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27. We thank A. M. Prochera, S. Hope, and P. Lopez for technical assistance, E. Silva for editorial assistance, and NIDA for services provided under contract #271–89–8159. Supported by Houghten Pharmaceuticals Inc. (San Diego, CA) (C.T.D. and R.A.H.); the Medical Research Council of Canada (grant MT-10131 to P.W.S.); and NIDA (grant DA-03742 to J.M.B., grants DA-02615 and DA-000138 to G.W.P., and grant DA-04443 to P.W.S.).

27 June 1994; accepted 25 October 1994

TECHNICAL COMMENTS

A Unified Polymerase Mechanism for Nonhomologous DNA and RNA Polymerases

Extrapolating from the co-crystal structure of rat DNA polymerase β (pol β) complexed with primer-template, dideoxycytidine triphosphate (ddCTP), and two metal ions, H. Pelletier *et al.* (*1*) recently concluded that the orientation of the DNA primer-template in *Escherichia coli* DNA polymerase I Klenow fragment (KF) and the reverse transcriptase (RT) of human immunodeficiency virus–type 1 is opposite to that derived from published co-crystal structures (*2*, *3*). We disagree with this conclusion and suggest an alternative interpretation of the structural data, namely, that there is no contradiction between the orientations of the DNA inferred from these structures; rather, the apparent inconsistency is the result of an inappropriate alignment of the pol β structure with the other polymerase structures. While the crystal structures of KF, RT, and T7 RNA polymerase (RNAP) can be aligned by superposition of a homologous "palm" subdomain, pol β is not homologous to these other polymerases, and therefore should not be aligned with them by superimposing protein structures. Instead, we suggest that pol β can be oriented relative to this family only by superposition of the functionally important entities in the polymerase reaction, namely, the two catalytic divalent metal ions and the 3′ terminus of DNA primer strand. This alignment is achieved by rotating the entire pol β complex by about 180° (Figs. 1 and 2) from the structural alignment proposed by Pelletier *et al.* (*1*). The alignment we suggest allows all four polymerases to use the identical polymerase mechanism on similarly oriented primer-template molecules without the need to re-orient the primer-templates from their previously determined positions and is therefore consistent with structural, biochemical, and molecular genetic studies of polymerase-substrate complexes. [By contrast, the proposal of Pelletier *et al.* (*1*) that the direction of primer-template binding to KF, RT, and RNAP should be reversed contradicts the conclusions drawn from a substantial body of existing data.] A further advantage of our proposed alignment is that it reveals additional analogies between pol β and the other three polymerases in the overall structure of the polymerase domain.

A dominant and consistent feature of all four polymerase structures determined thus far (*4*–*8*) is the existence of a large cleft comprised of three subdomains dubbed "fingers," "palm," and "thumb" by virtue of the anatomical similarity of the polymerase domain to the structure of a right hand (*4*, *5*). The palm subdomain lies at the bottom of the cleft and contains the catalytically essential acidic residues, whose function has been established to be that of binding the metal ions involved in catalysis of the polymerase reaction (*1*, *7*, *9*). The substantial structural similarity in the palm subdomain structures of KF, RT, and RNAP implies that this subdomain is homologous in these three polymerases. By contrast, the corresponding subdomain of pol β is not homologous to those of the three other polymerases, as Davies *et al.* (*7*) and Sawaya *et al.* (*8*) have documented. The palm subdomain of pol β shows some limited structural analogy to the other polymerases in that they all contain a multistranded β sheet with two α helices on one side; however, the topology of the whole pol β palm subdomain is different. Superposition of the Cα positions of the pol β palm subdomain on any of the palm subdomains in the three other structures results in superimposed β strands with opposite orientations. Moreover, the relative positions of the three-metal-ion–binding carboxylates in the ami-

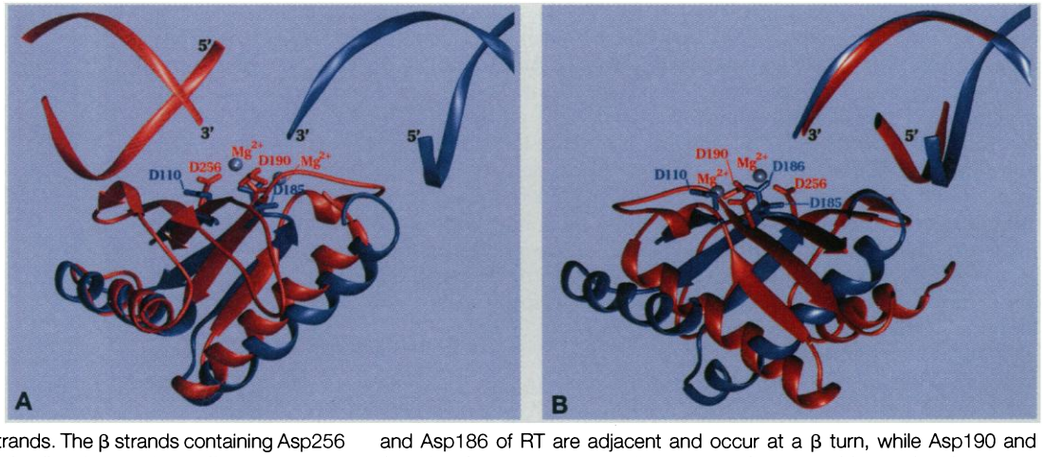

Fig. 1. Alignment of the "palm" subdomains of reverse transcriptase (blue) and pol β (red) and their respective co-crystallized DNAs (*1*, *3*) by (**A**) superimposing the corresponding Cα positions of two α helices and two β strands in the two enzymes, as done by Pelletier *et al.* (*1*), and (**B**) superimposing the 3′ ends of the DNA primer strands in the two complexes. The alignment in (B) was achieved by superimposing the corresponding phosphorous positions at the 3′ end of the DNA primer strands, which puts the metal-binding carboxylates in similar positions, in spite of the lack of superposition of the protein strands. The β strands containing Asp256 in pol β and Asp110 in RT run in opposite directions and are differently ordered relative to the other two carboxylates on the protein sequence. The Asp185 and Asp186 of RT are adjacent and occur at a β turn, while Asp190 and Asp192 of pol β are necessarily separated by one residue so that they occur on the same side of an extended β strand.

no acid sequence of pol β are different from the positions of the corresponding carboxylates in the other three polymerases (*7*, *8*). Both groups working on the pol β structure have therefore concluded, on the basis of these differences in connectivity and topology of the palm subdomains, that it is unlikely that the palm subdomains of pol β and the other three polymerases arose from a common ancestor [that is, they are not homologous (*7*, *8*)]. Moreover, Sawaya *et al.* (*8*) have pointed out that the core folding motif (α-β-β-α), which appears to be common to pol β and the other three polymerase structures (Fig. 1A), is also found in other unrelated protein structures. Given the lack of homology between the palm subdomains of pol β and the other three structures. There is no compelling justification for superimposing one small structural motif and therefore no reason to favor this alignment over the alternative (Fig. 1B), in which the protein structures appear completely dissimilar but the trio of catalytic carboxylates are nevertheless similarly positioned.

In KF, RT, and RNAP, the similarities among the finger and thumb subdomains are less than that among the palm subdomain (Fig. 2). The fingers subdomain forms a long wall on one side of the cleft; although the structures of the fingers subdomains of KF and RNAP appear homologous, that of RT is unrelated. The thumb subdomains form the other side of the cleft and have analogous rather than homologous structures in these three enzymes. In each case, they are long, mostly α-helical protrusions that move in response to the binding of DNA (*4*, *5*). KF has an additional flexible subdomain at the tip of its thumb. Because alignment of pol β with the other polymerase structures by superposition of the DNA substrates rotates pol β through 180° from the relative polymerase orientation favored by Pelletier *et al.* (*1*), the names of the fingers and thumb subdomains of pol β should be interchanged to be consistent with the other polymerases. When this is done, a striking structural analogy is revealed. The (new) fingers subdomain of pol β (colored blue in Fig. 2) forms a wall on one side of the cleft and interacts with the template strand, as in the other polymerases, and the thumb (colored green in Fig. 2) consists of an α-helical protrusion with a flexible tip (containing the 8K subdomain) whose structure changes upon binding DNA. This subdomain in the thumb of pol β may be analogous to the flexible tip of the KF thumb, but the nature of its interactions with DNA is unknown, as its position in both crystal forms is influenced by major packing contacts between it and the end of a neighboring DNA molecule. Thus, although the alignment we propose for the pol β structure de-emphasizes the apparent structural similarities in the palm subdomain, it shows compelling structural analogies in the fingers and thumb subdomains, which may well reflect functional analogies between these subdomains among the polymerases.

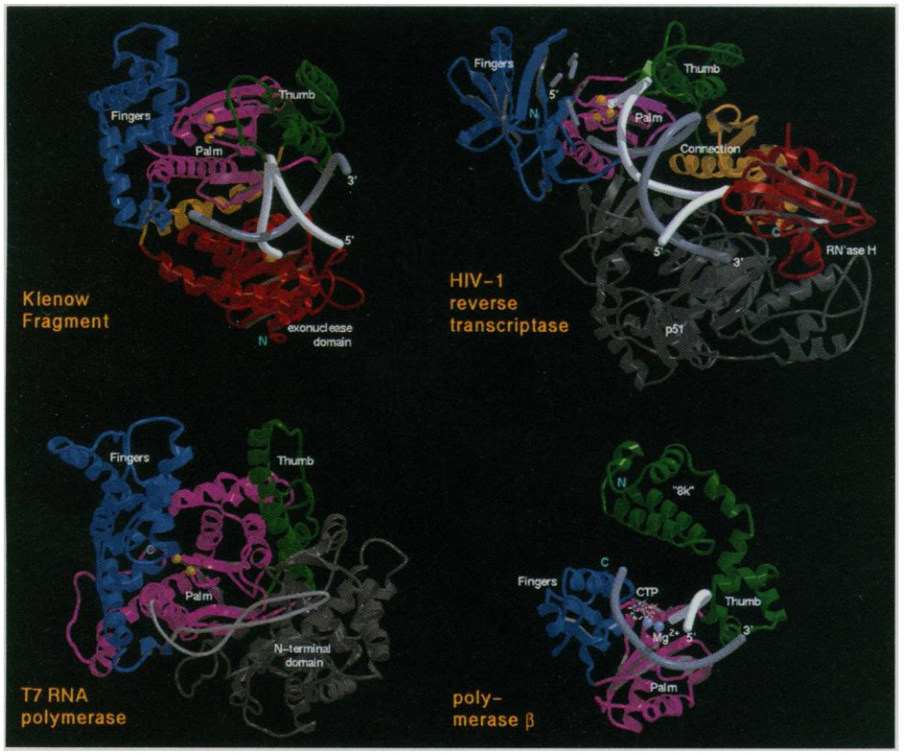

Fig. 2. The backbone crystal structures of HIV RT, KF, pol β, and RNAP proteins. RT, KF, and RNAP were aligned by superposition of their homologous palm subdomains. Pol β was aligned by superimposing the terminal three phosphates of the bound primer strand (*1*) on the corresponding three phosphates of the primer strand bound to RT (*3*) as shown in Fig. 1B. (The optimal alignment of other polymerases on pol β will only be possible when the positions of the two metal ions, the dNTP α phosphate, and the 3′-OH of the primer strand are known in each case.) The experimentally observed DNA backbones are shown as coils in white for the primer strands and gray for the template strands. The Cα positions of the catalytic carboxylic acid residues are shown as yellow spheres. Functionally analogous interactions are seen between the primer-template and the fingers and thumb subdomains in all four polymerases. The template strand bound to RT has been extended by model building (dashed line) to show that it interacts with the fingers subdomain, as is also the case for the template strand in the pol β complex (using the changed naming of subdomains resulting from our alternative alignment).

The important function of the palm subdomain in each of the four polymerase structures is to present two metal ions in the appropriate geometrical arrangement to catalyze a phosphoryl transfer reaction at the polymerase active site. A chemical mechanism of phosphoryl transfer catalyzed by two divalent metal ions spaced 3.8 Å apart was proposed initially from the structure of the 3′ to 5′ exonuclease domain of KF bound to single-stranded DNA (*10*) [and supported by molecular genetic experiments (*11*)] and from the structure of alkaline phosphatase (*12*). This mechanism was then extrapolated to the phosphoryl transfer reactions catalyzed by ribozymes and by polymerases (*10*, *13*, *14*). The structures of pol β complexed with Mn^{2+} and dTTP (*7*) and with Mg^{2+}, primer-template, and ddCTP (*1*) confirm the earlier proposal of a two-metal-ion mechanism for the polymerase reaction and also show that one of these metal ions interacts with all three phosphates of the deoxynucleoside triphosphate (dNTP), which was not anticipated (*13*). However, the more recent proposal by Pelletier *et al.* (*1*) that one aspartic acid is acting both as a metal ion ligand and a general base abstracting a proton from the 3′-OH is not possible. The available oxygen of the carboxylate (Asp^{256} Oδ1 in the case of pol β) is too far from the 3′-OH of the primer terminus and has the wrong orientation to allow interaction. Further, the pK_a of a metallated carboxyl group is inappropriate for a role as general base. We suggest, therefore, that only the positioning and chemical properties of the two metal ions are necessary to effect catalysis. Thus, an important feature of a two-metal-ion mechanism is the absence of a requirement for the direct participation of protein side chains in the chemistry of catalysis (Fig. 3).

It can also work in either direction, as happens in the two-step alkaline phosphatase reaction. Thus, it is of no consequence that the protein environment of the metal ions, including the positions of the carboxylate ligands, will be very different in our proposed alignment of pol β from that in KF, RT, or RNAP. Indeed, the three catalytically important carboxylates in KF are liganded to the two metal ions in a manner different from that in the case of pol β (*13*, *15*). Even within the group of three homologous structures, the metal ion coordination must differ, as KF uses two aspartate side chains and one glutamate, RT uses three aspartates, and RNAP uses only two aspartates. What is important for catalysis is that the metal ions be positioned identically relative to the primer terminus and the dNTP α-phosphate in all of the polymerase structures.

The relationship of the catalytic mechanism of pol β to that of KF, RT, and RNAP thus provides an illustration of convergent evolution, analogous to the now classic example of subtilisin, whose relation to the mammalian serine proteases was elucidated by Joseph Kraut and his co-workers (*16*). In the bacterial serine protease, subtilisin, the catalytic triad of Asp-His-Ser side chains and the enzyme binding sites that stabilize the substrate oxyanion formed in the transition state (*17*) have the same structures and perform the same catalytic functions as in the mammalian serine proteases, even though the surrounding protein scaffold is entirely different. In an analogous way, the two metal ions are the catalytically important entities in polymerases, and the rest of the enzyme needs only to present the substrate properly to these catalytic groups. Indeed, as has been previously pointed out (*10*, *13*, *14*), a phosphoryl transfer reaction could be achieved by this mechanism even if the rest of the enzyme were made of RNA.

While the DNA orientations previously proposed for KF and RT were derived from co-crystal structures in addition to other considerations, the orientations proposed by Pelletier *et al.* (*1*) are not supported by any experimental data. The suggestion (*1*) that the DNA co-crystal structures of KF and RT are to be ignored because the crystallization conditions were unphysiological does not appear to be justifiable. Very few of the protein structures determined in the past four decades have been done under physiological conditions, including that of the pol β complex with DNA, which was crystallized from 9% polyethylene glycol and 75 mM lithium sulfate. Many complexes of protein with nucleic acid have been crystallized from the high ionic strengths of 1 M to 2 M ammonium sulfate, including those of the Gln-tRNA synthetase complexed with $tRNA^{Gln}$ (*18*) and the bacteriophage 434 repressor complexed with operator DNA (*19*). In fact, in many cases, increasing ionic strength increases the ratio of specific to nonspecific binding because specific interactions often include a significant hydrophobic component, while nonspecific interactions are largely electrostatic in nature. Finally, the suggestion (*1*) that the relevance of protein-ligand complex structures is suspect if crystals of the complex are isomorphous with those of the apo-protein, even when co-crystallized as the complex, is without precedent. Substrate and inhibitor complexes whose crystals were isomorphous with those of the apo-protein were found initially with myoglobin, lysozyme, carboxypeptidase A, α-chymotrypsin, and ribonuclease S in the 1960s and have occurred in hundreds of complexes since. In short, there is no reason to expect that the co-crystal structure of pol β with DNA is any more or less relevant to the complex that exists in solution than the structures of KF and RT co-crystallized with DNA.

The direction of DNA binding that Pelletier *et al.* (*1*) propose for KF, RT, and RNAP is also inconsistent with a number of biochemical experiments. As they point out, the placement of the ribonuclease H domain in RT demands that an RNA-DNA hybrid substrate must be bound in the orientation originally proposed (*5*) and observed in the DNA co-crystal structure (*3*). Furthermore, footprinting of a bound DNA-DNA duplex is also consistent with this mode of binding [rather than that suggested by Pelletier *et al.* (*1*)], as the extent of protection upstream of the primer terminus (*20*) is more extensive than would be achieved with the opposite orientation of binding (Fig. 2). Moreover, the proposal (*1*) of two possible orientations of primer-template binding by RT raises substantial problems at the polymerase active site, requiring two overlapping binding sites for the phosphodiester backbone, because switching directions also switches strands. For RNAP, the previously proposed direction of DNA binding [figure 5 in (*6*)] is supported by footprinting data [for both initiation (*21*) and elongation complexes (*22*)], which show extensive protection upstream of the site of RNA synthesis, and by mutational data identifying the protein contact to the −11 position of the promoter (*23*). The opposite orientation, proposed by Pelletier *et al.* (*1*), would give no contact between the protein and most of the promoter.

The crystallographic evidence for the orientation of a primer-template bound with its primer terminus at the polymerase active site of KF is less direct than for RT. The co-crystal structure (*2*) is of an editing complex with the 3′ end of the DNA in the 3′ to 5′ exonuclease active site rather than the polymerase active site. Nevertheless, the duplex portion of the DNA is observed to make interactions with the few residues in the thumb subdomain that are highly conserved in the pol I family, consistent with the conclusion (*2*) that the observed duplex position is the binding site for the product of DNA synthesis. Moreover, the homology between the palm subdomains of KF, RT, and RNAP demands that the orientation of primer-template relative to this subdomain must be the same in all three of these polymerases. Now that the position of the polymerase active site within the cleft of KF is established, it is also clear that the

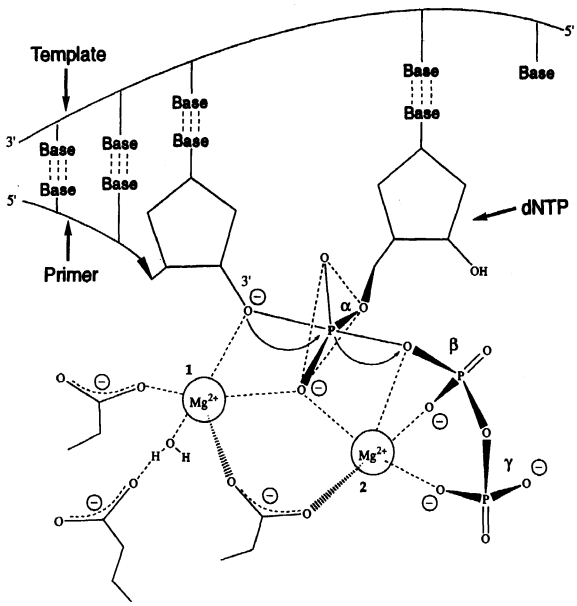

Fig. 3. The intermediate (or transition state) of the two-divalent-metal-ion mechanism for the polymerase reaction. A detailed proposal (*13*) of a two-metal-ion mechanism for the polymerase reaction reproduced here has been altered to reflect the interactions of the β and γ phosphates with Mg^{2+} number 2, as observed in the pol β complex with dTTP and Mn^{2+} (*8*) and the pol β complex with primer-template and ddCTP (*1*). The carboxylate ligands are in generic positions not intended to represent any specific polymerase. The roles proposed for Mg^{2+} number 1 are to lower the pK_a of the 3′-OH in order to form the 3′-O^- and to stabilize the 90° 3′-O-P-O bond angle between the apical and equatorial oxygens in the transition state (*13*). The role of Mg^{2+} number 2 is likewise to stabilize the pentacovalent transition state geometry and to facilitate the leaving of the pyrophospate (*25*).

extent of protection by KF of DNA upstream of the primer terminus (*24*) is more consistent with the model deduced from the co-crystal (*2*) than with the earlier model (*4*).

Thus, we conclude that all four of the polymerases of known structure will be able to use the same two-metal-ion mechanism of catalysis in spite of the detailed differences in the way that these metal ions are anchored to the protein in each of the four polymerases. While the 3′-OH of the primer terminus, the phosphates of dNTP (or ribonucleoside triphosphate), and the two-metal ions should show the same relative orientations in all polymerases, the precise structure of the product duplex as it emerges from the site of synthesis may well differ. High-resolution crystal structures of other polymerases with primer-template and dNTP [like the ternary complex of pol β reported by Pelletier *et al.* (*1*)] will be necessary to allow more precise relative alignments of polymerases and detailed mechanistic comparisons to be made.

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11 August 1994; accepted 25 October 1994

Polymerase Structures and Mechanism

The comment by T. Steitz *et al.* (*1*) disagrees with the conclusions in our paper (*2*) that were based on our structural studies of rat DNA polymerase β (*2*, *3*). Nevertheless, given the current state of the literature on DNA and RNA polymerases, every proposal presented in our paper is reasonable and can be tested experimentally (*2*).

The proposals of Steitz *et al.* (*1*) depend on the idea that pol β is nonhomologous with other polymerases (that is, that pol β arose from a different ancestor), and Steitz *et al.* (*1*) cite our report (*3*) as taking the view that the palm subdomain of pol β is a result of convergent evolution. However, our discussions (*3*) favor neither convergence nor divergence because there appears to be equally strong evidence supporting both sides of the debate over the evolution of pol β.

In contrast to the effect it has on the proposals of Steitz *et al.* (*1*), the evolutionary history of pol β has no bearing on our structural alignments and proposals (*2*, *3*). Regardless of whether pol β converged or diverged, there are three catalytically important, highly conserved carboxylic acid residues located in the palm subdomain, and only one unambiguous orientation of pol β superposes these residues with those of all other polymerase crystal structures (*2*–*7*) (Fig. 1). [Even in the case of covergent evolution (*8*), it is expected that the catalytic residues, at the very least, superpose. Otherwise, it is not really convergent evolution.] That the rest of the palm subdomain of pol β then happened to superpose well with the other polymerase palms was additional evidence that the structural alignments (*3*) were correct.

Instead of relying on structural alignments of conserved active site residues, Steitz *et al.* (*1*) suggest that a greater weight be placed on functional alignments of nonhomologous entities in the fingers and thumb subdomains of the polymerase structures. However, a "functionality" approach can still favor our current pol β structural alignments (*2*, *3*). The thumb subdomain of pol β, for instance, shows greater flexibility than the fingers subdomain (*2*), as is also the case for other polymerases (*5*, *6*). In addition, structural elements that are similar to alpha helices M and N of pol β only in that they protrude into the active site from the same "thumb" side of the DNA binding channel are present in all other polymerase structures and are proposed to

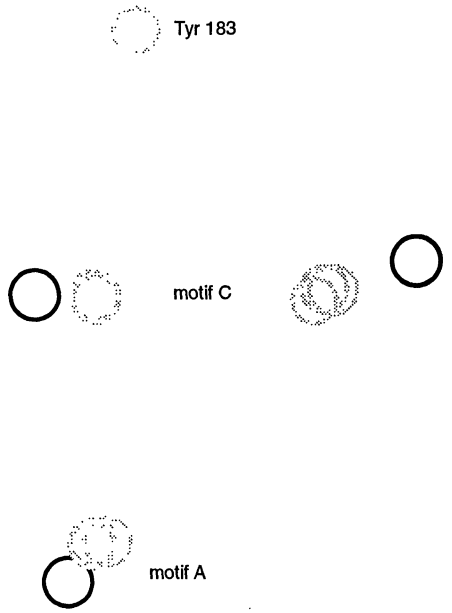

Fig. 1. Least squares superposition of the Cα's for the catalytically critical carboxylate residues of four different polymerase active sites (*2*–*7*). Dark circles represent Asp^{190} and Asp^{192} (motif C), as well as Asp^{256} (motif A), of pol β, and lighter circles represent active site carboxylates of the other three polymerases for which crystal structures are known [see (*3*, figure 2) for a listing of the residue numbers]. The 3′-OH of the DNA primer terminus approaches the active site from the direction of the bottom of the page (*2*). In keeping with previous proposals (*2*), the Cα position of Tyr^{183} of RT is included as an integral part of the active site for that polymerase and allows the 3′-OH of the DNA primer terminus to approach the active site from the direction of the top of the page, as is the case when RT is in an anti-pol β mode of DNA binding (*2*). The protein side chains [not shown (*14*)], extend out of the plane of the page toward the viewer.