | 0.063 (1.0X) |
| -7              | 0.044 (0.70X) | 0.015 (0.24X) | 0.063 (1.0X) | 0.088 (1.4X) |
| -6              | 0.0093 (0.15X) | 0.066 (1.0X) | 0.063 (1.0X) | 0.050 (0.79X) |
| -5              | 0.13 (2.1X) | 0.059 (0.94X) | 0.32 (5.2X) | 0.063 (1.0X) |
| -4              | 0.45 (7.1X) | 0.063 (1.0X) | 0.29 (4.6X) | 0.088 (1.4X) |
| -3              | 0.063 (1.0X) | 0.055 (0.87X) | 0.044 (0.7X) | 0.13 (2.1X) |
| -2              | 0.20 (3.2X) | 0.11 (1.7X) | 0.10 (1.6X) | 0.063 (1.0X) |

*Numbers in parentheses show k<sub>cat</sub>/K<sub>d</sub> expressed as a ratio to the value obtained with ddAMP-terminated A23 P/T.
position −2, dT at position −3, dG at position −4 or −5, and possibly dT at position −6 (with the corresponding changes in the template strand) confer an increased rate of ATP-dep-

ent removal of dDMP from the primer terminus, whereas the presence of a dA at position −6 or dC at position −7 produces a primer-template that is less susceptible to this reaction.

**Effects of Upstream Sequence on ATP-dependent Removal of ddCMP, ddGMP, ddTMP, and AATMP**—To compare the removal of different chain-terminating residues, A23P/T pairs were prepared that differed only at the site of chain termina-

tion. This was accomplished by annealing A23P with different templates that were identical to A23T except that the dT at position 35 (from the 3′-end) was changed to dG, dC, or dA. When the 34-mer oligonucleotide A23P was annealed to these templates, they could be terminated with ddCMP, ddGMP, or ddTMP/AATMP, respectively. Additional P/T pairs were synthesized that contained single base substitution at position −4, −5, or −6, in addition to the altered base pair at the primer terminus. The catalytic efficiency of ATP-dependent removal for each of these P/T pairs is shown in Table VI. Comparison of rates of removal when only the chain terminator was changed (compare shaded entries in Tables II and VI) shows that re-

moval of ddCMP or ddGMP in this sequence context occurred with about equal efficiency (top two parts of Table VI), removal of ddAMP occurred about 2.5 times more efficiently (Table II), and removal of ddTMP occurred about 13 times more efficiently (third part of Table VI). In contrast to the other experiments in

**Table III**

**Effects of multiple base pair substitutions in the upstream region of A23 P/T on ATP-dependent removal**

Oligonucleotides (34-mer/50-mer) corresponding to the A23 sequence or containing the indicated base substitutions at the indicated positions in the primer and corresponding complementary base substitutions in the template were chain-terminated with [32P]ddAMP and incubated with HIV-1 RT

**Table IV**

**Upstream primer sequences of termination sites that are poor versus good substrates for ATP-dependent removal**

Shown are the sequences of the 10 primer positions immediately upstream of the primer terminal ddAMP in M13 that were poor (<30% ATP-dependent removal in 40 min; Table I) (top) or good removal sites (>50% ATP-dependent removal in 5 min; Table I) (bottom) for ATP-dependent removal. The residues are numbered from the 3′-end of the primer, with the chain-terminating residue defined as position −1.

| Termination site | Upstream primer sequences of poor removal sites |
|------------------|-----------------------------------------------|
|                  | −11 | −10 | −9 | −8 | −7 | −6 | −5 | −4 | −3 | −2 | −1 |
| A1 | 5′- | A | C | G | A | C | G | T | T | G | T | ddA |
| A5 | 5′- | T | G | T | A | A | A | A | C | G | ddA |
| A9 | 5′- | C | C | A | A | G | C | T | G | C | ddA |
| A10 | 5′- | G | C | T | G | C | T | G | C | ddA |
| A12 | 5′- | G | G | T | C | G | A | C | T | C | ddA |
| A23 | 5′- | T | C | A | T | G | G | T | C | A | ddA |

| Termination site | Upstream primer sequences of good removal sites |
|------------------|-----------------------------------------------|
|                  | −11 | −10 | −9 | −8 | −7 | −6 | −5 | −4 | −3 | −2 | −1 |
| A15 | 5′- | A | T | C | C | C | C | G | G | G | T | ddA |
| A16 | 5′- | C | C | G | G | G | T | A | C | C | G | ddA |
| A20 | 5′- | C | G | A | A | T | G | T | A | A | T | ddA |
| A21 | 5′- | A | T | G | G | T | A | T | G | G | T | ddA |
| A22 | 5′- | T | C | C | T | G | T | G | T | G | ddA |
| A24 | 5′- | T | C | G | T | G | T | G | T | G | ddA |
| A26 | 5′- | T | G | A | A | A | T | G | T | G | ddA |
| A27 | 5′- | T | C | C | G | C | T | C | A | C | ddA |
| A30 | 5′- | T | C | C | G | C | T | C | A | C | ddA |

**Table V**

**Summary of the upstream sequences of ddAMP-termination sites in M13 that are poor versus good substrates for ATP-dependent removal**

Shown is the prevalence of each base at each of the 10 positions upstream of the primer terminus in six termination sites that are poor substrates for removal (<30% ATP-dependent removal in 40 min; Table I) (top) or 10 termination sites that are good substrates for removal (>50% ATP-dependent removal in 5 min; Table I) (bottom). The residues are numbered from the 3′-end of the primer, with the chain-terminating residue defined as position −1.

| Base | Summary of upstream sequences of six poor removal sites |
|------|-----------------------------------------------------|
|      | −11 | −10 | −9 | −8 | −7 | −6 | −5 |
| A    | 1   | 0   | 3  | 2  | 1  | 2  | 1  |
| C    | 1   | 4   | 0  | 1  | 1  | 2  | 2  |
| G    | 2   | 1   | 2  | 0  | 4  | 2  | 0  |
| T    | 2   | 1   | 1  | 3  | 0  | 3  | 4  |

| Summary of upstream sequences of 10 good removal sites |
|-----------------------------------------------------|
| −11 | −10 | −9 | −8 | −7 | −6 | −5 |
| A   | 3   | 1   | 2  | 2  | 2  | 0  | 2  | 2  | 1  | 4  |
| C   | 3   | 4   | 4  | 4  | 2  | 2  | 2  | 1  | 2  | 2  |
| G   | 0   | 2   | 1  | 3  | 3  | 2  | 3  | 6  | 2  | 2  |
| T   | 4   | 3   | 3  | 1  | 3  | 7  | 3  | 3  | 5  | 2  |
this study, these results may be influenced by the structural differences in the group being transferred in the reaction as well as by the change in sequence context.

Substitutions at position -4, -5, or -6 had effects on ddCMP and ddGMP removal similar to those shown in Table II for ddAMP removal. Replacement of the nucleotide at position -4 with dA or dG in the primer strand (and the complementary base in the template strand) resulted in a 4–7-fold increase in the rate of removal for all three chain terminators; replacement of the nucleotide at position -5 with dG increased the rate of removal by 5–6-fold; and substitution at position -6 with dA decreased the rate of removal by 7–11-fold. In contrast, the effect on the rate of removal of ddTMP was much less (at most a 1.6-fold increase for substitutions at position -4 or -5 and a 2.2-fold decrease for substitution at position -6). These comparisons are shown in Fig. 4. It is apparent that removal of ddAMP, ddCMP, or ddGMP. As with ddTMP-terminated P/Ts, there was little effect of upstream sequence changes on removal efficiency of AZTMP.

### DISCUSSION

Our data have revealed large differences (several hundred-fold) in the efficiencies of removal of nucleotide analogues from blocked primer-templates due to sequence context. We show that the main determinants of the sequence effect are located at the 3'-end of the primer, with the chain-terminating residue defined as position -1.

### TABLE VI

Effects of single base pair substitutions in the upstream region on ATP-dependent removal of ddCMP, ddGMP, ddTMP, or AZTMP

Oligonucleotides (34-mer/50-mer) corresponding to the A23 sequence, but with a base change at the first single-stranded position of the template to allow termination with the indicated chain terminator and containing a single base substitution at the indicated position in the primer (and complementary change in the template) were chain-terminated with [32P]ddCMP (top part), [32P]ddGMP (second part), [32P]ddTMP (third part), or [32P]AZTMP (bottom part) and incubated with HIV-1 RT<sup>MQR</sup> and ATP as described in the legends to Figs. 1 and 2 to obtain the catalytic efficiency (the average of three experiments) for each P/T pair and the relative catalytic efficiency (in parentheses) compared to the P/T pair without substitutions at primer positions -4, -5, or -6 (shaded) terminated with ddCMP (A), ddGMP (B), ddTMP (C), or AZTMP (D). S.D. for all $k_{exc}/K_d$ values was ±40%. The residues are numbered from the 3'-end of the primer, with the chain-terminating residue defined as position -1.

#### Table VI

| Primer position | A         | C         | G         | T         |
|-----------------|-----------|-----------|-----------|-----------|
| -4              | 0.18 (6.7X) | 0.027 (1.0X) | 0.16 (5.9X) | 0.044 (1.6X) |
| -5              | 0.081 (3.0X) | 0.061 (2.3X) | 0.17 (6.3X) | 0.027 (1.0X) |
| -6              | 0.0025 (0.093X) | 0.032 (1.2X) | 0.027 (1.0X) | 0.010 (0.37X) |

#### Table VI

| Primer position | A         | C         | G         | T         |
|-----------------|-----------|-----------|-----------|-----------|
| -4              | 0.10 (4.2X) | 0.024 (1.0X) | 0.11 (4.6X) | 0.024 (1.0X) |
| -5              | 0.056 (2.3X) | 0.028 (1.2X) | 0.13 (5.4X) | 0.024 (1.0X) |
| -6              | 0.0024 (0.10X) | 0.029 (1.2X) | 0.024 (1.0X) | 0.013 (0.54X) |

#### Table VI

| Primer position | A         | C         | G         | T         |
|-----------------|-----------|-----------|-----------|-----------|
| -4              | 0.44 (1.3X) | 0.33 (1.0X) | 0.37 (1.1X) | 0.54 (1.6X) |
| -5              | 0.33 (1.0X) | 0.37 (1.1X) | 0.30 (0.91X) | 0.33 (1.0X) |
| -6              | 0.15 (0.45X) | 0.23 (0.70X) | 0.33 (1.0X) | 0.18 (1.6X) |

#### Table VI

| Primer position | A         | C         | G         | T         |
|-----------------|-----------|-----------|-----------|-----------|
| -4              | 0.69 (1.5X) | 0.46 (1.0X) | 1.1 (2.4X) | 0.46 (1.0X) |
| -5              | 0.25 (0.54X) | 0.26 (0.56X) | 1.0 (2.2X) | 0.46 (1.0X) |
| -6              | 0.41 (0.89X) | 0.92 (2.0X) | 0.46 (1.0X) | 0.61 (1.3X) |
ence was observed for excision of ddAMP, ddCMP, and ddGMP, the efficiency of excision of ddTMP or AZTMP was much less dependent on the upstream sequence. PPi- and ATP-dependent removal reactions showed similar dependence on sequence context. Most experiments in this report were carried out with RTMDR because of the highly efficient nucleotide-dependent excision by this enzyme; however, parallel results were seen with RTAZT.

These results show that comparison between quantitative results obtained with different P/Ts must be interpreted with caution; however, we observed less than 4-fold variation in removal of ddTMP or AZTMP because of the highly efficient nucleotide-dependent excision by this enzyme; however, parallel results were seen with RTAZT.

The effect of RT active site relative to the primer terminus in the complex is also important. For removal to occur, the primer terminus has to be positioned in the dNTP binding site of the enzyme (termed the N-complex by Boyer et al. (6)). On the other hand, for dNTP incorporation to occur, the RT has to move forward by one base to accommodate dNTP binding (termed the P-complex (6)). Crystal structures for both the N- and P-complexes have been reported (36). When the primer lacks a 3'-OH group due to incorporation of a chain terminator, the presence of the next complementary dNTP leads to formation of a dead end complex (3, 6, 10, 23, 37–39) with the primer terminus in the P site and the dNTP in the N site, and the removal reaction is blocked.

In the absence of the incoming dNTP, it is thought that the enzyme rapidly equilibrates between the pre- and post-translocation positions, as has been proposed for RNA polymerase (40, 41). The distribution of RT molecules complexed with chain-terminated P/T in either the N or P-complexes have been reported (36). When the primer lacks a 3'-OH group due to incorporation of a chain terminator, the presence of the next complementary dNTP leads to formation of a dead end complex (3, 6, 10, 23, 37–39) with the primer terminus in the P site and the dNTP in the N site, and the removal reaction is blocked.

In the absence of the incoming dNTP, it is thought that the enzyme rapidly equilibrates between the pre- and post-translocation positions, as has been proposed for RNA polymerase (40, 41). The distribution of RT molecules complexed with chain-terminated P/T in either the N or P position may depend on several factors including the structural properties of the enzyme (10, 13), the structure of the chain terminator at the primer terminus (6, 38), and concentrations of an acceptor substrate for removal (nucleotide or PPi) and of dNTP substrates for chain elongation. There is also evidence that different P/T sequences can favor either N- or P-site occupancy by RT.

### FIG. 4. ATP-dependent removal of ddAMP, ddCMP, ddGMP, ddTMP, or AZTMP from synthetic oligonucleotides P/T pairs.

Oligonucleotide pairs (34-mer/50-mer) corresponding to the M13 sequences for incorporation of ddAMP (A23 P/T) or with a nucleotide change at template position +1 to accommodate incorporation of ddC (designated C23 P/T), ddG (G23 P/T), ddT, or AZT (T23 P/T) and with either no change in the upstream sequence or containing a single nucleotide change at upstream position −4, −5, or −6 in the primer (and corresponding change in the template) were synthesized, annealed, and chain-terminated with the appropriate 32P-labeled nucleotide analogue. The chain-terminated P/Ts were incubated with HIV-1 RTMDR and varying concentrations of ATP. The rates of Ap4ddA synthesis were determined and plotted versus ATP concentration to obtain the catalytic efficiency ($k_{cat}/K_m$) of removal for P/Ts containing the indicated nucleotide in the primer at position −4 (left panel), −5 (middle panel), or −6 (right panel). The bases in the primer strand of A23 at these positions are underlined. The symbols indicate removal efficiency for ddAMP-terminated primer-templates (closed circles), ddCMP-terminated primer-templates (open circles), ddGMP-terminated primer-templates (closed triangles), ddTMP-terminated primer-templates (open triangles), and AZTMP-terminated primer-templates (closed squares).
(23). A favorable P/T sequence could enhance the removal reaction by increasing the residency time of RT in the N position by impeding the translocation from N to P or enhancing the translocation from P to N. An increase in the length of time that the RT remains in the N-complex would increase the likelihood that removal will take place.

From crystallographic data, most DNA-protein contacts consist of weak interactions between residues forming a 60-Å DNA binding groove in HIV-1 RT (39, 42–44) and the phosphodiester backbone of the P/T. These interactions are broken and reformed during translocation by the enzyme along the P/T during processive DNA polymerization. However, the effects of sequence on pausing (18), misincorporation (19), and discrimination against nucleotide analogues (20) as well as the removal of chain terminators described in this report suggest that certain interactions between RT and P/T are sequence-specific. For the removal reaction to occur, the primer terminus must lie in the N site, where it interacts with Asp113, Tyr115, Gln151, and Asp185 in the 66-kDa subunit of RT, whereas the corresponding template nucleotide interacts with Gly352. If a “closed” configuration is induced upon binding the excision acceptor substrate (15), additional contacts can occur, including Arg72 (primer) and Leu74, Asp76, and Phe61 (template). Upstream contacts include Tyr183, Met184, and Met230 (primer positions –2 and –3) and Asn81, Glu88, and Pro157 (template positions –2 and –3). The minor groove binding track residues make contacts with template positions –4 and –5 (Ile94) and primer positions –4 to –6 (Trp566, Gly629, and Gln258) (45). Most of these interactions should occur with any P/T sequence, but sequence context may influence the strength of the interactions, e.g. through effects on the dimensions of the minor groove or the flexibility of the duplex DNA.

How can we explain the strong negative effect of dAMP at primer position –6 on removal of chain terminators? The crystal structures show changes in DNA conformation along the DNA binding groove (36, 39, 43, 44). From the primer terminus to about position –6, the DNA has an A-like structure, and upstream from there it changes to B-like structure. The transition from A-like to B-like structure is accompanied by a 40° bend in the DNA centered near base pair −9. In the binary N-complex of RT and AZT-terminated P/T (36, 42), primer position –6 interacts with RT residue Asn255. Mutations at this position confer greatly decreased processivity on both RNA and DNA template-primers (46), suggesting that Asn255 plays an important role in translocation along the P/T or in binary complex stability. From extensive studies of DNA binding proteins, it has been shown that asparagine and glutamine can form strong hydrogen bonding interactions with adenine (47). Perhaps dAMP at position –6 interacts with Asn255 in a manner that alters the conformation of the P/T, forming a complex that is deficient in the removal reaction.

Mutations in HIV-1 RT that induce resistance to phosphonoformic acid (foscarnet), a PPi analogue, confer decreased PPi-removal ability of HIV-1 RT containing foscarnet resistance mutations in vivo in vitro. This could explain why the removal reaction plays a greater role in the mechanism of resistance to T-analogues than for most other chain terminators.

Our data show the importance of sequence context for the ability of HIV-1 RT to remove chain terminators from blocked DNA chains. Whereas the mechanism of this effect is still not clear, it is evident that care must be taken to control for P/T sequence differences when comparing the removal of different chain terminators and when evaluating the susceptibility of new drugs to this activity of RT.

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Sequence Effects on ATP-dependent Removal by HIV-1 RT
Effects of Primer-Template Sequence on ATP-dependent Removal of Chain-terminating Nucleotide Analogues by HIV-1 Reverse Transcriptase
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