Probing Structure/Function Relationships of HIV-1 Reverse Transcriptase with Styrene Oxide N²-Guanine Adducts

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Details of the interactions between the human immunodeficiency virus (HIV-1) reverse transcriptase and substrate DNA were probed both by introducing site-specific and stereospecific modifications into DNA and by altering the structure of potential critical residues in the polymerase. Unadducted 11-mer DNAs and 11-mer DNAs containing R and S enantiomers of styrene oxide at N²-guanine were ligated with two additional oligonucleotides to create 63-mers that served as templates for HIV-1 reverse transcriptase replication. Oligonucleotides that primed synthesis 5 bases 3′ to the adducts could be extended up to 1 base 3′ and opposite the lesion. However, when the positions of the 3′-OH of the priming oligonucleotides were placed 1, 2, 3, 4, 5, and 6 bases downstream of the styrene oxide guanine adducts, replication was initiated, only to be blocked after incorporating 4, 5, 6, and 7 bases beyond the lesion. The sites of this adduct-induced termination corresponded to the position of the DNA where α-helix H makes contact with the DNA minor groove, 3–5 bases upstream of the growing 3′ end. In addition, mutants of the polymerase in α-helix H (W266A and G262A) alter the termination probabilities caused by these DNA adducts, suggesting that α-helix H is a sensitive monitor of modifications in the minor groove of newly synthesized template-primer DNA several bases distal to the 3′-OH.

During the last 10 years, the use of site-specifically and stereospecifically modified oligonucleotides has played a major role not only in defining which DNA adducts are mutagenic but also in instructing us how different DNA polymerases interact at those sites. We have expanded these studies to use DNA lesions as a probe of polymerase structure and function. The decision to use HIV-1 RT as the polymerase of choice is warranted not only because the cocrystal structure is known for an intact enzyme/DNA complex (1) but also because of the tremendous biological significance of RT. Previously using this strategy, we demonstrated that the major groove DNA adducts, N²-adenine SO lesions, were readily bypassed by wild type RT, only to experience termination/pause sites 3–5 bases beyond the site of the lesion (2). These sites of enhanced termination probabilities corresponded to the adduct being positioned in the template-primer duplex near the thumb subdomain of the polymerase, where the DNA is in close contact with an α-helix and is making the transition from A to B form DNA. Our results could be rationalized because in this bend, the minor groove is widened with a compensatory narrowing of the major groove. The presence of an adduct in the major groove at this position was hypothesized to be the likely source of the enhanced termination through alteration of the minor groove α-helix H contacts, 3–5 bases upstream of the 3′-OH.

To further test whether DNA adducts can give insight into the structure and functioning of HIV-1 RT, we have carried out a study in which monocyclic, minor groove adducts known to cause no significant perturbation to the local DNA structure (3) were used to probe DNA-protein contacts. This study was designed to accomplish the following: 1) determine the effects that these minor groove DNA lesions might have on HIV-1 RT replication, 2) monitor the movement of DNA at a distance from the catalytic site as it traverses through the polymerase, and 3) identify key amino acid residues important for adduct bypass when the adducts are positioned in the template-primer duplex. To accomplish these objectives, we chose to build site-specific and stereospecific SO N²-guanine minor groove adducts into 63-mer oligonucleotides (Fig. 1). The R enantiomer of SO is oriented in the 3′ direction relative to the template strand such that it is pointing toward the 3′-OH of the elongating primer (3). The S enantiomer has the opposite orientation such that it is pointing in the same direction as DNA synthesis. Consequently, although both R and S adducts report specific interactions within the minor groove, the two enantiomers have nearly opposite spatial orientations. This stereospecificity in the SO lesions offers an opportunity to probe different polymerases-DNA contacts within the context of an otherwise identical template-primer.

Also, throughout these studies, we used three forms of HIV-1 RT: the wild type heterodimer (i.e. p66/p51) and two mutant heterodimers, W266A and G262A, whose amino acid alterations are located in α-helix H of the thumb subdomain. Analysis of the cocrystal structure suggests that this α-helix H makes contact with the minor groove of the newly synthesized DNA as it undergoes a 45° bend and transition from A to B form DNA (Fig. 2). Alanine-scanning mutagenesis has revealed that these two residues make important DNA contacts and that alanine substitution drastically elevates koff and reduces processivity and frameshift fidelity (4, 5). In contrast, alanine-scanning mutagenesis of α-helix I, which sits over the template phosphate backbone (Fig. 2), has suggested that these residues do not make critical DNA contacts (6).
MATERIALS AND METHODS

Expression and Purification of Wild Type and Mutant Forms of HIV-1 RT—Wild type HIV-1 RT and mutants were prepared as described previously (4).

Preparation of the SO-adducted Templates—The 11-mer oligonucleotides adducted with \( R \) and \( S \) enantiomers of styrene oxide at the second position of codon 12 (N2-guanine) of \( N\)-ras were synthesized, purified, and supplied by Thomas and Connie Harris (Vanderbilt University) as described previously (7). Two 63-mer templates were constructed by enzymatic ligation (Fig. 1). The SO-adducted 11-mer was ligated to a 20-mer at the 3' termini and to a 32-mer oligomer at the 5' termini, in the presence of a 46-mer scaffold. A nonadducted, control 63-mer was also synthesized. Purification of the 63-mers was performed on a 12% denaturing polyacrylamide gel. The sequence of the 63-mer template was as follows, in which the underlined G is the site of adduction:

\[
5'\text{-GAATGTGGAAGATACGTG-}3', \; 5'\text{-GGCGAGGTGGAATGGTCTGGCAATGTGACTGGGAACAC-}3' \\
20\text{-mer} \\
11\text{-mer} \\
32\text{-mer} \\
\]

Phosphorylation with T4 DNA kinase

Anneal with 46-mer scaffold

5'\text{-GAATGTGGAAGATACGTG-}3', \; 5'\text{-GGCGAGGTGGAATGGTCTGGCAATGTGACTGGGAACAC-}3' \\
3'\text{-ACCTCCTATGGCACCCGTTACAGGCACGC-}5' \\
46\text{-mer scaffold} \\

T4 DNA ligase

Gel purify 63-mer

Fig. 2. Model of the protein-DNA interactions occurring between \( \alpha \)-helix H and \( \alpha \)-helix I of HIV-1 RT and the template-primer duplex. The model structure was generated as described previously (5). Helix H (e.g. Gly262 and Trp266) is observed to make contact with nucleotides of the primer strand in the DNA minor groove 3–5 nucleotides from the primer terminus. In contrast, helix I sits above the phosphate backbone of the template strand. The 3' termini of the primer and template strands are indicated. Template nucleotides 4 and 7 base pairs from the 3'-primer terminus are represented as ball-and-sticks and designated n-3 and n-6, respectively. This figure was made with MOLSCRIPT (13).

Fig. 1. Construction and sequences of DNA substrates and primers.

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**Primer Extension Assays**—The oligonucleotide primers, ranging in length from 17- to 21-mers, were 5' end-labeled with \([\gamma-32P]\)ATP (6000 Ci/mmol, DuPont NEN) using T4 DNA kinase (New England Biolabs, Beverly, MA). A 12-fold excess of template over the labeled primer was annealed by heating the template-primer mix in the polymerization reaction buffer (33 mM Tris-OAc pH 7.5, 66 mM KOAc, 10 mM MgOAc) for 2 min at 65 °C, followed by slow cooling to <35 °C. Native polyacrylamide gels (10%) were run to confirm that greater than 90% of the primers were annealed to their respective templates. Deoxynucleotides were added to the template-primer mix to a final concentration of 500 \( \mu \text{M} \) in a reaction buffer, (33 mM Tris-OAc pH 7.5 at 37 °C, 66 mM KOAc, 10 mM MgOAc, 0.2 mg/ml bovine serum albumin, 1 mM dithiothreitol). Wild type HIV-1 RT and the mutant polymerases were added to the reaction mix (10 \( \mu \text{l} \) of total volume) at concentrations unique for each enzyme. All reactions were carried out at 37 °C. Extensions were terminated by the addition of 5 \( \mu \text{l} \) of stop buffer (95% (v/v) formamide, 20 mM EDTA, 0.05% (w/v) bromphenol blue, 0.05% (w/v) xylene cyanol)2.5 \( \mu \text{l} \) of reaction mixture. DNAs were separated through 15% polyacrylamide-8 M urea gels and were run at 2000 V for 3 h. The relative intensities of the termination sites were determined by PhosphorImager technologies.
RESULTS

R- and S-SO N2-Guanine Adducts Block HIV-RT Replication—Using unadducted R and S styrene oxide-adducted 63-mers as templates, HIV-1 RT replication was initiated on the primers shown in Fig. 3. The primers are referred to by the position of the 3'-OH relative to the adduct site in the complementary strand, such that the −5 primer initiates synthesis five nucleotides 3' to the adduct site, the 0 primer is located opposite the lesion, and all the positively numbered primers contain the adducted base within the template-primer duplex.

Under replication conditions defining single hit encounters (i.e., conditions leaving most of the primer unextended, 20% utilization) between the polymerase and the primer, wild type HIV-1 RT was readily able to extend the −5 primer (Fig. 4) on unadducted templates to full-length products (Fig. 4, lane 1), whereas under these experimental conditions, there was no evidence that either the R- or S-SO lesions could be bypassed (Fig. 4, lanes 2 and 3, respectively). Addition of greater amounts of the polymerases revealed that all primers could be extended consistent with the ratio of template and primer being 10:1. The major pause site occurred 1 base prior to the lesion, with only slight incorporation opposite the lesion. However, using vast excesses of enzyme under multiple hit conditions allowed for modest levels of bypass of these lesions (8). The reduced processivity of the W266A and G262A mutants replicating unadducted templates is apparent by numerous pause sites at positions less than full-length DNAs (Fig. 4, lanes 4 and 7, respectively). These pause sites were generally in runs of template purines, and both of these mutants are sensitive to premature termination at the same sites. Similar to the wild type enzyme, under these experimental conditions, neither the W266A or G262A were able to replicate past the R or S adduct site but terminated predominantly 1 base 3' to the lesion (Fig. 4, lanes 5 and 6 and lanes 8 and 9, respectively). No major differences were noted in the extent or site of termination between the oppositely oriented R- or S-SO enantiomers.

Minor Groove Adducts Result in Strong Termination 4–7 Bases beyond the Adduct Site When Primers Are Positioned 0 to +2—Because both the R and S enantiomers of SO blocked replication 3' and opposite the lesion, this result prevented an analysis of the effects that these minor groove adducts might have as they translocate through the HIV-1 RT. To get around this problem, we designed oligonucleotide primers 0 to +2 (Figs. 3 and 4) to “mimic” synthesis opposite and beyond the template lesion. Lanes 1–9 of Fig. 5 show the results of replication reactions using reaction conditions consistent with multiple hit conditions with wild type enzyme on unadducted (U) or contained R or S enantiomers of SO at the N2-guanine (R and S, respectively) were annealed with 5' end-labeled primer oligonucleotides (17-mer) (15 fmol) whose 3'-OH was positioned 5 bases upstream of the base of interest. Lanes 1–3 show replication initiated by wild type RT (5 fmol) on unadducted, R-SO, and S-SO N2-guanine adducted templates, respectively. Lanes 4–6 and 7–9 show replication of the same set of templates by W266A (5 fmol) and G262A (5 fmol) RT mutants, respectively. The arrows indicate the sites of the primer-adducted base and full-length product.
FIG. 5. In vitro replication of DNAs containing SO N²-guanine adducts: effect of primers that initiate synthesis opposite and 1 and 2 bases beyond the lesion site. Oligonucleotide templates (63-mers) (187 fmol) were assembled that contained either no lesion (U) or a R or S enantiomer of SO at the N²-guanine (R and S, respectively) and are presented in groups of threes (lanes 1–3, 4–6, 7–9, etc., respectively). Oligonucleotides (17-mers) (15 fmol), which primed these templates, were annealed such that their 3'-OHs were 2 bases beyond the lesion (lanes 1–3, 4–6, 7–9, 10–12, and 19–21), 1 base beyond the lesion (lanes 1–3, 4–6, 7–9, 10–12, 19–21), and opposite the lesion (0 primer) (lanes 7–9, 16–18, and 22–24), and opposite the lesion (0 primer) (lanes 7–9, 16–18, and 22–24). Three different HIV-1 RT polymerases were used for synthesis: wild type (10 fmol) (lanes 1–9), W266A (125 fmol) (lanes 10–18), and G262A (125 fmol) (lanes 19–27).

enzyme. Because all of these primers are 17-mers, the length of the full-length products on the unadducted templates increases by 1 base each (Fig. 5, lanes 1, 4, and 7). In strong contrast to the data presented in Fig. 4, the replication beyond the R enantiomer shows dramatic termination at positions 4, 5, 6, and 7 beyond the lesion (Fig. 5, lanes 2, 5, and 8 for the +2, +1, and 0 primers, respectively). Under the reaction conditions used in these experiments, it is of interest to note that the R-SO-containing template could be readily extended using the 0 primer (Fig. 5, lane 9), whereas no synthesis on the S-SO-containing template was observed (Fig. 5, lane 8). It is also of interest to note that the insertion of the seventh base beyond the adduct always results in a very diffuse band, suggesting that different nucleotides can be incorporated at that site (Fig. 5, lanes 2, 5, and 8).

Qualitatively similar results were obtained for extension of primers using S-SO-containing templates. However, the following significant differences were observed. Using the +2 and +1 primers, termination occurred primarily at 5 and 6 bases 3' to the lesion with minor termination at the 4th base position. Additionally, no extension was observed 7 bases beyond the lesion as was seen with the R enantiomer, and the diffuse band was located 6 bases beyond the lesion rather than 7 bases beyond the lesion.

The identical series of experiments was carried out using alanine mutants of HIV-1 RT, W266A (Fig. 5, lanes 10–18) and G262A (Fig. 5, lanes 19–27). Using the W266A mutant to replicate beyond the R-SO enantiomer, significant differences were observed relative to the wild type enzyme. Strong termination was observed only at 4, 5, and 6 bases beyond the lesion and the extent of primer utilization decreased significantly from the +2 to 0 primer, such that no synthesis could be observed using the 0 primer. Even more dramatic effects were observed with the templates containing the S enantiomer. Replication was observed only with the +2 primer with termination occurring 4, 5, and 6 bases beyond the lesion. With both the R and S enantiomers, a diffuse band was observed 6 bases beyond the lesion, indicating that replication was being differentially modulated by the alanine substitution at position 266.

With the G262A mutant polymerase, termination sites were again qualitatively similar to W266A in that only the +2 primer was effectively utilized (Fig. 5, lanes 20, 21, and 23). No replication was ever observed beyond 6 bases past the lesion, and primer utilization decreased dramatically because the 3'-OH was positioned near the lesion (Fig. 5, lanes 24, 26, and 27).

Minor Groove Adducts Positioned within the DNA Bend 3–6 Bases beyond the Catalytic Site Inhibit Primer Extension—The data presented in Fig. 6 show the results that were obtained when primers were positioned at sites 3, 4, 5, and 6 bases beyond the unadducted site (U) or the R enantiomer (R) with the wild type enzyme. The 5' ends of these primers were held constant relative to the +2 primer, and thus their lengths increased from 18 to 21 bases. The amount of enzyme and time of reaction were sufficient for the wild type polymerase to completely utilize all primers on unadducted templates and resulted in full-length products. However, using the identical
Replication of DNAs Containing Minor Groove Adducts That Facilitate a Conformational Change in the DNA Such That Processive Synthesis Is Reestablished

reaction conditions, the +3 to +6 primers on the adducted templates were extended very poorly (Fig. 6, lanes 2, 4, 6, and 8, respectively). In most cases, a single base addition represents >95% of the synthesis that occurred. This result suggests that the polymerase is able to load onto the template-primer duplex but that translocation relative to the DNA did not occur. The exception to this trend was obtained using the +3 primer, in which synthesis was very poor overall, but when it was initiated, the termination sites were at 4, 5, 6, and 7 bases beyond the lesion (Fig. 6, lane 2), a result consistent with data obtained with the 0 to +2 primers.

Mutations in α-Helix H of HIV-1 RT Allow for More Facile Replication of DNAs Containing Minor Groove Adducts That Are Positioned within the Bent DNA—In the previous section, it was demonstrated that wild type HIV-1 RT only very poorly utilized primers in which a minor groove adduct was positioned within the template-primer stem 3–5 bases from the 3′-OH. To ascertain whether mutations in the α-helix that contact this bend might affect replication, we utilized the +3 and +4 primers on unadducted and R-SO enantiomer-containing templates, comparing wild type (1-h primer extension) (Fig. 7, lanes 1 and 2, +3 primer; lanes 3 and 4, +4 primer), W266A (5-min primer extension) (lanes 5 and 6, +3 primer; lanes 7 and 8, +4 primer), and W266A (1 h primer extension) (lanes 9 and 10, +3 primer; lanes 11 and 12, +4 primer). Under the multiple hit conditions used here, all unadducted templates were extended to completion with both sets of primers. In contrast to what was observed using the wild type enzyme, the +3 primer supported significant DNA synthesis at 5 min and 1 h by W266A (Fig. 7, lanes 6 and 8, respectively). Termination occurred at 4, 5, and 6 bases downstream of the lesion. Although extension of the +4 primer by W266A in 5 min was equal to the wild type after 1 h (Fig. 7, lanes 4 and 8, respectively), the W266A mutant extended this primer efficiently after 1 h (lane 12).

**DISCUSSION**

The overall processivity of the HIV-1 RT can be modulated by a number of factors including both the structure of the polymerase (4, 6) and the sequences that are being actively replicated (9) or those that are located in the template-primer stem (10, 11). More specifically, it has been established that many of the pause sites often occur in homopolymeric runs and synthesis through these sites can lead to frameshift and base substitutions through a template-primer slippage intermediate (9–11). Although the fidelity of these reactions was originally expected to be associated with the templating base at the catalytic site, these studies suggested that single nucleotide differences in the template-primer double-stranded region, which were as far away as 6 nucleotides, could influence fidelity (10). The x-ray crystallographic structure of a RT-DNA complex suggests that α-helix H contacts the minor groove of the template-primer DNA (4–6 base pairs from the catalytic site). This “action at a distance” has been previously observed as HIV-1 RT replicated past DNA major groove site-specific and stereospecific styrene oxide lesions on N6 adenines (2, 8). In these experiments, it was shown that termination probabilities were enhanced up to 6-fold at positions 3–5 base pairs beyond the site of the lesion. These data suggested that the presence of the lesions in the major groove modulated important polymerase contacts with the DNA.

To gain understanding of the effects that specific minor groove base alterations might have on the polymerase processivity, we chose to monitor the effects that monocyclic adducts might have on termination and the previously described action at a distance. The data presented in Fig. 5 dramatically display the strong termination of wild type replication when the R and S adducts are positioned 4–7 and 4–6 base pairs downstream of the catalytic site, respectively. It is also obvious that these lesions affect the ability of the various primers to be used effectively for replication such that initiation of synthesis from the +2 primer was greater than the amount of replication observed with the +1 primer and decreased further with the 0 primer. This decreased primer utilization was further enhanced when either of the mutant forms of the polymerase (i.e., G262A and W266A) were used. There was no synthesis using the S-SO enantiomer template with either the +1 or 0 primer or the R-SO enantiomer-containing template with the 0 primer. These data suggest that alanine substitution perturbs important DNA contacts within the region where the bend in the DNA is observed to occur. The perturbations are probably residue-specific, because in one case the alanine substitution is increasing the size of the side chain (i.e., G262A) and in the other case dramatically reducing it (i.e., W266A). The exact identity of these two side chains is probably crucial because these residues are highly conserved within the retroviral reverse transcriptases (12). Thus, one of the roles of α-helix H may be to monitor the integrity of the newly replicated DNA by facilitating a conformational change in the DNA such that unmodified DNAs are sufficiently malleable to allow progression of processive synthesis, whereas modified DNAs are conformationally constrained to cause pausing and replication termination. For this model and consistent with our data, the presence of monocyclic minor groove adducts near or at the catalytic site are insufficient distortions to completely block the initiation of replication, but adducts near the active site, coupled with α-helix mutations, are sufficient to effectively prevent either initiation or elongation.

The question arises whether specific contacts between specific residues in α-helix H and the various SO lesions can be
example, the distance between the cleoside were significantly different for the two lesions. For distances between the lesion and the terminally incorporated nucleoside spans ~8 bases, whereas this distance is predicted to be only 5 bases on the S-SO lesion. Resolution of this unexpected finding will require the solving of the co-crystal structure of HIV-1 RT with each of these adduct-containing DNAs or solving the NMR solution structure of the complexes.

Finally, we have consistently noted that the S-SO-containing templates support lower levels of overall synthesis relative to the R-SO or unadducted templates. Preliminary studies suggest that the $k_{\text{on}}$ rate is roughly equivalent but that the $k_{\text{off}}$ is distinctly different when the S-SO adduct is in the template-primer stem:\(^2\) This suggests that these adducted oligonucleotides could potentially be used as competitive inhibitors of the action of HIV-1 RT.

Thus, overall we conclude that it is possible that replication of a lesion-containing DNA can be used in conjunction with wild type and mutant polymerases to not only gain insight into the parameters that define the molecular interactions between the two macromolecules but also to understand the structure-function relationships of the HIV-1 RT.

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