

Exploiting Structurally Diverse Nucleoside Analogs as Probes of Reverse Transcription Complexes

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Abstract: Crystal structures of HIV-1 reverse transcriptase (RT) in complex with either duplex DNA or an RNA/DNA hybrid have provided significant insights into the manner in which this highly versatile enzyme accommodates the conformationally-distinct nucleic acid substrates encountered during the reverse transcription cycle. Biochemical data likewise suggest that unique structural features of the nucleic acid substrates contribute towards recognition by their cognate RT. While site-directed mutagenesis of catalytically- and structurally-critical protein motifs is relatively facile, understanding how nucleic acid structure contributes to its recognition presents a greater challenge. The relative ease with which large DNA and RNA fragments can now be chemically synthesized, in conjunction with the increased availability of ribo- and deoxyribonucleoside analogs, allows nucleic acid structure to be examined with respect to the role of hydrogen bonding, nucleobase stacking, sugar ring geometry and charge of the phosphodiester backbone. This review summarizes our use of nucleoside analogs to understand how the structure of *cis*-acting regulatory signals mediates their recognition by structurally diverse retroviral and retrotransposon enzymes.

INTRODUCTION

Human immunodeficiency virus type 1 (HIV-1) reverse transcription requires completion of a series of specialized catalytic events (reviewed in [58]). These include (a) initiation of minus strand DNA synthesis from a host-coded tRNA, (b) minus-strand transfer, (c) initiation of plus-strand synthesis from the central and 3' polypurine tracts (c and 3' PPTs), (d) plus-strand transfer, (e) strand displacement synthesis and (f) termination of plus-strand synthesis mediated by the central termination sequence (CTS). The entire process is catalyzed by the viral reverse transcriptase (HIV-1 RT), a multifunctional enzyme possessing DNA and RNA-dependent DNA synthesis and ribonuclease H (RNase H) activities. This enzyme is remarkably flexible, utilizing RNA/RNA, DNA/RNA, RNA/DNA, and DNA/DNA template/primer combinations as DNA synthesis substrates at various stages of reverse transcription. In addition, RT-associated RNase H is required to digest the RNA genome of the RNA/DNA replication intermediate, thus facilitating plus-strand synthesis as well as the two strand transfer events. Among the most stringent requirements of HIV RT is to accurately initiate plus-strand DNA synthesis from, and ultimately remove, the 3' and cPPTs, with failure to do so resulting in truncation of the viral genome or synthesis of a DNA intermediate that cannot be integrated into the genome of the infected cell.

Clearly, HIV RT is a complex enzyme of diverse function, and has accordingly received a great deal of scientific attention. While *in vitro* site-directed mutagenesis has provided a wealth of information on critical residues of the DNA polymerase and RNase H catalytic centers [58], it is important to recognize that the structure of the nucleic acid

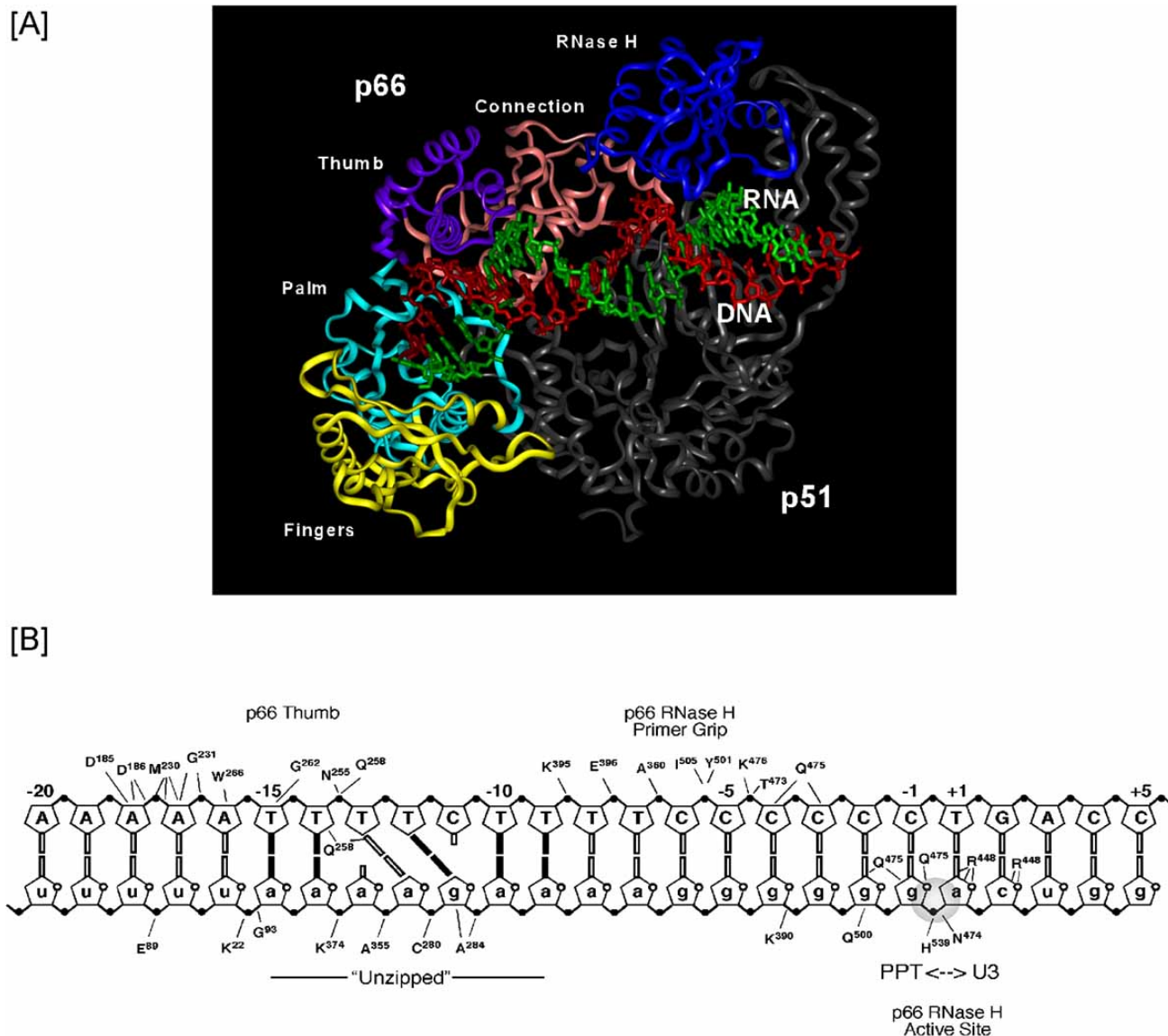
substrate contributes to its recognition and processing. In this review, a brief synopsis of select aspects of RT structure and function will be presented, with special emphasis on plus-strand primer selection and termination of plus strand synthesis at the CTS. In particular, the recent use of nucleoside analogs in oligonucleotide-based model systems designed to probe RT-substrate interactions will be discussed. In addition to retroviral RT, comparative studies with the counterpart from the long terminal repeat retrotransposon Ty3 [20] are presented to highlight how structurally divergent *cis*-acting regulatory signals are recognized by their cognate enzyme.

RT STRUCTURE

During HIV maturation, RT is cleaved from the *gag-pol* precursor by the viral protease (PR) [58]. Both polypeptides are cleaved at the amino- and carboxy-terminal PR-RT and RT-integrase (IN) junctions to generate a homodimer of 66 kD subunits. Subsequent processing removes a C-terminal 15 kD peptide from one of these subunits to produce the mature p66/p51 heterodimeric form of RT (Fig. 1A) [14]. Despite extensive sequence identity between the two subunits, p66 and p51 RT play vastly different roles in the structure and function of the enzyme. Whereas the larger subunit houses both the DNA polymerase and RNase H active sites at its amino and carboxy-terminal domains, respectively, and forms the majority of the template-primer binding cleft, p51 assumes a more globular, less extended tertiary structure, serving primarily as a support for the catalytic subunit [30].

Within the polymerase domain of p66, active site residues are situated within the fingers, palm, and thumb subdomains, so named for the resemblance of the domain to a grasping right hand [30]. The p66 thumb is especially important in template-primer binding and translocation, containing several residues involved in contacts between RT and template/primer, primarily along the sugar-phosphate backbone of the primer strand on the minor-groove side of the helix. The C-terminal RNase H domain comprises a series of α -

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helices flanking a central β -sheet, the overall structure of which shares considerable homology with *Escherichia coli* (*E. coli*) RNase H1 [13, 28]. A notable difference between the HIV-1 RNase H domain and the bacterial enzyme is that the former lacks a large, central α -helical motif (α -helix C). The absence of this element, believed in the bacterial enzyme to play a role in substrate binding, likely explains why the isolated HIV-1 RNase H domain is catalytically inactive and does not bind RNA/DNA efficiently [11]. The compensatory mechanism by which RT recognizes, binds, and cleaves hybrid substrate in the absence of the bacterial element is one of the principle topics of this review. Finally, the

polymerase and RNase H domains are linked by a connection subdomain. This subdomain shares considerable sequence homology with the RNase H domain [2], and most likely arose *via* gene duplication of the latter during the course of evolution.

THE RT-PPT/DNA COMPLEX

The structure of HIV-1 RT in complex with an RNA/DNA hybrid containing the 3' PPT and flanking viral sequences has been reported by Sarafianos *et al.* [53]. Although the global structure of RT in this context does not significantly differ from those in complex with double-stran-

ded DNA (dsDNA), a number of new contacts were observed between the connection subdomain and RNase H domain of p66 and the DNA strand of the hybrid. The involved residues, collectively referred to as the RNase H primer grip (RHPG), are believed to play a specific role in stabilizing the hybrid substrate in the vicinity of the RNase H catalytic center. Virus containing mutations in the RHPG were shown to be defective in minus-strand initiation, minus-strand primer removal, and PPT processing [27, 42]. *In vitro* biochemical studies showed that residue Tyr501 is especially critical for general RNase H function and PPT primer removal [50].

The structure of the PPT-containing hybrid is also remarkable. First, the overall configuration of the RNA/DNA was found to be asymmetric, with the RNA strand assuming an A-form geometry, while the DNA strand was in a mixed A/B configuration. This is in agreement with various NMR and molecular modeling studies of RNA/DNA hybrids [10, 17, 47]. In most instances, the RNA strand is found to be more static, deviating less from the 3' endo sugar configuration and helical structure typical of A-form nucleic acid. In contrast, the DNA strand is most often in dynamic equilibrium between A- and B-forms, with individual ribose moieties frequently toggling between 2' endo and 2' exo states, and exhibiting much more fluid base-stacking and inter-strand hydrogen bonding interactions [10].

While the overall conformation is typical of hybrid duplexes, additional structural features of the PPT-containing RNA/DNA were identified. Perhaps most intriguing was a localized region of altered base pairing approximately 11 bp upstream from the PPT/U3 junction. Significant A-tract-induced minor groove compression typical of dA/dT homopolymers was also detected in this region [1]. It was later demonstrated by Kvaratskhelia *et al.* [33] that altered base pairing within the HIV-1 PPT was neither a crystal-packing artifact nor enzyme-induced. In this work, locally enhanced thymine sensitivity to oxidation by KMnO_4 was observed within one of the rA/dT tracts of a PPT-containing hybrid in the absence of RT. Taken together, these structural peculiarities within the PPT/DNA structure may contribute to resistance of the purine-rich element to RNase H-mediated cleavage (see below). Variations in minor groove width have also been proposed as a mechanism by which bacterial RNases H distinguish between RNA/DNA and RNA/RNA duplexes as potential substrates [16].

While the RT-PPT/DNA structure [53] provides important insights into how HIV-1 RT accommodates a nucleic acid hybrid, the RNase H active site was positioned ~11 nt from the PPT/U3 junction. Fig. 1B shows the sequence of PPT/DNA duplex highlighting in particular the region of altered base pairing and minor groove compression. However, in order to define regions of the hybrid which might contribute to its recognition and processing, it has been necessary to re-locate protein:nucleic acid contacts to approximate an enzyme with its RNase H catalytic center (and in particular the catalytic His539) positioned over the scissile PPT/U3 junction. This model, including position designations, will serve as a reference for the remainder of this review.

INITIATION OF PLUS-STRAND DNA SYNTHESIS

Following minus-strand transfer, HIV-1 RT copies the portion of viral RNA containing, sequentially, the U3, 3' PPT, *env* gene, *pol* gene (which contains the cPPT), and upstream regions of the viral genome to create the RNA/DNA hybrid substrate required for selection and extension of the plus-strand primers [58]. During and immediately following minus-DNA synthesis, the genomic RNA template is hydrolyzed into small fragments, many of which either spontaneously dissociate from nascent minus-strand DNA or are easily displaced during subsequent plus-strand synthesis. Of the remaining fragments, the 3' and cPPTs appear to be specifically recognized by HIV-1 RT, which cleaves at sites immediately flanking, but not within, the purine-rich elements - most critically at the PPT/U3 junction [22]. Thereafter, plus-strand synthesis initiates from the two PPT primers to the exclusion of other RNA fragments, although a few secondary initiation sites have been observed [29].

Genetic manipulation of the PPT has proven important for exploring its function *in vivo* and in model systems mimicking various stages of plus-strand primer selection. Complete removal or extensive mutagenesis of the 3' or cPPTs drastically impairs virus replication [9, 43, 52], with specific substitutions at positions -2 or -5 of the G-tract (Fig. 1B), or at multiple positions within the upstream A-tracts being especially detrimental [26, 41]. Specific defects were detected in both initiation of plus-strand synthesis and plus-strand primer removal. More ambiguous is the importance of the rU:dA tract immediately 5' to the HIV-1 3' and cPPTs (Fig. 1B). This element is highly conserved among retroviruses, and in Moloney murine leukemia virus (MoMLV) [3, 52] and simian immunodeficiency virus (SIV) [23], its deletion drastically reduces virus titer, although it was not clear that the interruption reflected failure to initiate plus-strand synthesis. Instead, PCR analysis of reverse transcription products generated from SIV containing mutations in the rU:dA tract indicated impaired minus-strand DNA synthesis over the affected region [23]. Furthermore, serial passaging of mutant viruses selected for variants containing long poly-rA insertions at the 5' terminus of the PPT, suggesting duplication of the upstream A-tract resulting from slippage and/or pausing during minus-strand synthesis as a potential explanation for the mutant phenotype. Although an intriguing possibility, no slippage or pausing was observed when *in vitro* transcribed HIV-1 RNA lacking a rU:dA tract was reverse transcribed by recombinant HIV-1 RT (J. Rausch, unpublished observations).

A number of *in vitro* model systems have been developed to examine PPT processing in greater detail. In some instances, RNA/DNA hybrids 80 nt or longer are incubated with RT and dNTPs, and DNA synthesis products detected by internal [^{32}P]-labeling or unidirectional PCR. Huber and Richardson [22] used such a methodology to show not only that HIV-1 RT will initiate DNA synthesis from a PPT embedded within a considerably longer hybrid, but also that the enzyme cleaves within the rU:dA tract to generate plus-strand primers of either 17 or 19 nt in length. Similarly, Powell *et al.* [48] demonstrated that HIV-1 RT will initiate

from a PPT placed into a foreign sequence context. In agreement with *in vivo* studies, the effects of mutating the rG:dC tract of the HIV-1 or Mo-MLV PPTs were greater than those of similar changes in the rA:dT tracts [49]. Specifically, extending the rG:dC-tract by one nucleoside displaced the initiation site to the same extent, while the efficiency of DNA synthesis was substantially reduced in mutants containing rG → rA substitutions at position -2 or -4. Conversely, no changes in plus-strand synthesis were observed with any of several rA → rG changes in the interrupted A-tract.

Advances in chemical RNA synthesis have allowed oligonucleotide-based model systems for PPT-processing and plus-strand DNA synthesis to be developed. For example, using an HIV-1 model system, Powell *et al.* [48] demonstrated that mutating the rG:dC tract induces internal cleavage of the PPT and decreased efficiency of plus-strand priming. In contrast, introducing substitutions within the interrupted rA:dT tract has little effect on enzymatic processing. Cleavage downstream from the PPT (especially at position +5), as well as at position -1, was shown to be essential for efficient initiation of plus-strand DNA synthesis [54], and removal of the primer was shown to occur after incorporation of only 12 nt into the nascent DNA strand [19]. Finally, although the affinity of HIV-1 RT for PPT and non-PPT-containing hybrids is similar [55], initiation of DNA synthesis from a PPT primer is more efficient [18].

EXPLORING RT-PPT/DNA INTERACTIONS USING NUCLEOSIDE ANALOGS

An important feature of the HIV-1 RT/PPT co-crystal [53] was a region of the RNA/DNA hybrid which deviated significantly from standard Watson-Crick base pairing - the so-called “unzipped” region (Fig. 1B). While the significance of such anomalous base pairing was not immediately clear, it raised the possibility that flexibility of the hybrid might contribute to its recognition and accurate processing by RT. Understanding how PPT geometry might mediate its recognition requires that the RNA and DNA strands can be altered in a manner that preserves both the length and sequence context of the hybrid. As a step in this direction, our laboratory developed a model oligonucleotide-based system incorporating nucleoside analogs, either individually or in tandem, at strategic positions of either the PPT plus strand RNA primer or minus strand DNA template. This approach has proven useful for probing, at a sub-molecular level, substrate recognition by a number of different nucleic acid binding proteins [56, 59]. Such PPT-containing hybrids can be used to determine how specific changes to nucleic acid architecture, as opposed to simple base substitutions, affect substrate recognition and cleavage by the cognate RT. Other examples in which the fluorescence properties of an analog were exploited, or alternative model systems were utilized, will also be discussed.

Non-Hydrogen-Bonding Pyrimidine Isosteres 2,4-difluoro-5-methylbenzene (dF) and 2-fluoro-4-methylbenzene (dD) are isosteric (shape) mimics of thymine and cytosine, respectively, in which the amino and carbonyl oxygens at positions 2 and/or 4 of pyrimidines have been replaced by fluorine [45]. While sterically equivalent to their naturally occurring counterparts, these analogs do not participate in

hydrogen bonding in the context of duplex nucleic acid (Fig. 2B), making them particularly advantageous for exploring the importance of such interactions in substrate recognition by DNA-binding enzymes. For example, dF has been used to demonstrate that the shape of a templating base, rather than its hydrogen bonding potential, is the critical fidelity determinant in DNA synthesis catalyzed by Klenow fragment [45]. Conversely, the more promiscuous human DNA polymerase κ requires inter-strand hydrogen bonding for efficient incorporation of an incoming nucleotide [60].

In our studies of plus-strand primer processing, we introduced single or tandem dD or dF substitutions into the PPT minus-strand DNA template (Fig. 2C) in order to investigate how locally removing hydrogen bonding affected RNase H cleavage specificity [51]. Hydrolysis profiles of HIV-1 RT on these substrates are shown in Fig. 2D, with the WT profile shown in lane C. Remarkably, in apparent competition with normal PPT binding determinants, the RNase H catalytic center could be relocated to cleave 3 nt downstream from the site of analog substitution (Fig. 2D). This was especially apparent in the case of tandem dD substitutions between positions -1 and -5. Although aberrant hydrolysis was also apparent with dT → dF changes from positions -7 and -10, the isostere-induced cleavage determinants become increasingly incompatible with enzyme binding in substrates containing substitutions close to the PPT 5' terminus, culminating with an almost complete absence of cleavage at the PPT/U3 junction in substrates substituted at positions -14 and -15.

It is clear that local disruption of inter-strand hydrogen bonding, perhaps with concomitant electronic changes in the minor groove or related to base stacking, is sufficient to relocate the RNase H catalytic center to induce alternative site(s) of hydrolysis. Although the exact mechanism by which this occurs has yet to be determined, the 3 bp separation between the novel cleavage sites and sites of analog substitution suggest the structural anomaly is recognized by a protein motif close to the RNase H active site. Based on the crystal structure of Sarafianos *et al.* [53], a good candidate would be the RNase H primer grip, which X-ray crystallography indicates contacts the DNA strand of an RNA/DNA hybrid 3-4 bp from the RNase H active site (Fig. 1B).

Fig. 2E shows the sequence of the PPT from the *Saccharomyces cerevisiae* LTR-retrotransposon Ty3, which lacks both rA:dT and rG:dC tracts, yet is cleaved with the expected specificity by Ty3 RT (Fig. 2F, left) [36]. Surprisingly, a dual -1/-2 dT → dF substitution eliminated cleavage at the PPT/U3 junction, instead positioning the RNase H active center ~12 bp downstream into the U3 sequence (Fig. 2F, lanes 4-6). Although structural information is not available for the monomeric, 55 kDa Ty3 RT, the data of Fig. 2 suggests the isostere-induced structural anomaly was recognized by different domains of the HIV-1 and Ty3 enzymes. In the latter case, the site of novel cleavage suggests the isostere-induced structural anomaly was recognized by the thumb of the DNA polymerase domain rather than the RNase H primer grip. While structural studies with the Ty3 PPT are still underway in our laboratory, the data of Fig. 2 illustrates how nucleoside analogs can be exploited to examine two enzymes which catalyze equivalent reactions yet differ in their quaternary structure.

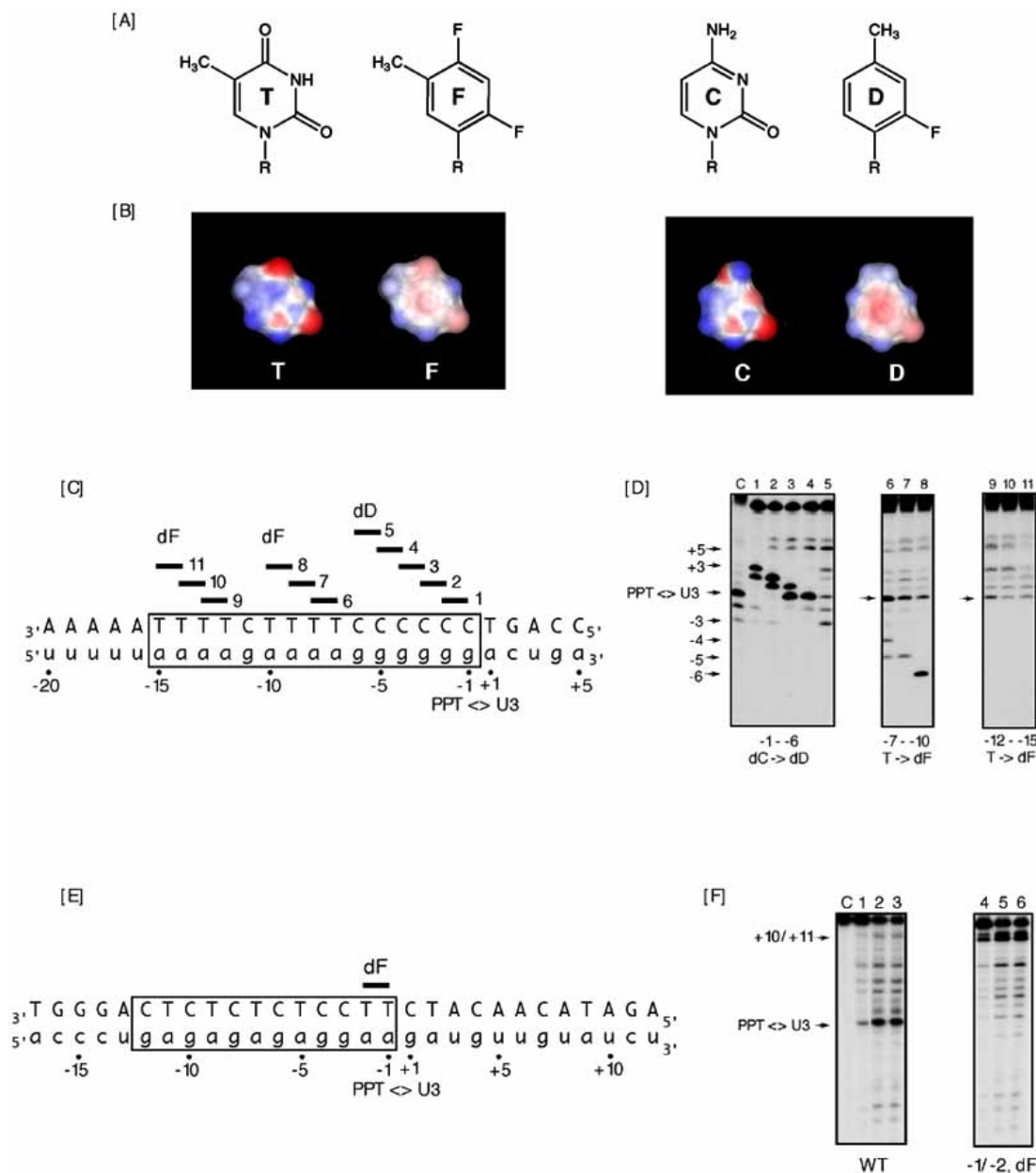


Fig. (2). Using pyrimidine isosteres to explore the effects of interstrand hydrogen bonding on substrate recognition. Chemical structures [A] and surface electrostatic potentials [B] of thymine (T), 2,4-difluoro-5-methylbenzene (F), cytosine (C), and 2-fluoro-4-methylbenzene (D). Because of the reduced polarity of the synthetic analogs (reflected in muted coloring relative to the natural bases), D and F are unable to effectively participate in interstrand hydrogen bonding. Consequently, substituting natural pyrimidines with synthetic analogs in the DNA strand of an RNA/DNA hybrid affects how these substrates are cleaved by HIV-1 or Ty3 RT. [C], sequence of the HIV-1 PPT indicating sites of dual dD (lanes 1 - 5) and dF (lanes 6 - 8 and 9 - 11) substitutions of the (-) DNA template. The PPT sequence is indicated by box. [D], Cleavage specificity of modified substrates. Lane 1, -1/-2 dD; Lane 2, -2/-3 dD; Lane 3, -3/-4 dD; Lane 4, -4/-5 dD; Lane 5, -5/-6 dD; Lane 6, -7/-8 dF; Lane 7, -8/-9 dF; Lane 8, -9/-10 dF; Lane 9, -12/-13 dF; Lane 10, -13/-14 dF; Lane 11, -14/-15 dF. Lane C, wild type (-) DNA template. The hydrolysis product corresponding to cleavage at the PPT/U3 junction is indicated. [E], Sequence of the Ty3 PPT indicating the site of a dual dT → dF substitution in the (-) DNA template. [F], Cleavage of the wild type (Lanes 1 - 3) and dF-substituted Ty3 PPT (Lanes 4 - 6) by p55 Ty3 RT.

Locked Nucleic Acid (LNA) LNAs are nucleoside analogs in which the 2' hydroxyl group of the ribose moiety is replaced by a 2'-O-4'C methylene linkage (Fig. 3A) [31]. This molecular tether serves to "lock" the sugar ring in the "north" or C3'-endo configuration, which in turn has the effect of constraining LNA-containing duplexes to the A-form configuration typical of RNA. A related effect of these changes is to

enhance duplex stability with increases of 3-5°C per LNA insertion having been reported [32]. Not surprisingly, the primary use of LNAs to date has been to increase the affinity of antisense oligonucleotides for their mRNA targets [25], although they have also been investigated as potential antiviral therapeutic agents [8].

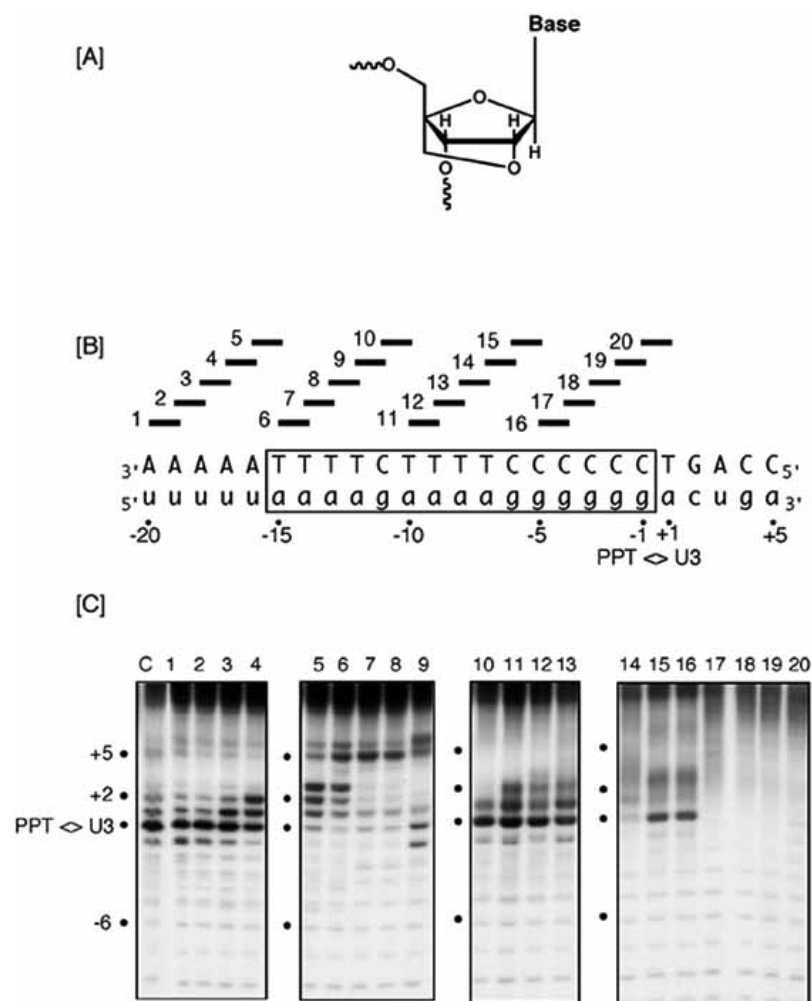


Fig. (3). Effect of dual LNA substitutions of the minus DNA template on cleavage at the HIV-1 PPT/U3 junction. [A] Chemical structure of locked nucleic acid showing 2',4' methylene linkage. [B], experimental strategy. Using a series of overlapping LNA doublets, the HIV-1 PPT (-) DNA template was substituted between positions -20 (doublet 1) and +1 (doublet 20). [C], Cleavage at the PPT/U3 junction as a function of LNA substitution of the (-) DNA template. Lane notations correspond to the position of LNA insertion, i.e., Lane 1 is a -20/-19 substitution and Lane 20 a -1/+1 substitution. Lane C, unsubstituted DNA template. The hydrolysis product corresponding to cleavage at the PPT/U3 junction is indicated.

We, however, wanted to examine how introduction of LNA into the DNA strand of the PPT- containing RNA/DNA hybrid affected the specificity of RNase H-mediated cleavage. Early work of Jacobson and co-workers [7, 46] showed that introducing a single LNA into duplex DNA induces a localized B-form to A-form transition extending one base pair to either side of the substitution, such that LNA substitution at every third nucleotide of one of the strands completely transforms the duplex. We therefore anticipated that in the context of the PPT/DNA hybrid, LNA would (a) introduce a small, well-defined region of A-form structure into a non-A/non-B environment and (b) remove the structural flexibility from the DNA strand at defined positions.

HIV-1 RT cleavage profiles of PPT/DNA variants containing tandem, overlapping LNA substitutions in the DNA strand are shown in Fig. 3. Most notably, when LNAs were introduced between positions -16 and -12, or between -4 and +1, cleavage is either displaced downstream from the PPT/U3 junction or is almost completely ablated (Fig. 3C).

Interestingly, these positions approximately correspond to predicted contacts between the DNA sugar-phosphate backbone and residues of the N-terminal p66 thumb and C-terminal RNase H primer grip, respectively, for an enzyme whose RNase H catalytic center is positioned for hydrolysis at PPT/U3 junction. Failure to cleave at this site, therefore, suggests disruption of these contacts, probably due to LNA-induced changes to the sugar pucker in the DNA strand and/or overall flexibility of the nucleic acid in the vicinity of the substitution(s). Such a conclusion is supported by NMR studies indicating that changes in sugar-pucker may serve as binding determinants for PPT recognition [17, 62]. Also noteworthy is the observation that placing LNAs within the unpaired portion of the PPT (positions -8 to -11) does not significantly alter cleavage at the PPT/U3 junction, indicating that neither helical geometry nor DNA flexibility in this region are critical to PPT function.

Abasic Tetrahydrofuran Linkages Abasic analogs were developed largely for use in model systems designed to mimic genomic DNA damaged by chemical or photolytic

processes [37], and disrupts both base stacking and inter-strand hydrogen bonding in the context of dsDNA. Both RNA and DNA variants (Fig. 4A) are tetrahydrofuran derivatives containing either a hydroxyl group or saturated carbon at the 2' position, respectively. Unlike their natural counterparts, however, the 1' carbon is saturated to prevent open-chain aldehyde formation along with the associated hydrolysis of the nucleic acid strand under mildly alkaline conditions.

PPT-containing RNA/DNA hybrids with abasic substitution at positions -1, +1, or +2 of either strand were used to assess the importance of individual nucleobases within the RNase H catalytic center (Fig. 4B) [63]. Although relatively few direct contacts between HIV-1 RT and nucleobases in duplex DNA or an RNA/DNA hybrid have been reported, those that are observed frequently involve highly conserved residues believed to play an important role in DNA polymerase or RNase H activity. Remarkably, only modest changes in cleavage at the PPT-U3 junction resulted from eliminating nucleobases -1, +1, or +2 from the DNA strand (Fig. 4C, D[i] - [iii]) indicating they are not essential for recognition by RT-associated RNase H, and maintenance of overall helical geometry by flanking regions of the hybrid is sufficient for correct enzyme binding.

In contrast, hybrids containing abasic substitutions in the RNA strand at positions -1 or +1 were not cleaved at the PPT/U3 junction (Fig. 4D, [iv] - [v]). Removing an RNA base may locally alter nucleobase stacking or induce deformation of the nucleic acid structure that precludes proper accommodation of the RNA strand at the RNase H catalytic center. Alternatively, abasic substitution in the RNA strand may interfere with catalytically important contacts between one or more RNase H residues and the RNA nucleobase(s). The residues most likely to be involved are Gln475 and Arg448. These are conserved residues of the RNase H primer grip and were shown in the RT-PPT/DNA co-crystal structure to come in close proximity to RNA nucleobases on either side of the scissile phosphate, with Arg448 forming a hydrogen bond with the base at position +1 and Gln475 a hydrogen bond with nucleobase -2 [53].

Pyrollo dC Pyrollo dC (pdC, Fig. 5A) is an environmentally sensitive fluorescent variant of deoxycytosine (dC) that allows for *in situ* detection of altered base pairing between pdC and dG (or rG) [61]. Specifically, whereas pdC is maximally fluorescent (Ex 350/Em 460) in its monomeric state, it is partially quenched by adjacent bases within an oligonucleotide or by participation in standard Watson-Crick base pairing with dG (or rG) in double-stranded nucleic acid. Consequently, in an oligonucleotide-based experimental system, the extent to which pdC is base paired can be determined by comparing the fluorescence of the relevant pdC-containing oligonucleotide in the experimental system to that of equivalent single- or double-stranded controls. Such an approach has been used to detect the boundaries of transcription "bubbles", as well as to establish conditions that promote/inhibit bubble formation [38-40].

We have exploited these properties of pdC to examine rG:dC base-pairing at multiple locations throughout the HIV-1 PPT/DNA hybrid [12]. The environment of dC at position -11 was particularly important. Located within the

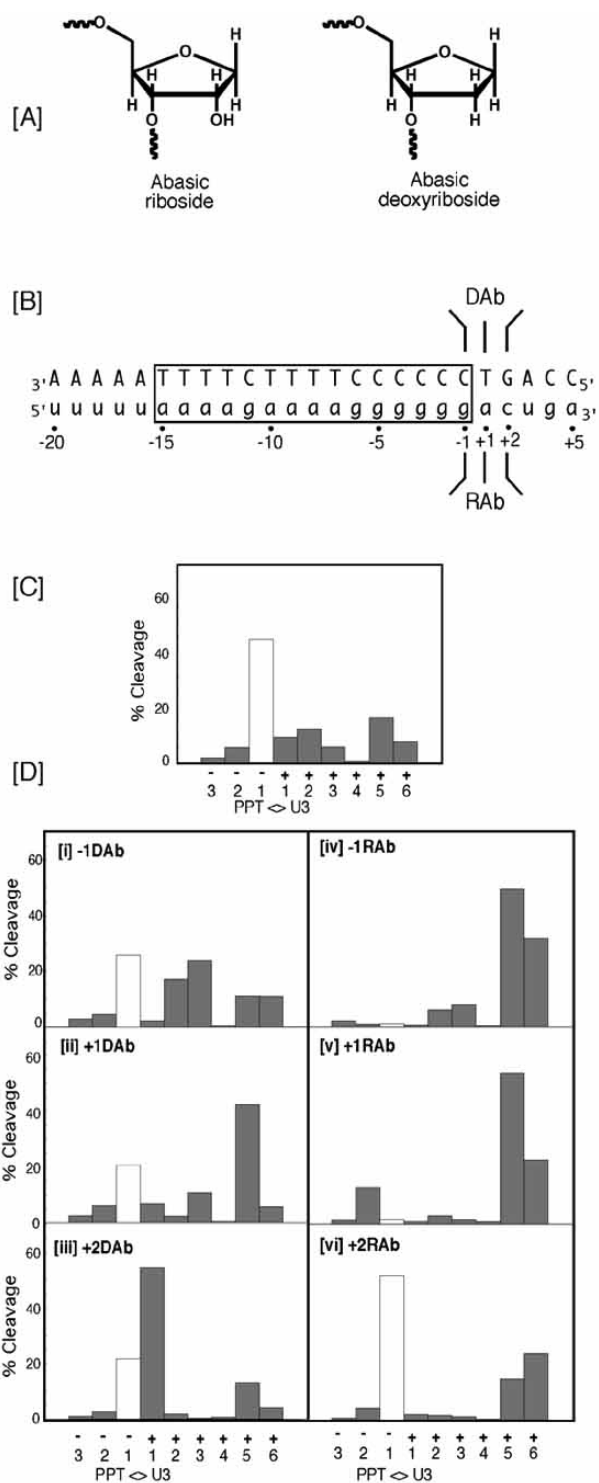


Fig. (4). Examining nucleobase requirements at the HIV-1 PPT/U3 junction *via* targeted insertion of abasic lesions. [A] Chemical structures of abasic ribosides (RAb) and deoxyribosides (DAb). These analogs were substituted at positions -1, +1 and +2 of either the RNA or DNA oligonucleotides utilized in the experimental system presented in [B]. [C], Cleavage profile for the unsubstituted RNA/DNA hybrid. Cleavage within the PPT and downstream of the PPT/U3 junction is represented as a fraction of the total hydrolysis products, with the open box indicating the PPT/U3 junction. [D] Cleavage profiles for RNA/DNA hybrids containing abasic deoxyriboside and riboside lesions.

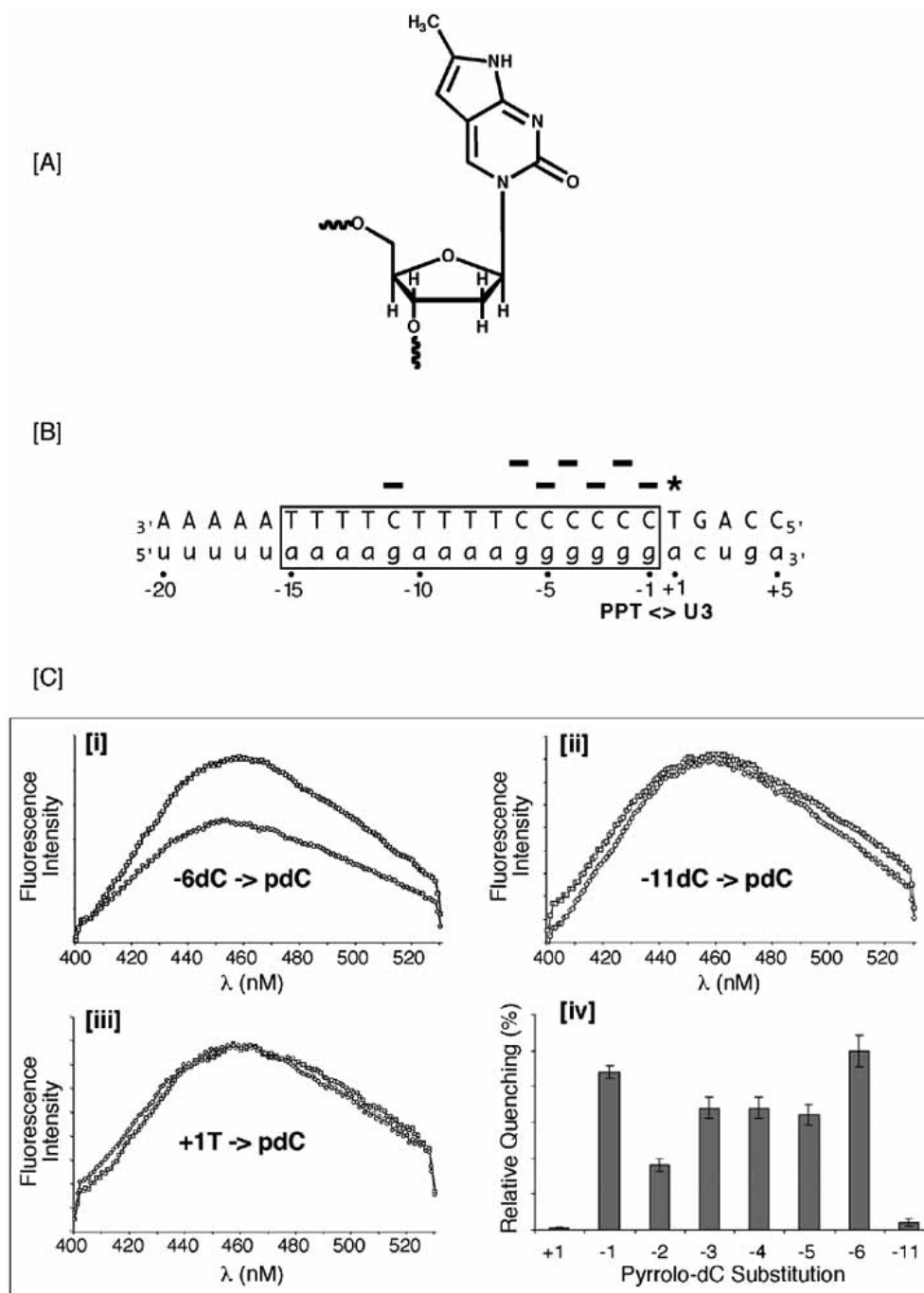


Fig. (5). Examination of HIV-1 PPT structure *via* quenching of pdC fluorescence. [A] Chemical structure of pyrrolo-dC. [B] Experimental strategy. pdC was substituted for dC between positions -1 and -6 (horizontal bars), as well as position -11, of the HIV-1 minus DNA template. As a control, pdC was introduced at position +1 (asterisk) to create an rA:pdC mismatch. [C] [i] - [iii], Representative spectra of pdC-substituted PPT minus DNA templates. [C] [iv], Summary of quenching data for pdC-substituted HIV-1 PPT DNA templates.

“unzipped” portion of the hybrid, this nucleoside has been reported as unpaired in the RT-PPT/DNA co-crystal structure (Fig. 1B; [53]). The environment of dC residues within the rG:dC tract was also of particular interest, since this region was unresolved in the RT-PPT/DNA co-crystal structure.

DNA oligonucleotides containing individual dC→pdC substitutions throughout the PPT rG:dC tract (Fig. 1B), as well as position -11, were hybridized to complementary,

PPT-containing RNA. As a control, a +1 dT→pdC variant was also generated to create a rA:pdC mismatch. The fluorescence of each hybrid was then measured and compared to that of the appropriate single-stranded pdC-containing DNA. The emission spectrum of DNA containing pdC at position -6 (Fig. 5C, panel [i]) illustrates the extent to which fluorescence can be quenched by normal Watson-Crick base pairing following hybridization of complementary RNA. Conversely, in panel [iii], the absence of quenching reflects a lack of base

pairing of the +1 rA:pdC mispair. Fig. 5, Panel [ii] results likewise suggest that pdC at position -11 is unpaired, indicating that the localized unzipping of the hybrid observed in the RT-PPT/DNA co-crystal structure may also occur in solution. Finally, among rG:pdC pairs within the rG:dC tract, base pairing strength was variable, with the most significant deviation from average observed at position -2. The last observation is particularly interesting, since two groups have noted that nucleoside substitutions at this location are particularly detrimental to PPT function [26, 49].

RELIEVING MINOR GROOVE COMPRESSION WITH 2,6-DIAMINOPURINE

Although the biological significance remains controversial, a number of retroviruses [57] harbor a second copy of the PPT at the center of their genome (the cPPT), downstream of which is a sequence of A-tracts defined as the central termination sequence (CTS). Studies with HIV suggested that A-tract induced groove compression, coupled with strand displacement synthesis, served to halt the replication machinery at a late step in (+) strand DNA synthesis prior to nuclear import of the pre-integration complex [34, 35]. In order to study central termination in equine infectious anemia virus (EIAV), an experimental system was developed in which DNA synthesis initiating from the cPPT traverses the CTS (Fig. 6B). In the presence of the four dNTPs, cPPT-mediated DNA synthesis terminated abruptly in the middle of the template following synthesis over a (dT)₆ sequence, i.e. following formation of a (dA)₆:(dT)₆ tract (Fig. 6C, left). This motif has previously been demonstrated to alter DNA curvature and induce minor groove compression [4, 5, 44], which most likely compromised important contacts to the thumb subdomain of the polymerizing enzyme(s). To test this hypothesis, the same DNA synthesis experiment was performed, substituting a 2,6-diaminopurine-containing deoxyribonucleoside triphosphate (dDAPTP) for dATP. Fig. (6C, right) illustrates that this substitution led to synthesis of the full-length cDNA product, demonstrating how the additional Watson-Crick base pairing afforded by 2,6-DAP, which is readily incorporated as an alternative to dATP, can be exploited to study how nucleic acid architecture controls an important event in the retroviral reverse transcription cycle.

PROBING TY3 RT STRUCTURE WITH NUCLEOSIDE ANALOGS - BIOCHEMICAL COMPLEMENTATION

Finally, data of Fig. 7 illustrate how nucleoside analogs can be exploited to provide high resolution information on related RTs for which structural information is presently unavailable. To map contacts between Ty3 RT and duplex DNA, a series of single nucleotide extension experiments was performed on substrates containing single LNA substitutions between positions -3 and -7 of either the template or primer (Fig. 7A). Under these conditions, we observed that introducing an LNA into the primer at position -3, -4 or -5 induced a steric clash that interfered with DNA synthesis, while this clash was alleviated when the LNA was relocated to position -6 or -7 (Fig. 7B). Conversely, LNA analogs at the equivalent positions of the DNA template indicated they could be tolerated between positions -3, -4 and -5, but not at positions -6 and -7. Collectively, the nucleoside analog inter-

ference data of Fig. 7B highlight the importance of asymmetrically-distributed contacts with the DNA template and primer between positions -3 and -7 for efficient polymerization by Ty3 RT.

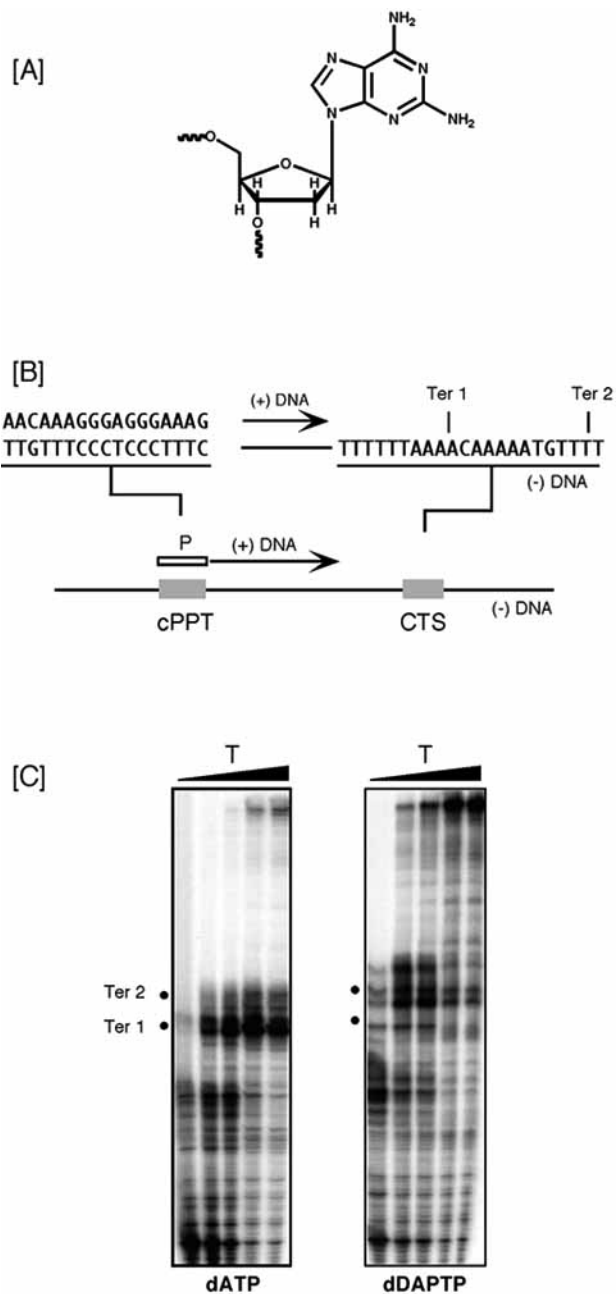


Fig. (6). 2,6-Diaminopurine relieves A-tract-induced minor groove compression at the EIAV central termination sequence. [A] Chemical structure of diaminopurine deoxyriboside (DAP). [B], Experimental strategy. The substrate for DNA-dependent DNA polymerase analysis is a minus DNA template containing the complement of the EIAV central PPT and downstream central termination sequence. DNA synthesis from a plus strand PPT primer is evaluated using a dNTP mixture containing either dATP or dDAPTP. [C] Relief of EIAV central termination by substituting dDAPTP (right) for dATP (left) in the nascent plus DNA. A time course for each DNA-dependent DNA synthesis reaction is presented. The major termination sites Ter 1 and Ter 2 have been indicated.

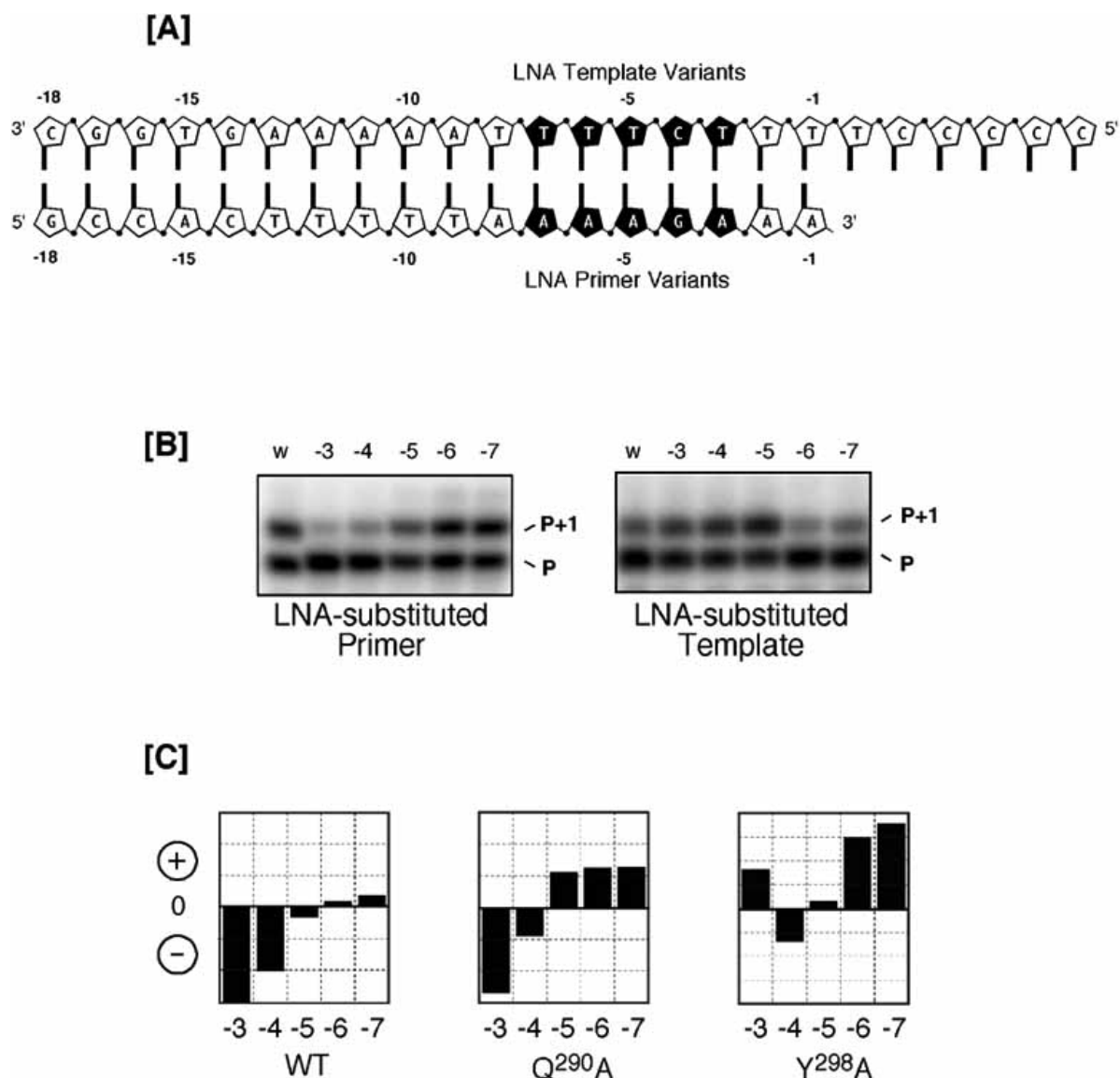


Fig. (7). Exploiting LNAs to map contacts between duplex DNA and the thumb subdomain of Ty3 RT. [A], Experimental strategy. DNA duplexes containing individual LNA substitutions (filled pentamers) between positions -3 and -7 of either the DNA template or primer (defining -1 as the base pair adjacent to the single-stranded template overhang) were used to evaluate single nucleotide addition to the primer terminus by Ty3 RT. [B], Effect of LNA substitutions of the DNA primer (*left*) or template (*right*) on single nucleotide addition to the primer terminus. The position of the substitution is indicated above each panel. Lanes w, unsubstituted template and primer. [C] Fine mapping of contacts between the thumb subdomain of Ty3 RT and duplex DNA by biochemical complementation. The histograms of each panel indicate the efficiency of single nucleotide addition by either wild type Ty3 RT and enzymes containing mutations in the thumb subdomain. Notations “+” and “-“ indicate whether single nucleotide addition was stimulated or inhibited, respectively by the wild type or mutant enzyme.

By analogy with the structure of the HIV-1 enzyme [21, 24], Ty3 RT contacts with the primer between positions -3 and -5 are most likely mediated by its thumb subdomain, which we tentatively identified with the help of computer modeling [6]. Subsequently, residues equivalent to the “translocation track” of HIV-1 RT [15] were selected for alanine scanning mutagenesis [6]. The histogram for wild type Ty3 RT in Fig. 7C again indicates that LNA substitution of primer nucleotides -3, -4 and -5 is incompatible with efficient DNA synthesis. In contrast, Ty3 RT mutant Gln290Ala is active on a duplex containing a -5 LNA-substituted primer, while mutant Tyr298Ala is active on a

duplex containing a -3 LNA substitution. Since the methylene linker of the LNA is oriented into the minor groove, this could potentially result in a steric clash with bulky amino acids of the thumb. However, in the case of Gln290 and Tyr298, this clash can be partially compensated by their substitution with a smaller residue such as alanine. Thus, consistent with our structural model [6], the biochemical complementation data of Fig. 7C indirectly suggested contact between Gln290 of the Ty3 thumb subdomain and the DNA primer at position -5, while residue Tyr298 contacts this at position -3.

CONCLUSION

Clearly, the use of nucleoside analogs as structural probes can be extremely useful for enhancing our understanding of how RTs recognize their various nucleic acid substrates. In addition to the examples mentioned here, many other fluorescent and structural analogs, as well as photocrosslinkable nucleobases, are available for chemical or enzymatic incorporation into RNA or DNA, and have been used as probes in a variety of other model systems [56, 59]. With greater availability and decreasing cost, introducing chemical modifications into nucleic acid should increasingly complement site-directed mutagenesis as a tool for studying protein-nucleic acid interactions.

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