Using pyrrolo-deoxycytosine to probe RNA/DNA hybrids containing the human immunodeficiency virus type-1 3′ polypurine tract

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ABSTRACT

Recent structural analyses indicate that localized regions of abnormal base pairing exist within RNA/DNA hybrids containing the HIV-1 polypurine tract (PPT) and that these distortions may play a role in PPT function. To examine this directly, we have introduced pyrrolo-deoxycytosine (pdC), a fluorescent, environmentally sensitive analog of deoxycytosine (dC), into the DNA strand of PPT-containing hybrids. Steady-state fluorescence analysis of these hybrids reveals that the DNA base 11 nt from the PPT–U3 junction is unpaired even in the absence of reverse transcriptase (RT). Unstable base pairing is also observed within the (rG:dC)₆ tract in the downstream portion of the duplex, suggesting that HIV-1 RT may recognize multiple pre-existing distortions during PPT selection. HIV-1 RT hydrolyzes pdC-containing hybrids primarily at the PPT–U3 junction, indicating that the analog does not induce a gross structural deformation of the duplex. However, aberrant cleavage is frequently observed 3 bp from the site of pdC substitution, most likely reflecting a specific interaction between the analog and amino acid residues within the RNase H primer grip. pdC substitution within the template strand of a DNA duplex does not appear to significantly affect RT-catalyzed DNA synthesis. Implications of these findings on the use of pdC to examine nucleic acid structure are discussed.

INTRODUCTION

Early in the life cycle of human immunodeficiency virus type 1 (HIV-1), viral RNA is converted into double-stranded DNA for integration into the genome of the infected cell (1). This process is catalyzed by reverse transcriptase (RT), a virus encoded, heterodimeric enzyme composed of 66 and 51 kDa subunits (p66 and p51), possessing DNA polymerase and ribonuclease H (RNase H) activities (2). Whereas minus-strand synthesis is primed by host-derived tRNA lys,3,5, and ribonuclease H (RNase H) activities (2). Whereas subunits (p66 and p51), possessing DNA polymerase encoded, heterodimeric enzyme composed of 66 and 51 kDa process is catalyzed by reverse transcriptase (RT), a virus for integration into the genome of the infected cell (1). This (HIV-1), viral RNA is converted into double-stranded DNA.

Although the mechanistic basis for plus-strand primer selection is not yet clear, some important features of this process are emerging. For example, local secondary structure does not appear to play a role in processing in HIV-1, since the PPT is utilized for plus-strand priming when placed in a foreign sequence context (7). In addition, selective PPT utilization is unlikely to require multiple viral components, since HIV-1 RT alone can catalyze specific plus-strand priming in vitro (5,6). These data suggest that structural features specific to PPT-containing hybrid duplexes must be sufficient to (i) direct HIV-1 RT to cleave precisely at the PPT 3′ terminus and (ii) selectively initiate plus-strand synthesis from the PPT(s). However, the nature of these structural features, as well as the means by which they are recognized, has yet to be determined.

Recent data suggest that the RNase H primer grip of HIV-1 RT may play a role in specific recognition of the PPT (8–10). Identified by crystallographic analysis of HIV-1 RT in complex with a PPT-containing RNA/DNA hybrid, these residues, located primarily within the connection and RNase H domains of the p66 subunit, contact the DNA strand 3–8 nt upstream from the scissile phosphate (10). Because RT in the crystallized complex is not positioned appropriately for cleavage at the PPT–U3 junction, contacts critical to primer selection cannot be specifically identified. However, since many residues within the RNase H primer grip do not appear to directly interact with duplex DNA (11,12), this motif may be involved in general recognition of RNA/DNA hybrids. Crystallographic analysis also reveals a region of unpaired, mispaired and weakly paired bases centered ~12 nt from the PPT–U3 junction (position –12; Fig. 1A). Unusual base pairing in this region was confirmed by chemical footprinting, which also showed that the distortion is intrinsic to the PPT-containing hybrid duplex, i.e. it is not induced by binding of RT (13). Chemical footprinting further showed unusual hydrogen bonding within the rA:dT pair at the PPT 3′ terminus (position +1; Fig. 1A), consistent with the previous observation that introducing a mispair at position +1 does not adversely affect PPT cleavage (14). Finally, mutational analysis of the PPT suggests that the distortions at positions

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Nucleic Acids Research, Vol. 32 No. 4. Published by Oxford University Press

Published online March 5, 2004

Received December 17, 2003; Revised and Accepted February 4, 2004
+1 and −10 to −14 are structurally interdependent and that their elimination induces inappropriate cleavage (13).

These and other studies (7,15) indicate that hybrid duplexes containing the HIV-1 PPT possess unusual structural features that may affect how the 3′ plus-strand primer terminus is created and selectively extended by HIV-1 RT. However, despite these advances, the (rG:dC)₆ tract within the PPT-containing hybrid remains particularly refractory to analysis. This region was disordered in the RT–PPT/DNA co-crystal and cannot be evaluated using potassium permanganate footprinting, which specifically targets the C5-C6 bond of thymidine. In order to examine the importance of base pairing at sites throughout the PPT-containing hybrid, non-hydrogen bonding pyrimidine isosteres, or shape mimics, were introduced into synthetic DNAs complementary to the PPT and hybrids containing these nucleoside analogs were subjected to RNase H-mediated hydrolysis by HIV-1 RT (16). The results suggest that disrupting base pairing at several sites within the hybrid adversely affect cleavage at the PPT–U3 junction. In addition, aberrant cleavage 3–4 nt downstream from the site of analog substitution was observed, a spacing suggesting involvement of the RNase H primer grip in determining cleavage specificity.

In the current study, we have exploited the fluorescent, environmentally sensitive deoxycytosine analog pyrrolo-deoxycytosine (pdC) (Fig. 1B) to directly examine hydrogen bonding within PPT-containing hybrid duplexes. This approach has previously been used to characterize transcription bubble formation in elongation complexes of T7 RNA polymerase (17,18), and use of 2-aminopurine (2-AP) in this manner is well documented (19). However, we are unaware of any previous report of the use of pdC to measure hydrogen bonding within a hybrid duplex. Our results indicate that analysis of pdC fluorescence can be used to detect the hybridization defects previously reported by Sarañanos et al. (10), indicating the sensitivity of the technique. In addition, we detected altered base pairing at position −2, suggesting that determinants within the (rG:dC)₆ tract may contribute to specialized PPT function(s). RNase H-mediated cleavage of substituted hybrids revealed an analog-directed cleavage pattern likely resulting from interaction between pdC and residues within the HIV-1 RT RNase H primer grip. Structural implications of this finding are discussed. Finally, our results demonstrate that deoxyguanosine is incorporated opposite pdC efficiently and with high fidelity during DNA-dependent DNA synthesis.
MATERIALS AND METHODS

Oligodeoxyribonucleotides and oligoribonucleotides

pdC-substituted 40 nt oligonucleotides were synthesized at 1 µmol scale on a PE Biosystems Expedit 8909 synthesizer by standard phosphoramidite chemistry, except pdC coupling times were extended to 15 min. Sequences and sites of substitution are illustrated in the figures and described in the text. pdC phosphoramidite was purchased from Glen Research (Sterling, VA). Deprotection and cleavage of oligonucleotides from the CPG support were carried out by incubation in 30% ammonium hydroxide for 36 h at 25°C. Oligonucleotides were purified by preparative polyacrylamide gel electrophoresis and quantified spectrophotometrically (260 nm), assuming a molar extinction coefficient equal to the sum of the constituent deoxynucleotides. Incorporation of pdC into pdC was confirmed by fluorescence spectrometry. A complementary 30 nt RNA containing the HIV-1 3′-PPT and flanking sequences at its 5′ and 3′ termini was purchased from Dharmacon Research (Boulder, CO). Hybrids were prepared by heating a 1.5:1 RNA–DNA mixture to 95°C in 10 mM Tris–HCl, pH 7.8, 25 mM KCl for 5 min, followed by slow cooling to 4°C. Samples were stored at −20°C.

Steady-state fluorescence measurements

Fluorescence measurements were taken using an AVIV fluorimeter equipped with a 75 W arc lamp and both emission and excitation monochromaters. Samples containing 1 μM RNA/DNA hybrid or single-stranded DNA in 50 mM Tris–HCl, pH 7.8, 10 mM NaCl were pipetted into a 100 µl ultramicro cell and fluorescence emission recorded at 460 nm, using an excitation wavelength of 350 nm at 25°C. Slat widths on both channels were set to 5 nm. All reported values represent the average of measurements obtained from three different samples.

Polypurine tract selection

PPT selection was evaluated as previously described (8) using recombinant RT and the previously described RNA/DNA hybrids. Briefly, substrates were generated by 5′-end-labeling the PPT-containing 30 nt RNA with [γ-32P]ATP and annealing it to each of the pdC-substituted 40 nt DNAs, as well as to an unsubstituted control oligonucleotide. Hydrolysis was initiated by adding wild-type pdC/p51 HIV-1 RT or one of several RNase H primer grip mutants (8) to RNA/DNA hybrids in 10 mM Tris–HCl, pH 8.0, 80 mM NaCl, 5 mM dithiothreitol and 6 mM MgCl2, with enzyme and RNA/DNA present at final concentrations of 50 and 200 nM, respectively. HIV-1 RT and Ty3 RT reactions were carried out at 37 and 25°C, respectively, due to the different temperature requirements of the two enzymes. Reactions were terminated after 10 min by adding an equal volume of a formamide-based gel loading buffer (95% v/v formamide containing 0.1% w/v bromophenol blue and xylene cyanol) and the hydrolysis products fractionated by high voltage electrophoresis through 15% (w/v) polyacrylamide gels containing 7 M urea. Products were visualized by autoradiography and/or phosphoimaging and quantified using Quantity One software (Bio-Rad).

DNA-dependent DNA polymerase activities

DNA synthesis was measured on 40 nt DNA templates annealed to a 3′-end-labeled 13 nt DNA primer. To ensure an unbiased cross-section of reaction products, reactions were terminated prior to completion. Reactions were initiated by adding 10 nM enzyme to a mixture containing 50 nM template/primer, 200 μM dNTPs, 10 mM Tris–HCl, pH 8.0, 80 mM NaCl and 6 mM MgCl2 and terminated after 10 min by adding an equal volume of a formamide-based gel loading buffer. HIV-1 RT and Ty3 RT reactions were carried out at 37 and 25°C, respectively. Reaction products were fractionated by high-voltage electrophoresis through 10% (w/v) polyacrylamide gels containing 7 M urea in Tris/borate/EDTA buffer. After drying, gels were subjected to autoradiography and/or phosphoimaging analysis using a Molecular Imager FX phosphoimager (Bio-Rad, Hercules, CA). Fidelity of DNA synthesis was evaluated similarly, except that certain nucleotides were withheld to determine the extent of misincorporation opposite pdC embedded within the template.

RESULTS

Fluorescence analysis of pyrrolo-dC-substituted hybrids

Pyrrolo-dC (pdC) is a valuable fluorescent tool to study protein–nucleic acid interactions and nucleic acid structure. An analog of deoxyctydine (Fig. 1B), the excitation and emission maxima of pdC (355 and 460 nm, respectively) are far from the maximum absorbance wavelengths of either nucleic acid or protein and do not overlap significantly with the emission spectrum of tryptophan. In addition, pdC fluorescence is quenched upon hybridization, a feature which has proven useful in examining dC:dG hydrogen bonding in situ (17,18). We exploited these features of pdC to analyze the HIV-1 PPT structure. Individual dC→pdC substitutions were introduced into synthetic DNA oligonucleotides complementary to the 3′-PPT at positions −11, −6 to −1, +1, +4 and +5 (Fig. 1). These were annealed to a synthetic RNA and the resultant hybrids subjected to fluorescence analysis. In addition, a dT→pdC substitution was introduced at position +1 to provide an internal negative control for interstrand base pairing and to further explore nucleic acid substitution at this position with respect to cleavage at the PPT–U3 junction (14). For each hybrid, the extent of hydrogen bonding between individual pdC→dT pairs could be assessed by measuring the decrease in fluorescence relative to that of single-stranded pdC-containing DNA. By doing so, we could identify local distortions within the hybrid duplex that might serve as binding and/or cleavage determinants recognized by HIV-1 RT.

Sample fluorescence profiles are shown in Figure 2A–C. At the emission maximum of 460 nm, fluorescence of the hybrid duplex containing pdC at position −6 is reduced by 25% relative to that of the equivalently substituted single-stranded DNA control (Fig. 2A). Conversely, the profiles of hybridized and single-stranded DNA with pdC at position +1 are nearly superimposable (Fig. 2B), consistent with a mispair at this location. This is also true of DNA substituted at position −11 (Fig. 2C), near the center of the distortion previously identified by Sarafianos et al. (10). Although altered interstrand hydrogen bonding at adjacent positions within a PPT/DNA hybrid
has been shown by potassium permanganate footprinting (13), the absence of fluorescence quenching of pdC at position −11 constitutes the first evidence that the DNA base is most likely unpaired in solution and in the absence of RT.

A quantitative summary of fluorescence quenching upon hybridization of each of the substituted DNAs is presented in Figure 2D. The results show that the stability of rG:pdC pairs throughout the PPT and flanking regions varies according to the site of substitution. Notably, only modest fluorescence quenching is observed upon pdC substitution at position −2, suggesting destabilization of this base pair. Variation among fluorescence profiles may reflect stacking interactions between pdC and adjacent DNA bases, making fluorescence of this analog somewhat context specific. However, since pdC introduced at positions −2 to −5 is flanked by deoxyguanosines, the stacking environments at these locations should be similar. Therefore, it is likely that hydrogen bonding at position −2 is not as stable as that of adjacent rG:dC pairs. Furthermore, although the results depicted in Figure 2 reflect the conditions used by Liu and Martin (10 mM NaCl, 25°C) (17,18), the same relative fluorescence quenching is observed when pdC-containing samples are placed in 80 mM NaCl and/or at 57°C (data not shown), suggesting that the altered base pairing most likely exists under physiological conditions. Taken together, these data demonstrate that measuring pdC fluorescence is as effective a means of detecting even small changes in the hybridization state of rG:dC pairs within a RNA/DNA duplex.

Effect of pdC substitution on processing of the HIV-1 PPT

As previously reported (6,20), HIV-1 RT cleaves unsubstituted RNA/DNA hybrids primarily at the PPT–U3 junction (position −1) and to a lesser extent at position −2 (Fig 3A, lane U). pdC substitution does not appreciably alter the extent of hydrolysis at these positions, suggesting that a significant fraction of each hybrid is appropriately recognized by HIV-1 RT (Fig 3A, lanes −1 to −11). However, substrates containing pdC at positions −1, −2, −3, −5 or −6 are also cleaved 3 bp from the site(s) of analog substitution (Fig. 3A, asterisks). The same may also be true of substrate containing pdC at position −4, although the convergence of normal and aberrant cleavage in this case makes the effect(s) of analog substitution difficult to determine. A similar pattern of analog-directed hydrolysis was previously reported to occur upon incorporation of pyrimidine isosteres into minus-strand DNA opposite the PPT in a manner similar to that described here (16), suggesting that the two types of analog may similarly affect hybrid recognition by HIV-1 RT. Other changes in cleavage specificity appear to be more substitution specific. For example, substrates containing pdC at positions −1 and −3/−4/−5 are hydrolyzed at positions +1 and +5, respectively. In addition, pdC substitution at −5 or −6 results in cleavage at positions +1 to +3. The latter is consistent with the observations of Rausch et al. (16), who showed that perturbation of base pairing within the upstream portion of the (rG:dC)₆ tract results in relaxation of cleavage.
specificity during PPT selection. Other aberrant cleavages were observed with substrates containing pdC substitutions at positions ±5, ±4, ±1 or ±11 (data not shown). However, these were less consistent and appeared to be less closely related to analog positioning, so were not considered further.

To determine whether alterations in cleavage specificity were enzyme specific, we subjected the pdC-substituted hybrids to hydrolysis by RT encoded by the S. cerevisiae Ty3 (21,22) (Fig. 3B). As has been shown previously (23), Ty3 RT catalyzes specific hydrolysis of the HIV-1 PPT, but at sites 3' of the PPT–U3 junction (Fig. 3B, lane U). Notably, however, although the intensity of cleavage at these positions is somewhat reduced, cleavage specificity is unaffected by pdC substitution (Fig. 3B, lanes ±1 to ±11). Hence, either the structural motifs within HIV-1 RT interacting with pdC are not present in Ty3 RT or the structure of such elements differs between the two enzymes.

**PPT processing by HIV-1 RNase H primer grip mutants**

First characterized crystallographically by Sarafianos et al., the p66 RNase H primer grip of HIV-1 RT contacts the DNA strand of a PPT-containing RNA/DNA hybrid 3–8 bp from the scissile phosphate within the RNase H catalytic center (10). Given the 3 bp spacing between sites of pdC substitution and aberrant cleavage, we suspected that a specific interaction between pdC and one or more residues within the RNase H primer grip mediates this event. To test this hypothesis, pdC-substituted hybrids were subjected to RNase H-mediated cleavage by HIV-1 RT containing alanine substitutions at positions ±2/±3, as observed with the wild-type enzyme (compare Fig. 3A, lanes ±5 and ±6, with Fig. 4B–F, lanes ±5 and ±6). Furthermore, primer grip mutants appear to catalyze analog-directed cleavage of the remaining substrates at different rates (Fig. 4B–F, lanes ±1 to ±3). To measure this, analog-directed hydrolysis was quantified for each enzyme as a percentage of total cleavage. Only substrates containing pdC at positions ±1, ±2 or ±3 were considered, since pdC at position ±4 would be expected to direct RT to cleave at the PPT–U3 junction. Our results indicate that the percentage of analog-directed cleavage is lower for N474A, Q475A and PPT–U3 junction. Our results indicate that the percentage of analog-directed cleavage is lower for N474A, Q475A and Y501A mutants (5.9, 10.1 and 11.6%, respectively) than for the wild-type enzyme (15.9%), suggesting that these amino acid substitutions reduce the susceptibility of HIV-1 RT to redirection by pdC. In contrast, pdC-induced cleavage is catalyzed somewhat more frequently by T473A and K476A mutants (10.6% and 21.7%, respectively). Taken together, these data demonstrate that altering the RNase H primer grip can affect pdC recognition, although the mechanism by which pdC is recognized remains unclear.

**DNA synthesis on templates containing pdC**

We also examined how RT-catalyzed DNA-dependent DNA synthesis is affected by pdC substitutions in the template strand. Using the substrate described in Figure 5A, both the
HIV-1 and Ty3 RTs were evaluated to determine whether potential effects were enzyme specific. The results of this analysis are shown in Figure 5B and C, respectively. Although some pausing by each enzyme is observed at the site of pdC substitution, total DNA synthesis is not significantly affected. Furthermore, the synthesis profiles of HIV-1 and Ty3 RT are similar, indicating that pdC in the template strand is recognized similarly by the two enzymes.

A different approach was required to examine the fidelity of nucleotide incorporation opposite pdC. Specifically, HIV-1 RT was added to reaction mixtures containing (i) DNA duplex in which pdC (or a dC control) was embedded in the template strand 2 nt from the primer 3' terminus (Fig. 6A) and (ii) one of the nucleotide mixtures described in the legend to Figure 6. By restricting the type of nucleotide available to the active enzyme, the frequency of specific incorporation and misincorporation events could be evaluated. The results, depicted in Figure 6B, show that HIV-1 RT appears to incorporate dGTP opposite dC and pdC with similar efficiency (Fig. 6B, lanes a). Conversely, DNA synthesis is terminated after addition of a single nucleotide when dGTP is absent from the reaction (Fig. 6B, lanes b–e), whether pdC is present or not. Taken together, these data indicate that in the context of DNA-dependent DNA synthesis, polymerization complexes are only minimally perturbed by translocation over pdC in the template strand and that the fidelity of DNA synthesis in these reactions is maintained.

DISCUSSION

To initiate plus-strand synthesis, HIV-1 RT must distinguish between RNA/DNA hybrids that contain the cPPT or 3'¢-PPT and those that do not. There are several interrelated explanations for how this distinction is achieved. For example, it has been demonstrated that a PPT-containing hybrid possesses features more typical of B-form DNA than of the non-A/non-B helical structure observed in most RNA/DNA duplexes (7,15). This may render the purine-rich RNA more suitable for plus-strand priming, since it has also been shown that RT initiates DNA-dependent DNA synthesis more efficiently from a DNA than an RNA primer (24–26).

By measuring pdC fluorescence, we have determined that the deoxynucleoside at position –11 within a PPT/DNA hybrid (Fig. 1A) does not participate in interstrand hydrogen bonding, proving that the region of altered base pairing centered around positions –12/–13 originally detected by Sarañanos et al. (10) (Fig. 7) is neither a crystal packing artifact nor is it induced by RT binding. Our results, in conjunction with those of Kvaratskhelia et al. (13), also indicate that the distortion is contiguous, extending from position –10 to –14. Because similarly altered hydrogen bonding has not been detected in other RNA/DNA duplexes, it is tempting to speculate that this feature of PPT-containing hybrids serves as a binding determinant for HIV-1 RT, possibly via interaction with the minor groove binding tract, a motif within the p66 subunit implicated in nucleic acid binding and translocation (27–29). Alternatively, the region may contain a flexible 'hinge' connecting two rigid (rA:dT)4 tracts, allowing RT to
accommodate the duplex in a sterically and/or energetically favorable manner not possible with generic hybrids.

The data of Figure 2D also indicate a weakening of interstrand base pairing within the (rG:dC)6 tract of the PPT-containing hybrid, particularly at position ±2 (Fig. 1A). Although the role of a nucleic acid distortion within the downstream portion of the PPT is unclear, any protein–nucleic acid contact in this area is likely mediated by the RNase H primer grip. For example, were RT positioned for cleavage at the PPT 3'-terminus, RNase H primer grip residues T473, Q475, K476, Y501 and I505, as well as several residues in the p66 connection subdomain and p51 subunit, would be predicted to contact the RNA strand at positions −1 to +2 (Fig. 1A), with K390 of p51 forming a hydrogen bond with the phosphate at position −4. Interestingly, only two interactions with nucleic acid bases in this region would be expected (R448 and Q475 with RNA at positions ±1 and ±2, respectively) and only Q475 would be predicted to contact both strands simultaneously, interacting with the DNA strand 4–5 bp from the scissile phosphate as well as the RNA strand at positions −1 and −2.

Taken together, these data suggest that Q475 and the base pair at position −2 may play an important role in hybrid recognition by HIV-1 RT. In support of this notion, virus encoding a p66N474A/p51 RT mutant replicates with low efficiency and the equivalent recombinant enzyme exhibits altered PPT cleavage specificity (Fig. 4) (8,9). Specificity is likewise affected by introduction of an (rG:dC) tract at position −2 or −4 within the equivalent (rC:dG) tract in Moloney murine leukemia virus (14), as well as by more global disruption of the (rG:dC) tract in HIV-1 (7,13). Though speculative, we propose that reduced hydrogen bonding observed at position −2 reflects an unusual structure near the 3' end of the PPT and that this structure is recognized by the RNase H primer grip during PPT selection.

Alternatively, it may function as a binding determinant after PPT cleavage has occurred, serving to reverse the orientation of RT for initiation of plus-strand synthesis. The latter hypothesis is currently under investigation.

The sensitivity of PPT cleavage to local perturbations within the (rC:dG)6 tract is clearly evident from pdC substitution experiments. In competition with native structural determinants, pdC directs HIV-1 RT to cleave 3 bp downstream from the site of analog substitution (Figs 3A and 4). A similar hydrolysis pattern has been observed upon substitution of non-hydrogen bonding pyrimidine isosteres into the DNA strand of a PPT-containing hybrid (16), suggesting that it may result from an interaction between RT and an electronic or structural feature common to the two types of analog. For example, the enzyme might respond to locally enhanced base stacking, which is possible in DNA containing either a pyrimidine isostere or pdC.

The experimental evidence presented in Figures 3 and 4 suggests that analog-directed hydrolysis is mediated by the RNase H primer grip, although the effects of mutations in this region appear to be site specific. Measured as a percentage of total cleavage of pdC-containing substrates, p66N474A/p51, p66Q475A/p51 and p66Y501A/p51 RTs exhibit an overall reduction in pdC-directed hydrolysis relative to the wild-type enzyme, whereas analog-directed cleavage is catalyzed slightly more frequently by p66T473A/p51 and p66K476A/p51 (compare Fig. 3A with Fig. 4B–F). To explore how these primer grip residues might modulate pdC sensitivity, we examined their positioning relative to the deoxythymidine (dT) 3 bp from the scissile phosphate in the HIV-1 RT–PPT/DNA crystal structure (10). We anticipate that the structural environment of this nucleoside (referred to by the authors as Pri16Thy) may resemble that of pdC during analog-directed cleavage, since the distance between the former and the scissile phosphate in the crystal structure is the same as that between pdC and the site(s) of analog-directed cleavage in substituted hybrids.
Interestingly, there are no direct contacts between RT and Pri16Thy in the structure of Sarafianos et al. (10), although both N474 and Q475 are in close proximity (4–5 Å). The side chain of the former residue runs parallel to the minor groove of the hybrid, approximately equidistant from either strand. Though speculative, it may serve a minor groove tracking function, recognizing subtle changes in groove width that may result from analog substitution. As suggested above, Q475 makes several contacts with both strands of the hybrid in the vicinity of Pri16Thy and is one of only two residues in the RNase H domain to descend into the minor groove. Y501 contacts the deoxyribose–phosphate backbone 2–3 nt 3’ to Pri16Thy. This residue has previously been shown to be critical in plus-strand primer selection and removal (8). In vivo, a Y→F substitution at this position confers resistance to BBNH, an RNase H inhibitor (30). T473 and K476 both contact the deoxyribose–phosphate backbone 4–5 bp from the scissile phosphate, 1 nt removed from what would be the site of pdC substitution. Mutation of either of these residues has been shown to slow virus replication (9). Taken together, these data suggest that substitution of even a single amino acid within the RNase H primer grip can alter the manner in which cleavage substrates, including those containing pdC, are recognized.

For the first time, we report the use of pdC fluorescence analysis to determine the hybridization states of base pairs within an RNA/DNA duplex, an approach validated by detection of both a mispair at position +1 and an unpaired base at position –11 in related hybrids (Figs 1A and 2). pdC has also been used to characterize the transcription bubble generated by T7 RNA polymerase (17,18). In the latter studies, the use of pdC-containing substrates does not appear to significantly affect enzyme function. Such is not the case in the present analysis, although the effects of analog substitution appear to be context and enzyme specific. For example, while incorporation of pdC into the DNA strand of a PPT/DNA hybrid alters the cleavage specificity of HIV-1 RT, hydrolysis products resulting from analog-directed cleavage constitute <20% of those generated by the wild-type enzyme, and hydrolysis catalyzed by T7 RT is virtually unaffected (Fig. 3). Furthermore, although some minor pausing is observed, pdC in the template strand does not appear to adversely affect either the processivity or fidelity of DNA synthesis catalyzed by either enzyme (Figs 5 and 6). Taken together, these data suggest that while the effects of analog substitution must be considered, using pdC fluorescence to examine nucleic acid structure should prove useful in a variety of model biological systems.

REFERENCES


