Published in final edited form as:
J Am Chem Soc. 2000 February 16; 122(6): 1001–1007. doi:10.1021/ja993464+.

Varied Molecular Interactions at the Active Sites of Several DNA Polymerases: Nonpolar Nucleoside Isosteres as Probes

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Abstract

We describe a survey of protein–DNA interactions with seven different DNA polymerases and reverse transcriptases, carried out with nonpolar nucleoside isosteres F (a thymidine analog) and Z and Q (deoxyadenosine analogues). Previous results have shown that Z and F can be efficiently replicated opposite each other by the exonuclease-free Klenow fragment of DNA polymerase I from *Escherichia coli* (KF$^-$), although both of them lack Watson–Crick H-bonding ability. We find that exonuclease-inactive T7 DNA polymerase (T7$^-$), *Thermus aquaticus* DNA polymerase (Taq), and HIV-reverse transcriptase (HIV–RT) synthesize the nonnatural base pairs A–F, F–A, F–Z, and Z–F with high efficiency, similarly to KF$^-$. Steady-state kinetics were also measured for T7$^-$ and the efficiency of insertion is very similar to that of KF$^-$; interestingly, the replication selectivity with this pair is higher for T7$^-$ than KF$^-$, possibly due to a tighter active site. A second group comprised of calf thymus DNA polymerase α (Pol α) and avian myeloblastosis virus reverse transcriptase (AMV-RT) was able to replicate the A–F and F–A base pairs to some extent but not the F–Z and the Z–F base pairs. Most of the insertion was recovered when Z was replaced by the nucleoside Q (9-methyl-1-H-imidazo[(4,5)-*b*]pyridine), which is analogous to Z but possesses a minor groove acceptor nitrogen. This strongly supports the existence of an energetically important hydrogen-bonded interaction between the polymerase and the minor groove at the incipient base pair for these enzymes. A third group, formed by human DNA polymerase *β* (Pol *β*) and Moloney murine leukemia virus reverse transcriptase (MMLV-RT), failed to replicate the F–Z and Z–F base pairs. No insertion recovery was observed when Z was replaced by Q, possibly indicating that hydrogen bonds are needed at both the template and the triphosphate sites. The results point out the importance of DNA minor groove interactions at the incipient base pair for the activity of some polymerases, and demonstrate the variation in these interactions from enzyme to enzyme.

Introduction

Recently a sizable number of crystal structures of DNA polymerase enzymes have been solved, helping to gain considerable molecular understanding of their biochemical mechanism.[1] Cocrystals of polymerases with DNA have added much detail to the picture of how polymerase activities such as replication and proofreading are carried out.1a–i At the same time, an even broader array of mechanistic studies has been carried out with this family of enzymes, painting a dynamic and functional picture of the process as a whole.[2] A detailed understanding of polymerases is, of course, important because of the essential role that the fidelity of information transfer plays in replication and in disease states. This information takes on practical importance as well, since polymerases are targets for a number of useful therapeutic agents.

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Although most of the interactions between polymerases and their nucleic acid substrates occur primarily along the outer sugar–phosphate backbone of the DNA, the interactions which govern the selection of the appropriate nucleotide are made inside this radius and involve the bases themselves. The published ternary crystal structures of complexes of DNA polymerases with double-stranded DNA and an incoming nucleotide show specific interactions between the minor groove of the DNA and H-bond donor groups of the protein,1a–e and this finding has been suggestive of possibly important roles played by these interactions.[3] Although some of these interactions have been examined by mutagenesis of specific side chains in polymerases,[4] few studies exist in which the functional importance of H-bond acceptors in the DNA itself has been probed.

There exist only a few studies in which polymerase minor groove interactions in KF^{-} or Taq were evaluated with modified DNAs. Spratt used a 3-deazaguanine nucleoside to evaluate the possibility of an H-bond to the 3-position in a template strand.5 The elimination of this nitrogen donor was observed to cause a 170-fold decrease in insertion efficiency with Klenow polymerase. However, data with analogues from our laboratory suggest that there is no decrease in efficiency when N is replaced by CH at the same position (see data below). Two other modified nucleotides, dCTP and dTTP analogues lacking minor groove keto groups, were used by Guo et al. to test minor groove interactions at the primer strand with KF and Taq.[6] A complete lack of insertion, and even polymerase inhibition, was observed with these analogues, which was attributed to loss of a minor groove interaction. In contrast to this, preliminary data from our laboratory have found little or no effect of elimination of a minor groove H-bonding group on the incoming nucleotide (see below). In a related study, a 3-deaza-dATP analogue was found to inhibit KF,7 although whether the inhibition arose in the insertion or extension step is not known.

To avoid some of the problems associated with known nucleoside analogues, such as steric differences, tautomerization, and alteration of basicity relative to their natural counterparts, we have proposed the use of nonpolar analogues for thymidine (F) and for adenosine (Z) to study DNA–protein interactions.[8] These compounds maintain steric size and shape as closely as possible, and have all Watson–Crick hydrogen-bonding groups removed to eliminate complications from those polar groups. Importantly, recent NMR studies have shown that the Z–F pair in duplex DNA is closely analogous in structure to an A–T pair.8f We have found that analogue base pairs such as A–F, F–A, F–Z, and Z–F are efficiently and selectively synthesized by KF^{-}, supporting the idea that shape complementarity and solvation effects play as important a role as base–base hydrogen bonds.[9,10] In a preliminary study, the possible significance of interactions between KF^{-} and the minor groove of DNA was also investigated.[11] For this polymerase it was found that minor groove interactions have a qualitatively small effect for insertion, but a single interaction in the primer strand can make a 300-fold difference in extension efficiency.

Here, we report new studies with a varied series of DNA polymerases and reverse transcriptases to investigate the general importance of minor groove interactions, Watson–Crick hydrogen bonds, geometric constraints, and solvation at the insertion step of replication. We find that the polymerases can be grouped into three classes, depending on the number of minor groove interactions needed at the active site and the tightness of the active site for efficient base pair synthesis. In addition, for the majority of cases no hydrogen bonds between the bases appear to be needed during the insertion step if the requirements of correct geometry and specific minor groove interactions are fulfilled. The results also illustrate the general utility of these nonpolar base analogues as probes of protein–DNA interactions.

Results

Single-Nucleotide Insertion Studies

We carried out single-nucleotide insertion experiments on a synthetic 23nt primer– 28nt template duplex containing F or Z in the template, and also using dFTP and dZTP (the nucleoside triphosphates of F and Z, respectively) as substrates (Figure 1). Seven widely varied DNA polymerases were studied, and we also repeated previous experiments with KF^{-} for comparison. Nonforcing conditions were used for all insertion reactions, so that natural base mismatches were generally not observed. Experiments were carried out in the presence of a single nucleoside triphosphate, and successful insertion is indicated by elongation of the radiolabeled primer to 24nt length.

The results are shown in Figures 2–4, grouped into three broad classes of findings. Three enzymes, $T7^{-}$, Taq, and HIV-RT, show results very similar to the previously reported data for KF^{-} (Figure 2).[9] First we examine the case with the analogues in the template strand (lanes 5–12). Among the four natural nucleotides, dATP is inserted very efficiently opposite F (A→F) by these enzymes, and dTTP is inserted to some extent opposite Z. Note that base pairs A–F and T–Z are the closest mimics of the natural base pairs A–T and T–A. Also observed to a lesser extent is the insertion of T opposite F, especially for HIV-RT where it appears to be roughly equivalent to the efficiency of C misinsertion opposite A.

When the nonpolar nucleotides dFTP and dZTP are used in the polymerization reaction (Figure 2, lanes 13–24) several new base pairs are formed by this first group of enzymes. The nucleotide dFTP is inserted with high efficiency opposite A, opposite itself, and opposite Z, whereas dZTP is processed more selectively and is inserted preferentially opposite F in the template. Some small insertion of dZTP opposite T, and to a lesser extent A, is observed for Taq and HIV-RT, but not for KF^{-} and $T7^{-}$. All these base pairs can closely mimic Watson–Crick geometry in the active site, except the Z→A mismatch, which may be synthesized due to a change of conformation of Z or A to syn. The Z→T base pair is likely synthesized poorly because of the high cost of desolvation of T when nonpolar Z is placed next to it.[10,12] Taken together, the results in Figure 2 suggest that none of these four enzymes needs hydrogen bonds between the bases for efficient nucleotide insertion. In addition, hydrogen bonds between the minor groove of the incipient base pair and the protein do not seem to be important either, since F and Z lack H-bond acceptor groups at those positions and are replicated very efficiently opposite each other nonetheless.

A second grouping of results is constituted by Pol α and AMV-RT (Figure 3). These polymerases were found to synthesize only a subset of the new base pairs, and generally did so with less efficiency than the first group of polymerases. The pairs F→A and Z→F were formed with high efficiency by Pol α, while A→F and F→Z were synthesized with lower efficiency under these nonforcing conditions. With AMV-RT, only A→F synthesis is efficient, and F→F, F→Z, and Z→F are moderate to low. With the former enzyme it is interesting to note that two unexpected base pairs, F→C and Z→C, were partially synthesized, which may be due to a better fit of Z opposite cytosine (which possesses a smaller N at the 3-position, whereas thymidine has the sterically larger N–H) in a highly constrained active site.

The third group, consisting of Pol *β* and MMLV-RT, was the least accepting of the nonnatural nucleotides (Figure 4). There was very little synthesis of any of the new base pairs with F or Z in the template or with the triphosphates dFTP or dZTP. The figure shows that only the base pairs A→F (for Pol *β*) and F→T (for MMLV-RT) were obtained with low efficiency.

Several reasons can be argued to explain the differences between the three groups on replicating the nonpolar base pairs. First, it is possible that Watson–Crick hydrogen bonds between the

bases are indispensable for DNA synthesis for the last group of polymerases. We consider this less convincing because a polymerase has no direct way of sensing these bonds, which are internal to the DNA. It is possible that the lack of H-bonds can be indirectly sensed, by the resulting destabilization of the duplex; however, our analogue base pairs are actually not destabilizing at the end of the DNA (see discussion below). A second possibility is that the active sites become increasingly sterically restrictive in the second and third groups. The analogue Z is larger by about 0.7 Å than its natural analogue A because of the C–H group at the 3 position,[8e] and thus it may not be sterically well accepted by some of the enzymes. Third, specific minor groove interactions between the incipient base pair and the enzyme active site may be energetically necessary at the transition state for nucleotide insertion, at least for some in the latter two groups of proteins. To investigate this third possibility, the nonpolar isostere Q was compared to Z in insertion studies.

Probing Minor Groove Interactions at the Active Site for Nucleotide Insertion

Minor groove interactions at the active site could explain the low efficiency in the synthesis of some of the nonpolar base pairs by the second and third groups of polymerases. To investigate this hypothesis, we carried out single-nucleotide insertion experiments comparing all natural triphosphates and dFTP opposite analogues Z and Q in the template, as well as comparing the insertion of dZTP and dQTP opposite all natural bases and analogues F, Z, and Q. The structures of Z and Q are the same except that the CH group at position 3 of Z is replaced by a nitrogen in Q (Figure 1), thus placing an H-bond acceptor in the minor groove analogous to A.[11] These experiments were carried out for all the DNA polymerases and reverse transcriptases of the second and third groups. The results for the second group are given in Figure 5.

When Q or dQTP was substituted for Z or dZTP, new base pairs were synthesized for the polymerases of the second group. When Z and Q are placed in the template (Figure 5, lanes 6–15), dFTP is inserted with much higher efficiency opposite Q than opposite Z by Pol α, suggesting that a specific H-bond at the template side of the incipient base pair is necessary for replication with that enzyme. Interestingly, the results are quite different when the minor groove on the primer side of the incipient base pair is probed (lanes 16–29). We find that Pol α prefers to insert dZTP opposite F considerably more efficiently than dQTP opposite F. This suggests the possibility of a nonpolar side chain of the enzyme interacting with position 3 of the nucleotide. Both results are consistent with the observed very efficient synthesis of F→A base pair by this polymerase, since this pair would satisfy both of these proposed interactions.

In the case of AMV-RT, we observe only insertion of dQTP (not dZTP) opposite F (Figure 5, lane 27). No insertions are seen when either Z or Q is placed in the template. In contrast with Pol α, the results with AMV-RT suggest that a minor groove hydrogen bond between the incoming nucleotide and the enzyme, but not with the template, is required. The other pairs such as Q→T vs Z→T are not affected with either Pol α or AMV-RT, which may be due to the higher cost for desolvation of T relative to F, which may negate any benefit of an improved minor groove interaction.

When insertion experiments comparing Z and Q were carried out for Pol *β* and MMLV-RT (the third group), formation of nonpolar base pairs was not observed with either analogue. The same results were obtained when dZTP and dQTP were compared. The results suggest that these enzymes may require an extremely close fit to Watson–Crick geometry, and/or that minor groove interactions at both sides of the groove (template side and incoming nucleotide) may be energetically necessary. Significantly, the X-ray crystal structure of the complex of Pol *β* with double-standed DNA and a nucleotide shows interactions consistent with this latter possibility (see discussion below).[1e]

Quantitative Single-Nucleotide Insertion Studies with T7 DNA Polymerase

Previous steady-state kinetics studies of analogues F and Z were carried out with KF^{-}, a poorly processive repair enzyme.[9] We were interested, therefore, to know in detail how a highly processive replicative enzyme would process these analogues. For that reason we undertook quantitative studies with T7^{-} polymerase so that we could compare the results with previous data for KF^{-}. These values would also lend more detailed information about how similar the polymerases of the first group are.

We carried out studies of insertion of all natural triphosphates opposite Z, insertion of dZTP opposite all natural bases, as well as opposite itself, and finally insertion of base pairs involving F that will resemble an A–T base pair, such as A→F, F→A, F→Z or Z→F, and also F→F, to try to get information about the steric requirements of the active site. We also measured formation of a natural base pair (A→T) and a mismatch (A→C) as controls. The data are given in Table 1.

First we investigated replication with Z in the template. The results show that T7^{-} inserts dFTP opposite Z with high efficiency and preferentially over the natural nucleotides and dZTP by a factor of 30–2500. Similar to experiments with KF^{-}, dTTP is found to be the best inserted of all the natural nucleotides by a factor of 2–800, consistent with the idea of shape complementarity even though dATP is desolvated much more easily. When dZTP was used as the triphosphate by T7^{-} DNA polymerase, we found that it was inserted efficiently and very selectively opposite F over the natural bases by a factor of 230–880. Remarkably, T7^{-} shows an almost 2 orders of magnitude difference in selecting dZTP opposite F than dZTP opposite Z based (apparently) only on steric exclusion. At the same time, when dFTP is employed as the substrate for T7, we find that it is inserted better opposite itself than opposite Z with a 5-fold preference, and only less efficiently than a natural base pair by a factor of 32. The data suggest that this may reflect the larger size of the F–Z base pair compared to a natural base pair. Only A→F is inserted with higher efficiency than F→F, which may be due to small hydrogen bond interactions between the bases, or because the natural base is still a better substrate for the enzyme.

In summary, the data show that all the nonpolar base pairs mimicking an A–T base pair (A→F, F→A, F→Z, and Z→F) and F→F are synthesized quite efficiently by T7^{-}, and noticeable selectivity is found relative to “mismatched” base pairs in which Watson–Crick geometry is sterically excluded. These quantitative results are similar to the values found for KF^{-}, although better selectivity for all the cases is found when T7^{-} is used (see discussion below).

Discussion

The results from the qualitative insertion studies indicate three different classes of active site interactions between polymerases and DNA, depending on the minor groove interactions and steric fit around the incipient base pair. The first group, constituted by KF^{-}, T7^{-}, Taq, and HIV-RT, efficiently replicates base pairs containing analogues F and Z, despite their lack of Watson–Crick H-bonding ability. The base pairs A→F, F→Z, and F→F are formed only 3–150-fold less efficiently than a natural base pair A→T by T7^{-}, whereas a normal mismatch such as A→C is synthesized less efficiently by a factor of 69 000. In addition, these polymerases do not seem to require hydrogen bonds between the minor groove side of the incipient base pair and side chains at the active site to synthesize a pair with high efficiency. Neither difluorotoluene nor 4-methylbenzimidazole is capable of accepting hydrogen bonds at the minor groove positions. These observations agree with information obtained from the X-ray structures of T7[1c] and HIV-RT[1a] in complexes with double-stranded DNA and a nucleotide. No proton donor groups from the polymerase were found at the hydrogen-bonding distance

from the minor groove of the incipient pair template base or the triphosphate in either of these structures.

No structure of a ternary complex (polymerase + dsDNA + nucleotide) is available for KF⁻, and no specific interactions at the polymerase active site can be inferred from the binary complex (polymerase + dsDNA), because the existing KF⁻ structures show DNA at the exonuclease site.1h,i However, there is a structure of a binary complex of a thermostable bacterial (*Bacillus stearothermophilus*) DNA polymerase I large fragment (BF)[1f] which shows strong structural (0.65 Å root-mean-square deviation of Cα atoms) and sequence homology (49% identity) to KF⁻. Based on this high homology between BF and KF⁻, specific hydrogen bonds between Arg 668 and Gln 849 of KF⁻ and the minor groove of the bases at the triphosphate and the template base, respectively, have been proposed for the latter. This finding contrasts with our insertion results for KF⁻, which indicate that interactions through the minor groove of DNA with the enzyme at the incipient base pair are not necessary for efficient replication. To explain this, we suggest that either (1) although present, these H-bonds are not energetically important, or (2) the binary complex of BF reflects a post-incorporation conformation of the enzyme. Relevant to this is our previous finding that KF⁻ does not need minor groove interactions for synthesis of a base pair, but that they are energetically important for extension of that pair.[11] In case (2), the interactions might be more accurately described as being with the second base pair rather than the incipient pair. A similar situation may be observed with Taq polymerase and recent published structures. In general it is worth noting that the existence of hydrogen bonds in a protein structure does not guarantee that they will all be energetically important to the enzyme's activity.

In the case of Taq, hydrogen-bonded interactions at the polymerase active site between the DNA and side chains Arg746 and Gln754 of Taq were found in the X-ray structure of a binary complex.[1g] Recently, a structure of a ternary complex of Taq, expected to represent more closely the active conformation, has been published.[1b] No hydrogen bonds between the enzyme and the template base or the triphosphate are observed, results that are consistent with our insertion data. In general, we observe that the enzymes of the first group do not need interactions between protein side chains and the minor groove of the template base or the triphosphate at the incipient pair to support efficient replication.

The second group of polymerases, formed by Pol α and AMV-RT, does not appear to require hydrogen bonds between the bases for DNA replication, but a minor groove interaction at either the template base or the incoming triphosphate must be satisfied. In the case of Pol α, results with insertion of F opposite analogue Q relative to Z implicate a specific hydrogen-bonded interaction at the template in the incipient pair. Surprisingly, the Z→F base pair is formed with higher efficiency than Q→F, possibly due to a nonpolar van der Waals contact in the triphosphate binding site between the CH at position 3 of Z and the enzyme. No X-ray structure of Pol α is yet available to confirm this possibility.

The opposite case is found for AMV-RT, where a minor groove hydrogen-bonded interaction seems to be needed at the incoming triphosphate site. For example, we observe that dQTP is inserted opposite F, whereas dZTP is not. No effects of Q-for-Z replacement are observed on the template side. Again, no high-resolution structure of AMV-RT with DNA has yet been reported. The only other nonnatural base pairs that maintain the requisite minor groove interaction would be T→Z or T→Q, because they mimic a T–A base pair and T could fulfill the minor groove hydrogen bond at the triphosphate site. Apparently they are not formed because of the high cost of dehydration of thymidine.[12]

The enzymes Pol *β* and MMLV-RT fall in the third group of polymerases. Both of them failed to replicate any of the nonpolar base pairs involving F and Z, except A→F (Pol *β*) and F→T

(MMLV–RT) with low efficiency. Replacement of Z with Q (either in the template or triphosphate) did not rescue base pair synthesis. These results could indicate that these enzymes are able to sense the instability of the incipient base pair lacking Watson–Crick H-bonds, leading to dissociation rather than insertion; however, it should be noted that pairs such as A–F are actually more stable than A–T at the end of a DNA strand.[10] A second possibility is that these enzymes need minor groove hydrogen bonds at both minor groove sites, with the template base and the incoming nucleotide simultaneously. This possibility was previously noted by Benner,13 who observed that Pol β did not incorporate a pair consisting of nonnatural bases K and X, neither of which has minor groove H-bond acceptors. Consistent with all these results is the structure of the ternary complex of Pol β,[1e] which shows a hydrogen bond between Arg283 and the N3 of a guanine at the template position. Another hydrogen bond can also be made between Asn279 and the carbonyl group at position 2 of the incoming deoxycytidine triphosphate, although due to a longer distance it may be mediated by a water molecule. Mutagenesis studies carried out on Pol β also support our results. Substitutions of Asn279 or Arg283 with alanine reduced the catalytic efficiency of the mutants by factors of 17-fold and 5000-fold, respectively, relative to the wild-type enzyme.[4c] Of the present nonnatural base pairs studied, only the base pairs Q→T and T→Q would be able to make both hydrogen bonds with the enzyme, but they are not synthesized efficiently apparently due to the high cost of desolvation of thymidine and/or to the added size of Q relative to A. A nonpolar analogue similar to F but with a carbonyl at position 2 would be useful in analyzing the activity of this third group of enzymes.

Structural information is available for MMLV-RT,[1j] but not in a complex with DNA and/or nucleotide. However, Georgiadis et al.[1j] proposed minor groove interactions between the enzyme and the incipient base pair, based on a model of a ternary complex of MMLV-RT by superposition with the ternary complex of Pol β. Side chains Gln190 and the backbone NH of Gly191 were proposed to hydrogen bond to the incoming nucleotide and the template base, respectively. If this is the case, then our results with MMLV-RT are also consistent.

The single-nucleotide insertion kinetics carried out with $T7^{-}$ DNA polymerase show very similar results to the published data for KF^{-}.[9] The base pairs A→F, F→A, F→Z, Z→F, and F→F are synthesized quite efficiently by $T7^{-}$, although no significant hydrogen bonds between the template base and the triphosphate can be made. We also observe impressive selectivity for $T7^{-}$ when inserting these nonpolar bases, even higher than that found for KF^{-}. With $T7^{-}$ we find that dZTP is inserted opposite F more efficiently than opposite itself by a factor of 80, or 2-fold more selective than KF^{-}. The higher selectivity obtained by $T7^{-}$, which must be based on steric exclusion, suggests that $T7^{-}$ possesses an active site that is tighter than that of KF^{-}. This may also explain the large difference found in $T7^{-}$ when synthesizing the F→F base pair versus the Z→Z base pair (a 6500-fold difference for $T7^{-}$ vs 30-fold for KF^{-}).

In conclusion, we find three levels of interactions for these polymerases, depending on the number of minor groove interactions and steric environment in the active site. Importantly, no hydrogen bonds between the bases appear to be needed during the insertion step (at least for the majority of enzymes) if the requirements of correct geometry and specific minor groove interactions are fulfilled. Thus it appears that a close fitting to Watson–Crick geometry and satisfaction of specific minor groove interactions are among the most important factors in replication by these enzymes.

Experimental Section

Modified Nnucleosides and Triphosphate Derivatives

The phosphoramidite derivatives of F, Z, and Q were prepared as described.8e,11,14 The 5′-triphosphate derivative of F (dFTP), Z (dZTP), and Q (dQTP) were prepared following the published procedure.9b,9d,11

Single Nucleotide Insertion Reactions

Primer 5′ termini were labeled using [γ-^{32}P]ATP and T4 polynucleotide kinase. The labeled primer was annealed to the template in an "annealing buffer" by heating to 95 °C for 3 min and cooling to room temperature for 1 h. Polymerase reactions were started by mixing equal volumes of solution A containing the DNA–enzyme complex and solution B containing dNTP substrates. The reaction mixture was incubated at 37 °C and terminated by adding one and a half volume of stop buffer (95% formamide, 20 mM EDTA, 0.05% xylene cyanol and bromophenol blue). The reactions were incubated for different times depending on the enzyme used (see below) and extents of reaction were determined by running quenched reaction samples on a 15% denaturing polyacrylamide gel.

KF reactions

Solution A was made by adding Klenow fragment (exo-) (Amersham) diluted in annealing buffer (100 mM Tris·HCl (pH 7.0), 20 mM $MgCl_2$, 2mM DTT and 0.1 mg/mL BSA) to the annealed duplex DNA; solution B contained various concentrations of dNTPs in a buffer of 200 mM Tris·HCl (pH 8.0), 20 mM $MgCl_2$, and 6 mM mercaptoethanol; the concentrations used for single nucleotide insertions were primer/template 5 μM, KF 0.2 *u*/μL, dNTP 20 μM and incubation time 2 min.

T7$^-$ reactions

Solution A was made by adding T7 Sequenase version 2.0 DNA polymerase (Amersham) (denoted here as T7$^-$) and pyrophosphatase (Amersham), both diluted in annealing buffer (60 mM Tris· HCl (pH 7.5), 30 mM $MgCl_2$ and 75 mM NaCl) and 25mM DTT, to the annealed duplex DNA; solution B contained various concentrations of dNTPs in a buffer of 10 mM NaCl; the concentrations used for single nucleotide insertions were primer/template 150 nM, T7 0.1 *u*/μL, pyrophosphatase 0.15 u/mL, dNTP 2 μM and incubation time 4 min.

Taq reactions

Solution A was made by adding Taq DNA polymerase (Amersham) to the annealed duplex DNA (annealing buffer consists of 3mM $MgCl_2$); solution B contained various concentrations of dNTPs in a buffer of 200 mM Tris·HCl (pH 8.0), 20 mM $MgCl_2$, and 6 mM mercaptoethanol; the concentrations used for single nucleotide insertions were primer/template 150 nM, Taq 0.025 *u*/μL, dNTP 100 μM and incubation time 1 h.

HIV-RT reactions

Solution A was made by adding HIV-RT (Amersham) to the annealed duplex DNA (annealing buffer consists of 200 mM Tris·HCl (pH 7.9), 24 mM $MgCl_2$ and 20 mM DTT); solution B contained various concentrations of dNTPs in a buffer of 200 mM Tris·HCl (pH 8.0), 20 mM $MgCl_2$, and 6 mM mercaptoethanol; the concentrations used for single nucleotide insertions were primer/template 150 nM, HIV-RT 0.025 *u*/μL, dNTP 100 μM and incubation time 2 min.

Pol α reactions

Solution A was made by adding Pol α (Chimerx) diluted in annealing buffer (120 mM Tris·HCl (pH 7.9), 10 mM magnesium acetate, 0.2 mM spermine, 2 mM DTT and 0.6 mg/mL BSA) to the annealed duplex DNA; solution B contained various concentrations of dNTPs in a buffer of 200 mM Tris·HCl (pH 8.0), 20 mM $MgCl_2$, and 6 mM mercaptoethanol; the concentrations used for single nucleotide insertions were primer/template 150 nM, Pol α 0.1 *u*/μL, dNTP 100 μM and incubation time 1 h.

AMV-RT reactions

Solution A was made by adding AMV-–RT (Invitrogen) to the annealed duplex DNA (annealing buffer consists of 200 mM Tris·HCl (pH 8.3), 80 mM KCl, 20 mM $MgCl_2$ and 1 mM spermidine); solution B contained various concentrations of dNTPs in a buffer of 200 mM Tris·HCl (pH 8.0), 20 mM $MgCl_2$, and 6 mM mercaptoethanol; the concentrations used for single nucleotide insertions were primer/template 150 nM, AMV-RT 0.05 *u*/μL, dNTP 100 μM and incubation time 45 min.

Pol *β* reactions

Solution A was made by adding Pol *β* (Chimerx) diluted in annealing buffer (100 mM Tris·HCl (pH 7.9), 140 mM KCl, 10 mM $MgCl_2$, 10% glycerol, 2 mM DTT and 0.2 mg/mL BSA) to the annealed duplex DNA; solution B contained various concentrations of dNTPs in a buffer of 200 mM Tris·HCl (pH 8.0), 20 mM $MgCl_2$, and 6 mM mercaptoethanol; the concentrations used for single nucleotide insertions were primer/template 5 nM, Pol *β* 0.1 *u*/μL, dNTP 500 μM (except dZTP, 175 μM*)* and incubation time 6 h.

MMLV-RT reactions

Solution A was made by adding MMLV-RT (Amersham) to the annealed duplex DNA (annealing buffer consists of 100 mM Tris·HCl (pH 8.3), 150 mM KCl, 6 mM $MgCl_2$ and 20 mM DTT); solution B contained various concentrations of dNTPs in a buffer of 200 mM Tris·HCl (pH 8.0), 20 mM $MgCl_2$, and 6 mM mercaptoethanol; the concentrations used for single nucleotide insertions were primer/template 150 nM, MMLV-RT 0.25 *u*/μL, dNTP 100 μM and incubation time 5 min.

Steady-State Kinetics

Steady-state kinetics for standing-start single nucleotide insertions were carried out as described.[15] The conditions used were the same as for the qualitative insertion studies on $T7^-$. The final DNA (duplex) concentration was 5 μM. Amounts of polymerase used (0.005–0.1 *u*/μL*)* and reaction times (90 s to 70 min) were adjusted to give extents of reaction of 20% or less. The primer-template concentration was in excess over enzyme by 60- to 1200-fold in all cases. Extents of reaction were determined by running quenched reaction samples on a 15% denaturing polyacrylamide gel for analysis of radiolabeled primer extension, which was quantitated by phosphorimaging (Molecular Dynamics Storm 860). Relative velocities were calculated as the extent of reaction divided by reaction time and normalized to the lowest enzyme concentration used (0.005 *u*/μL).

Acknowledgments

This work was supported by the National Institutes of Health (GM52956) and by a fellowship to J.C.M. from the Spanish Ministry of Science and Culture.

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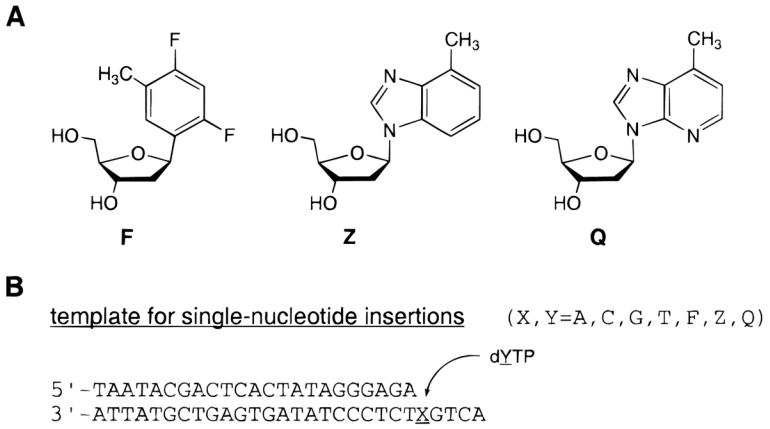

Figure 1.

Structures and sequences in this study. (A) Structures of natural DNA bases and analogues. (B) Sequence of primer and template DNAs used in the polymerase experiments.

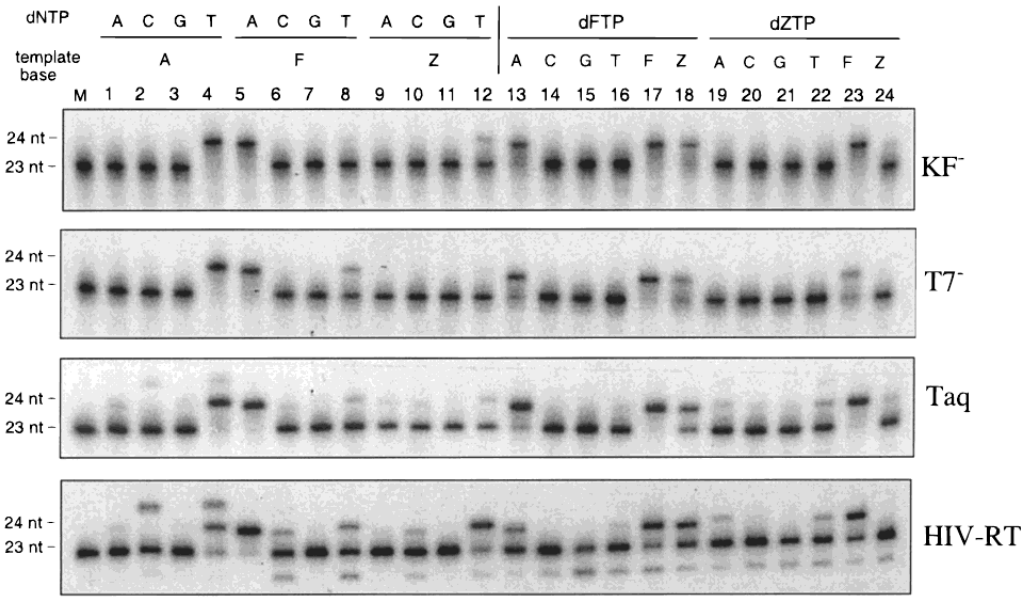

Figure 2.

Autoradiogram of denaturing PAGE gel showing single-nucleotide insertions with F or Z in the template strand and using dFTP or dZTP as the nucleotide substrates. The data were taken at 37 °C. Conditions used were as follows: KF 0.2 *u*/μL, 5 μM primer/template duplex, 20 μM dNTP and reaction time 2 min; T7 0.1 *u*/μL, pyrophosphatase 0.15 u/mL, 150 nM μM primer/template duplex, 2 μM dNTP and reaction time 4 min; Taq 0.025 *u*/μL, 150 nM primer/template duplex, 100 μM dNTP and reaction time 1 h; HIV-RT 0.025 *u*/μL, 150 nM primer/template duplex, 100 μM dNTP and reaction time 2 min.

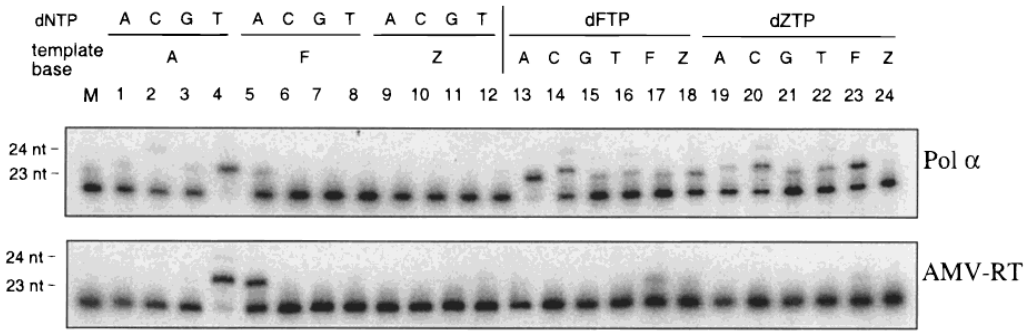

Figure 3.

Autoradiogram of denaturing PAGE gel showing single-nucleotide insertions with F or Z in the template strand and using dFTP or dZTP as the nucleotide substrates. The data were taken at 37 °C. Conditions used were as follows: Pol α 0.1 *u*/μL, 150 nM primer/template duplex, 100 μM dNTP and reaction time 1 h; AMV-RT 0.05 *u*/μL, 150 nM primer/template duplex, 100 μM dNTP and reaction time 45 min.

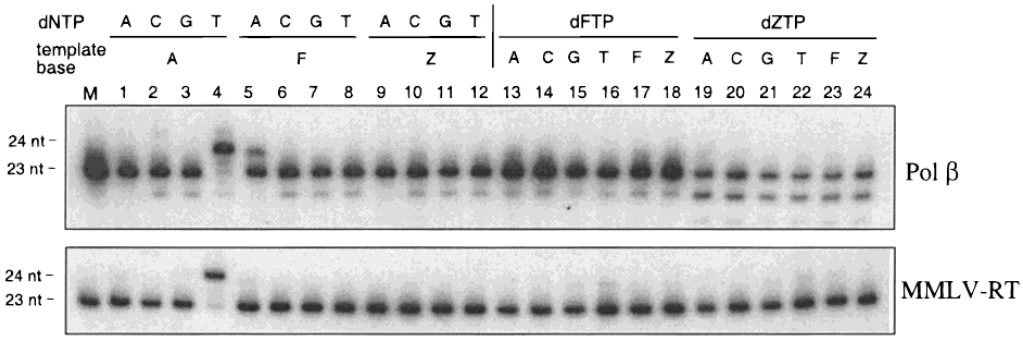

Figure 4.

Autoradiogram of denaturing PAGE gel showing single-nucleotide insertions with F or Z in the template strand and using dFTP or dZTP as the nucleotide substrates. The data were taken at 37 °C. Conditions used were as follows: Pol *β* 0.1 u/*μ*L, 5 nM primer/template duplex, 500 *μ*M dNTP (except dZTP, *μ*M) and reaction time 6 h; MMLV-RT 0.25 *u*/*μ*L, 150 nM primer/template duplex, 100 *μ*M dNTP and reaction time 5 min.

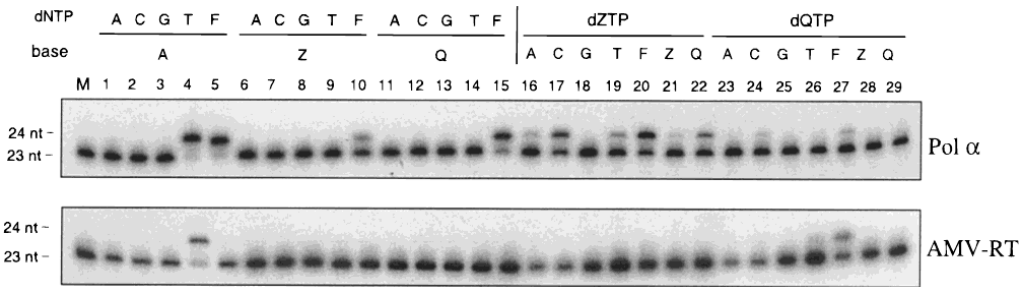

Figure 5.

Autoradiogram of denaturing PAGE gel showing single-nucleotide insertions with Z or Q in the template strand and using dZTP or dQTP as the nucleotide substrates. The data were taken at 37 °C. Conditions used were the same as in Figure 3.

Table 1

Steady-State Kinetics for Single Nucleotide Insertions with T7 DNA Polymeriase (exo-)[a]

nucleoside triphosphate	template base (X)	K_m (μM)	V_{max} (% min^{-1})	efficiency (V_{max}/K_m)	fidelity (selectivity)
dATP	Z	450 (35)	1.60 (0.17)	3.5×10^3	0.017
dCTP	Z	390 (110)	1.00 (0.40)	2.6×10^3	0.012
dGTP	Z	980 (20)	0.082 (0.03)	8.4×10^1	0.0004
dTTP	Z	500 (130)	3.5 (0.9)	7.0×10^3	0.033
dFTP	Z	17 (8)	3.5 (0.6)	2.1×10^5	1
dZTP	Z	240 (130)	0.040 (0.010)	1.6×10^2	0.0008
dZTP	A	280 (120)	0.007 (0.001)	2.5×10^1	0.0021
dZTP	C	240 (20)	0.003 (0.002)	1.3×10^1	0.0011
dZTP	G	100 (30)	0.005 (0.0006)	5.0×10^1	0.0042
dZTP	T	230 (150)	0.004 (0.0008)	1.7×10^1	0.0014
dZTP	F	20 (10)	0.25 (0.10)	$1.2 \times 10_1$	1
dATP	T	0.26 (0.10)	8.25 (0.5)	3.2×10^7	1
dATP	C	940 (760)	0.44 (0.086)	4.7×10^2	0.00001
dATP	F	0.55 (0.06)	5.90 (1.5)	1.1×10^7	0.30
dFTP	F	5.70 (4.3)	5.5 (0.90)	9.7×10^5	0.030
dFTP	A	29 (14)	2.3 (0.3)	7.9×10^4	0.0025

[a]Single-nucleotide insertions on 5 mM 23mer/28mer primer-template duplex, 0.005–0.1 units/mL T7 (exo-), 30 mM Tris·HCl (pH 7.5), 15 mM $MgCl_2$ and 42.5 mM NaCl and 12.5 mM DTT, incubated for 90 s to 70 min at 37 °C in a reaction volume of 10 μL. Standard deviations are given in parentheses. Values averaged from 3–5 data sets each except for the 3rd entry (2 data sets).

J Am Chem Soc. Author manuscript; available in PMC 2010 September 27.