Functional Hydrogen-Bonding Map of the Minor Groove Binding Tracks of Six DNA Polymerases[†]

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ABSTRACT: Recent studies have identified amino acid side chains forming several hydrogen bonds in the DNA minor groove as potentially important in polymerase replication of DNA. Few studies have probed these interactions on the DNA itself. Using non-hydrogen-bonding nucleoside isosteres, we have now studied effects in both primer and template strands with several polymerases to investigate the general importance of these interactions. All six polymerases show differences in the H-bonding effects in the minor groove. Two broad classes of activity are seen, with a first group of DNA polymerases (KF$^-$, Taq, and HIV-RT) that efficiently extends nonpolar base pairs containing nucleoside Q (9-methyl-1*H*-imidazo-[4,5-*b*]pyridine) but not the analogue Z (4-methylbenzimidazole), implicating a specific minor groove interaction at the first extension site. A second group of polymerases (Pol α, Pol β, and T7$^-$) fails to extend all non-H-bonding base pairs, indicating that these enzymes may need minor groove hydrogen bonds at both minor groove sites or that they are especially sensitive to noncanonical DNA structure or stability. All DNA polymerases examined use energetically important minor groove interactions to probe newly synthesized base pairs before extending them. The positions of these interactions vary among the enzymes, and only a subset of the interactions identified structurally appears to be functionally important. In addition, polymerases appear to be differently sensitive to small changes in base pair geometry.

DNA polymerases are critical to the stable transmission of genetic information. Longstanding efforts in many laboratories have focused on understanding the mechanism of these enzymes and how they achieve high fidelity in DNA replication (*1*−*3*). Recently, new insights have been added from structural studies of several polymerases. Cocrystal structures of ternary complexes consisting of DNA polymerase, double-stranded DNA, and a nucleotide substrate at the active site have revealed specific hydrogen bonding between the polymerase and the O2 and N3 atoms in the minor groove region of the duplex DNA (*4*−*8*). These hydrogen bond acceptors lie in the same position for all four correct base pairs but are in different geometric positions for mismatched base pairs. This raises one possible mechanism by which polymerases may test for correct base pairs in DNA. In the case of human DNA polymerase β (pol β),[1] Arg 283 and Asn 279 form hydrogen bonds to the N3 of guanine at the template and to the carbonyl at position 2 of the incoming deoxycytidine triphosphate, respectively (*5*). Thus, pol β may probe for correct base pairs by forming hydrogen-bonded contacts with both sides of the groove at the insertion step. In the structures of ternary complexes of T7 DNA polymerase (T7$^-$) (*6*) and Taq DNA polymerase (Taq) (*8*), Arg, Asn, Lys, and Gln side chains are making hydrogen bonds not only to the incipient base pair but also with up to five base pairs further downstream. On this basis it has been suggested that, even after formation of a mismatched pair, DNA polymerases may distinguish the incorrect geometry by sensing disruption of these minor groove interactions further along in the active site, leading to reductions of the efficiency of subsequent extension steps (*9*, *10*).

Mutagenesis studies of KF, pol β, and HIV reverse transcriptase (HIV-RT) have investigated the importance to DNA replication of protein side chains that may interact with the bases in the minor groove (*11*−*15*). Alanine and leucine substitutions of Asn 279 and Arg 238 in pol β markedly decrease catalytic efficiency and fidelity during the insertion step, an effect attributed to disruption of minor groove interactions. Alanine replacements of Arg 668 in KF and Gln 258, Gln 269, Trp 266, and Ile 94 in HIV-RT also decrease DNA-binding affinity and the k_{cat} for DNA synthesis. These results implicate minor groove interactions on insertion or extension, but it is not always clear which groups are contacted on the DNA.

There is a need, therefore, for defining the exact sites of the most important minor groove contacts in the DNA and

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[1] Abbreviations: F, 2,4-difluorotoluene deoxynucleoside; Z, 4-methylbenzimidazole deoxynucleoside; Q, 9-methyl-1*H*-imidazo[4,5-*b*]-pyridine; KF$^-$, Klenow fragment of *E. coli* DNA polymerase I (exonuclease free); Taq, *Thermus acquaticus* DNA polymerase; T7$^-$, T7 DNA polymerase (exonuclease free); HIV-RT, HIV reverse transcriptase; pol α, calf thymus DNA polymerase α; pol β, human DNA polymerase β; EDTA, ethylenediaminetetraacetic acid; DTT, dithiothreitol; BSA, bovine serum albumin; T_m, melting temperature.

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mapping their relative functional significance. An examination of the literature shows only a few existing studies of polymerase−DNA contacts from the DNA standpoint. Only one modified base has been investigated in the template to study the insertion step (*16*). Three modified triphosphates were examined recently to test interactions on the primer side of the groove, but they showed lack of insertion or inhibited the enzyme at an unknown step (*17*, *18*). Until very recently (see below), no modified bases have been used to study the significance of minor groove interactions at the extension steps which follow insertion, despite the fact that most of the structurally identified interactions are expected after insertion. Further, it is notable that only two polymerases (KF and Taq) have been examined with DNA analogues, so the generality of these effects remains largely unexplored.

We have synthesized a number of modified nucleosides that mimic the shapes of the natural bases but lack many or all of the standard hydrogen-bonding groups (*19*, *20*). Analogues of thymidine (F, a difluorotoluene deoxynucleoside) and deoxyadenosine (Z, a 4-methylbenzimidazole deoxynucleoside) (Figure 1), have been studied with DNA polymerases. We have found that nonpolar base pairs such as A-F, F-A, F-Z, and Z-F are efficiently and selectively synthesized by KF^- and other DNA polymerases and reverse transcriptases, supporting the idea that shape complementarity and solvation effects play as important a role as base−base hydrogen bonds (*21*−*24*).

These new analogues have also shown utility in studying minor groove interactions. Recently, we examined the possible significance of minor groove interactions in the extension step for KF^- by comparison of analogue F with T and analogue Z with A. In addition, we developed a new deoxyadenosine analogue, Q, which lacks Watson−Crick hydrogen-bonding groups but does have a minor groove acceptor nitrogen (*25*). For the Klenow fragment of *Escherichia coli* DNA polymerase I (lacking 3′ exonuclease actvity) (KF^-), it was found that minor groove interactions during nucleotide insertion have very little effect, but a single hydrogen bond in the primer strand at the first terminal base pair can make a 300-fold difference in extension efficiency.

We now report new studies with a group of five different DNA polymerases and HIV reverse transcriptase to investigate the general importance of minor groove interactions, geometric constraints, and DNA stability. These effects are examined at the several extension steps that follow formation of a given base pair. We find that the polymerases can be grouped into two general classes, depending on their capacity to extend nonpolar base pairs containing Z, Q, or F. All of the enzymes appear to use minor groove interactions to some extent to probe for incorrect base pairs. However, not all structurally identified interactions appear to be functionally important to DNA polymerization. We also describe steady-state extension kinetics of base pairs containing F, Q, and Z by KF^- polymerase and explain these results on the basis of minor groove interactions, base pair geometry, and DNA stability.

MATERIALS AND METHODS

Modified Oligonucleotides and Triphosphate Derivatives. Oligodeoxynucleotides were synthesized on a Perkin-Elmer/Applied Biosystems (ABI) 392 DNA synthesizer using standard β-cyanoethyl phosphoramidite chemistry. The phosphoramidite derivatives of F, Z, and Q were prepared as described (*20*, *25*, *40*) and were incorporated using the standard ABI coupling cycle. Oligonucleotides were deprotected in concentrated NH_4OH (55 °C, 14 h). After lyophilization the DNA was purified by preparative denaturing polyacrylamide gel electrophoresis and isolated by excision, crushing, and eluting into 0.2 M NaCl. The salts were removed by dialysis against distilled deionized water, and the DNA was quantitated by absorbance at 260 nm, using the nearest neighbor method to calculate molar extinction coefficients. The 5′-triphosphate derivatives of F (dFTP), Z (dZTP), and Q (dQTP) were prepared following the published procedure (*22*, *24*, *25*).

Primer Extension Reactions. Primer 5′ termini were labeled using [γ-^{32}P]ATP and T4 polynucleotide kinase. Radioactivity was measured for each labeled primer, and the same amount of counts per minute (cpm) was used for each experiment. 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 (dATP, dCTP, dGTP, and dTTP). The reaction mixture was incubated at 37 °C and terminated by adding 1.5 volumes of stop buffer [95% formamide, 20 mM EDTA (pH = 8.0), 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. (A) 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 $MgCl_2$, 2 mM 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 primer extension were primer-template, 150 nM, KF, 0.1 unit/μL, and dNTPs, 20 μM each, and incubation time 10 min. Unit definition: One unit catalyzes the incorporation of 10 nmol of total deoxyribonucleotides into an acid precipitate material in 30 min at 37 °C using poly d(A-T) as a template-primer. (B) $T7^-$ reactions: Solution A was made by adding T7 Sequenase version 2.0 DNA polymerase (Amersham) and pyrophosphatase (Amersham) both diluted in annealing buffer [60 mM Tris·HCl (pH 7.5), 30 mM $MgCl_2$, and 75 mM NaCl] and 25 mM DTT to the annealed duplex DNA; solution B contained various concentrations of dNTPs in a buffer of 20 mM NaCl; the concentrations used for primer extension were primer-template, 150 nM, T7, 0.1 unit/μL, pyrophosphatase 0.15 unit/mL, and dNTPs, 20 μM each, and incubation time 3 h. Unit definition: One unit catalyzes the incorporation of 1 nmol of total nucleotide into an acid-insoluble form in 30 s at 37 °C using 5 mg of primed M13mp18 DNA as the template under standard assay conditions. (C) Taq reactions: Solution A was made by adding Taq DNA polymerase (Amersham) diluted in annealing buffer (3 mM $MgCl_2$) 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 primer extension were primer-template, 150 nM, Taq, 0.1 unit/μL, and dNTPs, 100 μM each, and incubation time 3 h. Unit definition: One unit catalyzes the incorporation of 10 nmol of total nucleotide into an acid-insoluble product in 30 min at 70 °C using M13mp18(+) DNA as template. (D) HIV-RT reactions: Solution A was made by adding HIV-RT (Amersham) diluted in annealing buffer [200 mM Tris·HCl (pH 7.9), 24 mM $MgCl_2$, and 40 mM DTT] 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; concentrations used for primer extension were primer-template, 150 nM, HIV-RT, 0.1 unit/μL, and dNTPs, 100 μM each, and incubation time 1 h. Unit definition: One unit incorporates 1 nmol of dTMP into a high molecular weight product in 10 min at 37 °C using poly(rA)·oligo(dT) as template-primer. (E) 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; concentrations used for primer extension were primer-template, 150 nM, pol α, 0.1 unit/μL, and dNTPs, 100 μM each, and incubation time 3 h. Unit definition: One unit is the amount of enzyme required to incorporate 1 nmol of total nucleotide into an acid-insoluble form in 60 min at 37 °C. (E) 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 primer extension were primer-template, 150 nM, pol β, 0.1 unit/μL, and dNTPs, 100 μM each, and incubation time 3 h. Unit definition: One unit is the amount of enzyme required to incorporate 1 nmol of total nucleotide into an acid-insoluble form in 60 min at 37 °C.

Steady-State Kinetics. Extension kinetics were carried out as described for standing-start single nucleotide insertions (*41*). The conditions used were the same as for the qualitative extension studies, except only dGTP was added as the substrate. The final DNA (duplex) concentration was 5 μM. The amount of polymerase used (0.005−0.1 unit/μL) and reaction time (1−15 min) were adjusted to give extents of reaction of 20% or less. The gradient of concentration of dGTP was also adjusted to fulfill this requirement. 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 extent of reaction divided by reaction time and normalized to the lowest enzyme concentration used (0.005 unit/μL).

Thermal Denaturation Studies. Solutions for the thermal denaturation studies contained a self-complementary oligomer in a buffer solution of 1 M NaCl, 0.1 mM EDTA, and 10 mM sodium phosphate (pH 7.0). After the solutions were prepared, they were heated at 90 °C for 5 min and allowed to cool slowly to room temperature prior to the melting

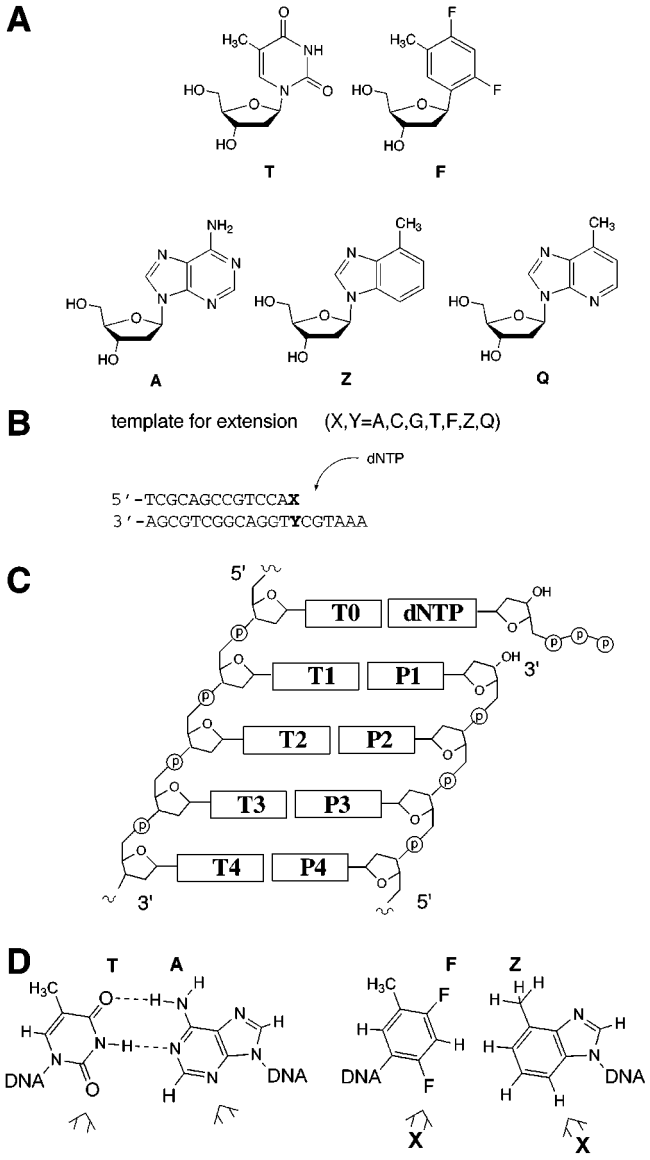

FIGURE 1: Structures and sequence in this study. (A) Structures of natural DNA bases and analogues in this study. (B) Primer-template sequence used for extension experiments. (C) Schematic diagram showing positions of H-bond acceptors in the polymerase active site. P refers to primer strand, and T refers to template strand. Position 0 is the incipient pair; these bases are shifted to position 1 after phosphodiester bond formation is complete. A pair is promoted further to positions 2−6 in the active site with successive extensions of the primer. (D) Diagram showing positions of minor groove acceptors on natural and analogue base pairs.

experiments. The sequences were monitored at 280 nm. Thermodynamic parameters were determined by plotting $1/T_m$ vs ln [oligonucleotide] using at least five different concentrations for each duplex. All of the measured complexes generated van't Hoff plots with good linear fits and displayed apparently two-state melting behavior.

RESULTS

Qualitative Extension Studies. We carried out qualitative extension experiments on a synthetic 14-nt primer−20-nt template duplex containing natural bases or nonpolar analogues in the primer or template strand (Figure 1). Extension efficiencies were monitored by use of radiolabeled primers in the presence of all four natural triphosphates, and

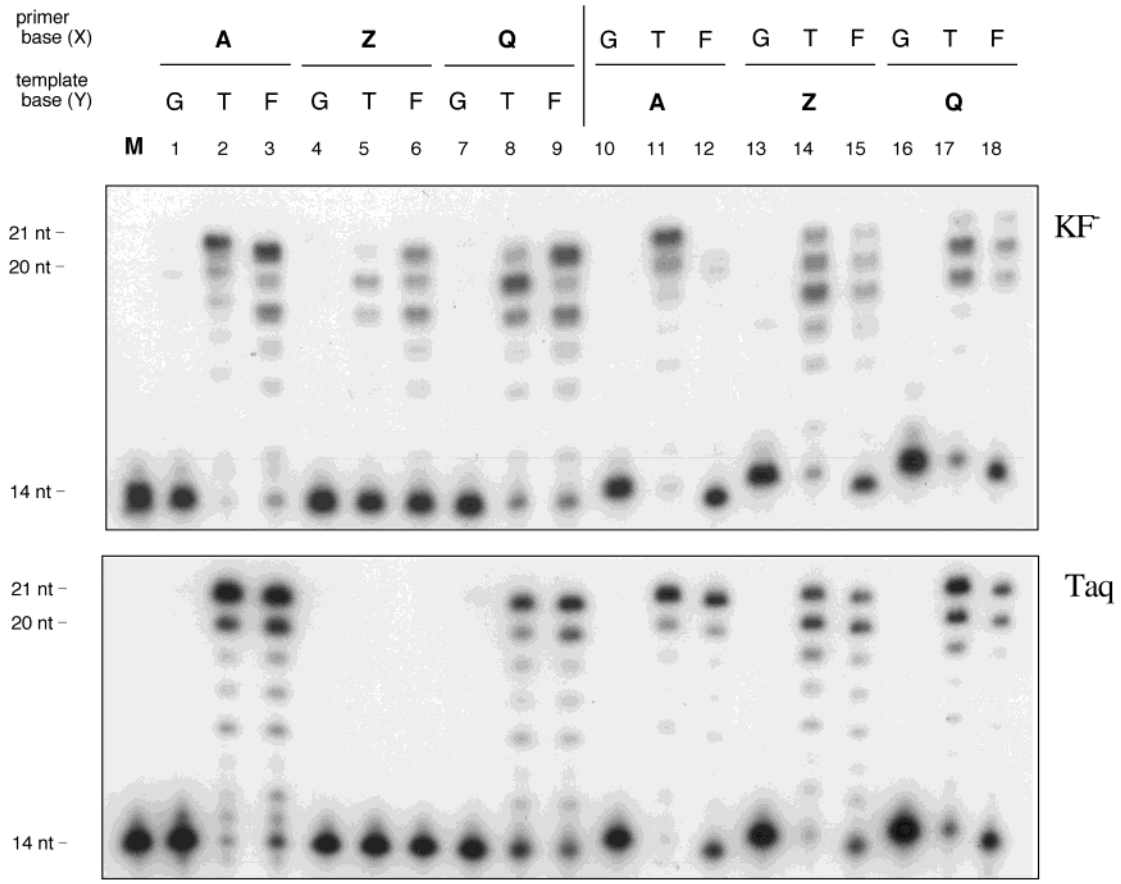

FIGURE 2: Autoradiogram of the denaturing PAGE gel showing minor groove interaction effects on extension of normal and modified base pairs at 37 °C. Conditions: KF⁻, 0.1 unit/μL, 150 nM primer-template duplex, 20 μM dNTP, and reaction time 10 min; Taq, 0.1 unit/μL, 150 nM primer-template duplex, 100 μM dNTP, and reaction time 3 h.

successful extension is indicated by elongation of the primer to 20-nt length. Some experiments also show extension to 21-nt length due to the tendency of some DNA polymerases to add an extra nucleotide (usually an adenine) after completion of the full length of the template (*26*, *27*). Five new structurally varied DNA polymerases were studied alongside previously studied KF⁻. Nonforcing conditions for primer extension were used in all cases to allow discrimination of kinetically small effects on qualitative gels. The high-resolution gels allow exact identification of positions of pauses (see schematic diagram, Figure 1C). For extension studies a probe base pair is placed at the terminus of the primer (position 1). A dark band at the first position arises from polymerase dissociation prior to extension of that pair, implicating an unfavorable interaction between polymerase side chain(s) and the minor groove groups in position 1. As the DNA is extended further, the original base pair at position 1 is moved through the active site, occupying positions 2−6 successively, and making specific interactions with any protein side chains at those positions. These later positions are also visible on the gel, and bands that are darker than controls at these positions also suggest specific unfavorable interactions. If no dark band is seen, this does not rule out a hydrogen-bonded interaction, but it does suggest that it may not be energetically important for that given extension step. Note that the convention used herein is to list the primer strand base in a pair first; e.g., A-T denotes A in primer and T in template.

The results of the primer extension studies fall into two general classes, depending on the polymerases' ability to fully extend the primers with modified nonpolar bases. One group includes three enzymes, KF⁻, Taq DNA polymerase, (Taq), and HIV reverse transcriptase (HIV-RT), that are found to be capable of fully extending some base pairs containing the nonpolar base Q, similarly to preliminary findings reported for KF⁻ alone (*25*). In the case of Taq, when Z and Q are compared in the primer strand, we observe efficient extension of base pairs Q-T and Q-F and no extension of pairs Z-T and Z-F (leaving the 14-nt primer unextended). The same appears to be true for KF⁻ (Figure 2). This strongly suggests the importance of a possible hydrogen bond at the extended base pair position. This minor groove interaction does not seem to be important in the template strand at the same position, since T-Z and T-Q are extended with similar efficiency. It is also notable that, when extending a Q-F pair, KF⁻ shows a small pause at the sixth base pair that is not observed for Q-T. This indicates a second possible functionally significant minor groove interaction with the template strand for KF⁻, and this same interaction does not seem to be important for Taq.

With HIV-RT we find results similar to, but not the same as, the above two enzymes. When Z and Q are compared in the primer strand, we observe small differences in full-length extension of Q-T versus Z-T and Q-F versus Z-F. This implicates a possible weak interaction at position 1 in the primer strand. In addition, both Q-T and Z-T are much more efficiently elongated than Q-F and Z-F, which suggests the existence of a strong minor groove interaction in the template strand at position 1. A putative hydrogen bond at that position would be possible through the O2 of thymidine but would

be greatly weakened or eliminated with the fluorine of difluorotoluene. This result is independently confirmed by comparison of Z and Q in the template strand. A strong pause is observed for T-Z, while T-Q is almost completely elongated. Moreover, the lower efficiency of extension of F-A and F-Q versus T-A and T-Q also suggests a possible minor groove interaction with the primer strand but less functionally important than the interaction with the template strand.

It should be noted that conservation of minor groove hydrogen bond acceptors alone is not sufficient for extension of the DNA through the polymerase active site. For the above three enzymes the base pair geometry also appears to be crucial, since mismatches Z-G, Q-G, G-Z, and G-Q are not extended at all, similarly to the natural purine−purine mismatches A-G and G-A.

A second group of enzymes, consisting of calf thymus DNA polymerase α (pol α), human DNA polymerase *β* (pol *β*), and T7 DNA polymerase lacking 3′ exonuclease activity (T7$^-$), was much more selective in extending nonpolar base pairs, even when they included the analogue Q or a natural base which maintains minor groove H-bonding ability. Pol α extends the base pair F-A by a small amount to the end of the strand and to an even lesser extent A-F, T-Q, and Q-T. Since the Q-T pair has the appropriate H-bond acceptors in the minor groove, this probably indicates the necessity for precise Watson−Crick geometry, so that the 3′-OH of the primer terminus is at the exact position for bond formation. Structural studies in DNA have suggested that Z (closely related to Q) causes small distortion because of its H1 proton, which is larger than the lone pair on N1 of adenine (*28*). Of pairs involving a nonnatural analogue, A-F and F-A are structurally very close mimics of the natural pairs (*29*), but we observe that they are extended only very weakly. One possibility is that, in addition to having a high degree of sensitivity to base pair geometry, pol α may also need to form interactions with both the primer and the template strands simultaneously for a pair to be extended.

In the case of pol *β*, base pairs A-F and Q-F are the best elongated, almost to full length, but still with low efficiency and with pauses at each new extension step. The difference between Q-F and Z-F suggests that a minor groove interaction is important with the primer strand, but this one minor groove H-bond acceptor is still not sufficient for efficient extension of these nonpolar base pairs by pol *β*. One possibility, as with pol α, is that H-bond acceptors are required in both sides of the groove (i.e., template and primer). The failure of extension with the Q-T and T-Q pairs, however, again suggests a strong sensitivity to small geometric differences from canonical pairs.

The final enzyme examined is T7$^-$. The base pairs Z-F, F-Z, and F-Q are not extended, but replacement with Q-F, T-Z, and T-Q, respectively, leads to efficient extension by one base with this enzyme. This provides strong evidence for an energetically important hydrogen bond with position 1 in the primer strand. The base pair A-F is the best extended, possibly because it is the closest structural mimic of a natural base pair. None of them is extended further by T7$^-$. This cannot be explained by a need for minor groove interactions at both sides of the DNA, since T-Q can make both interactions. Neither can it be explained by poor geometry for the incoming nucleotide, since a newly synthesized G-C base pair will be presented as the first base and the 3′-OH of the primer terminus should be at the correct position. Thus the modified pairs must have some influence to explain the inefficient extension to position 3. We attribute this to their low stability (see below).

Table 1: Steady-State Kinetic Parameters for Extension of an XY Pair Containing A, Z, and Q at the Primer and Template Strands of a DNA Duplex by the KF$^-$ Polymerase[a]

base pair (XY)	V_{max} (% min^{-1})	K_m (μM)	efficiency (V_{max}/K_m)	fidelity
AA	0.04 (0.004)	82 (50)	4.9×10^2	1.9×10^{-5}
AC	0.95 (0.1)	30 (15)	3.1×10^4	1.2×10^{-3}
AG	0.014 (0.005)	83 (25)	1.7×10^2	6.5×10^{-6}
AT	21 (2)	0.8 (0.06)	2.6×10^7	1
AF	8.2 (1)	36 (3)	2.3×10^5	8.8×10^{-3}
ZA	nd	nd	nd	nd
ZC	0.08 (0.002)	37 (9)	2.1×10^3	8.1×10^{-5}
ZG	nd	nd	nd	nd
ZT	0.06 (0.01)	64 (45)	9.4×10^2	3.6×10^{-5}
ZF	0.05 (0.01)	90 (50)	5.6×10^2	2.1×10^{-5}
QA	0.03 (0.007)	50 (38)	6.0×10^2	2.3×10^{-5}
QC	0.2 (0.04)	9 (5)	2.3×10^4	8.8×10^{-4}
QG	0.006 (0.002)	40 (40)	1.5×10^2	5.7×10^{-6}
QT	2.6 (0.2)	44 (10)	6.0×10^4	2.3×10^{-3}
QF	10.1 (1.5)	60 (15)	1.6×10^5	6.1×10^{-3}
TA	21 (4)	1.1 (0.1)	1.9×10^7	1
TZ	4.5 (0.3)	58 (37)	7.8×10^4	4.1×10^{-3}
TQ	6.6 (1.4)	54 (20)	1.2×10^5	6.3×10^{-3}
FA	0.53 (0.2)	80 (20)	6.4×10^3	3.3×10^{-4}
FZ	0.80 (0.2)	93 (35)	8.6×10^3	4.5×10^{-4}
FQ	1.2 (0.5)	78 (45)	1.5×10^4	7.9×10^{-4}

[a] Extension kinetics on 5 μM template-primer duplex (see Figure 1), 0.005−0.2 unit/μL KF$^-$, 0.4 M HEPES (pH 6.6), 0.4 mM Tris·HCl (pH 7.0), 0.02 mM EDTA, and 0.8 mg/mL BSA, incubated for 1−60 min at 37 °C in reaction volume of 10 μL in the presence of the dGTP and 100 mM Tris·HCl (pH 8.0), 10 mM $MgCl_2$, and 3 mM mercaptoethanol. Fidelity = efficiency/efficiency of correct base pair (A-T or T-A). nd = not determined. Standard deviations are given in parentheses.

Extension Kinetics for KF$^-$. We carried out steady-state measurements of extension kinetics of base pairs with A, Z, and Q in the primer strand and with all the natural bases and F in the template strand. In addition, we tested A, Z, and Q in the template strand with T and F in the primer strand. Relative efficiencies are measured by comparison of V_{max}/K_m data. The data are given in Table 1.

First we compared extension efficiency of A, Z, and Q in the primer strand, when placed opposite thymidine or its nonpolar analogue F. The analogue pair Q-F extends more efficiently than Z-F by a factor of 300, probably due to a hydrogen bond between the enzyme and the N3 in the analogue Q that is missing with Z. A smaller difference (65-fold) is observed on extension of Q-T when compared to Z-T, still showing the importance of minor groove interaction in the primer strand but also indicating that other factors could be affecting the extension efficiency. Significantly, the base pairs Q-F and Q-T are extended less efficiently than a natural base pair A-T by a factor of 160−400-fold, respectively. It could be argued that the slightly larger than canonical size of Q-T and Q-F is causing some misalignment of the 3′-OH primer terminus and therefore reducing the efficiency of extension. But, surprisingly, the base pair A-F, which can make the necessary minor groove interaction at the primer strand and resembles more closely a Watson−Crick geometry, shows an extension efficiency similar to Q-F, at 110-fold less efficient than the natural pair A-T.

These results clearly indicate that other effects are also very important for extension (see below). In any case, geometry effects are significant since all the natural purine−purine mismatches and their nonpolar analogues are extended very poorly, 4−5 orders of magnitude less efficiently than an A-T base pair. Kinetics of extension were not measured for the base pairs Z-A and Z-G due to their very poor extension even under more forcing conditions (0.2 unit/μL enzyme and 60 min reaction time).

Extension of base pairs containing A, Z, and Q in the template strand was investigated in the presence of T and F in the primer strand. We found that T-Z and T-Q were extended with very similar efficiency, with T-Q only 1.5 times better than T-Z. Analogous results were observed when F-Z and F-Q were compared, indicating that a minor groove interaction at the template side of the pair at position 1 does not seem to be important for extension. It is important to point out that although T-Z and T-Q resemble the Watson−Crick geometry and can fulfill the necessary minor groove interaction at the primer strand (similarly to Q-T, Q-F, and A-F), both are extended less efficiently than a T-A base pair by a factor of 160 and 240, respectively. This again suggests very high sensitivity to even small geometric differences or base pair stabilities (see below).

When T and F are compared in the primer strand, we find that T-A is extended more efficiently than F-A by a factor of 3000-fold, again strongly implicating the previously mentioned H-bond at this position. Interestingly, T-Q and T-Z are analogously extended better than F-Q and F-Z but by a much smaller factor of 8−10-fold. This may again reflect small geometric differences of Z and Q relative to A. It should be pointed out that the natural base thymidine and its nonpolar analogue F differ not only in the 2-position in the minor groove but also in the polarity of the Watson−Crick edge and in their stacking properties (*30*) (both of which affect duplex stability), and all these differences may influence the results.

Thermodynamic Stability of Base Pairs at the Primer-Template Terminus. The efficiency of extension of a pair depends largely on the affinity of the polymerase for the DNA as the primer-template is shifted in the active site; a mismatch leads to dissociation, or to partitioning to the exonuclease site, because of unfavorable interactions being incurred with the noncanonical DNA. Thus we next investigated the pairing properties of nucleosides Z and Q in order to try to correlate the rate of fraying of the DNA terminal pair of the different DNAs with their extension efficiency. We therefore examined DNA sequences with nucleosides F and Z at the terminus of blunt-ended self-complementary duplexes and also DNA sequences with two additional overhanging cytosines at the 5′-end that allow the primer-template complex to more closely resemble an active polymerase substrate DNA. We examined base pairs involving analogues Z and Q placed both in the primer and in the template strand. Thermodynamic stabilities of the duplexes were measured by UV-monitored thermal denaturation studies at pH 7.0 in the presence of 1 M NaCl, 10 mM NaH_2PO_4, and 0.1 mM EDTA.

In the case of the blunt-ended DNA oligonucleotides (Table 2), all of the base pairs containing Z and Q, at either 3′ or 5′ termini, were more stabilizing to the duplex than the natural base pair A-T, with T_m values 1−4 °C higher and free energies (37 °C) 0.05−0.15 kcal·mol^{-1} more favorable per base pair. This is consistent with the strong stacking affinities of nonpolar nucleosides (*30*). Purine−purine-like base pairs containing Z or Q were even more stable than purine−pyrimidine-like base pairs, with T_m values 1−2 °C higher and free energies (37 °C) 0.05−0.10 kcal·mol^{-1} more favorable. These differences between mismatches and correct pairs are lower than those seen for similar mismatches when placed in the middle of the strand, where pairing selectivity is 4−5 kcal·mol^{-1} (*31*).

Table 2: Free Energies and Melting Temperatures for Duplexes Containing Nonpolar Base Pairs Z and Q at the End of the Strand in Blunt-Ended Sequences and with Two Dangling Cytosines

DNA duplex	T_m [a]	$-\Delta G°_{37}$ [b]	$-\Delta G°_{37}$ [c]	$\Delta\Delta G$[d]
5′-TCGCGCGA[e] 3′-AGCGCGCT	54.4	10.3	10.3	--
5′-CCGCGCGA[e] 3′-AGCGCGCC	50.2	9.5	9.4	-0.45
5′-GCGCGCGZ 3′-ZGCGCGCG	57.4	10.6	10.6	0.15
5′-ZCGCGCGT[e] 3′-TGCGCGCZ	56.5	10.5	10.6	0.15
5′-TCGCGCGZ 3′-ZGCGCGCT	55.4	10.5	10.4	0.05
5′-GCGCGCGQ 3′-QGCGCGCG	58.5	10.6	10.4	0.05
5′-QCGCGCGT 3′-TGCGCGCQ	56.5	10.4	10.5	0.10
5′-TCGCGCGQ 3′-QGCGCGCT	56.7	10.6	10.6	0.15
5′-CCTCGCGCGA[e] 3′AGCGCGCTCC	58.3	11.4	11.4	--
5′-CCCCGCGCGA[e] 3′AGCGCGCCCC	50.9	9.5	9.6	-0.9
5′-CCZCGCGCGT[e] 3′TGCGCGCZCC	54.3	10.0	10.2	-0.6
5′-CCTCGCGCGZ 3′ZGCGCGCTCC	54.5	10.3	10.1	-0.65
5′-CCTCGCGCGQ 3′QGCGCGCTCC	56.4	10.5	10.6	-0.4
5′-CCQCGCGCGT 3′TGCGCGCQCC	56.8	10.6	10.4	-0.5

[a] Concentration of DNA strand, 7.5 μM. [b] Free energies (kcal·mol^{-1}) determined by averaged values from curve fitting. [c] Free energies determined from van't Hoff plot (ln C vs $1/T_m$) (kcal·mol^{-1}). [d] $\Delta\Delta G = (\Delta G - \Delta G_{AT})/2$. [e] Data taken from ref *39*.

However, the oligonucleotides with overhanging ends give different results. The cases that contain Z or Q analogues base-paired with T (Table 2), mimicking a Watson−Crick geometry, are less stable than an A-T base pair, with T_m values 2−4 °C lower and free energies (37 °C) 0.4−0.65 kcal·mol^{-1} less favorable. This indicates that base pairs of Z and Q with natural polar bases are less stable when shielded from the solvent in the middle of the DNA strand, an effect observed previously (*20*).

DISCUSSION

The present findings represent the first general survey of minor groove interactions from the DNA standpoint during the extension step. We recently carried out a study of the insertion step that precedes this, examining only the interactions with the incoming nucleotide and its partner in the template (*32*). We found that, for the insertion step, the enzymes KF$^-$, T7$^-$, Taq, and HIV-RT appear not to need any minor groove interactions with the incipient base pair.

Table 3: Minor Groove Contacts Predicted from Functional and Structural Studies

	functional[a]			structural[a]		
P/T	KF	TAQ	HIV-RT	BF[b]	TAQ	HIV-RT
dNTP/T0[c]	ø/ø	ø/ø	ø/ø	+/+	ø/ø	∗/∗
P1/T1	+/ø	+/ø	§/+	+/+	+/+	∗/ø
P2/T2	ø/ø	ø/ø	ø/ø	+/+	ø/ø	+/∗
P3/T3	ø/ø	ø/ø	ø/ø	+/+	+/ø	∗/∗
P4/T4	ø/ø	ø/ø	ø/ø	ø/ø	+/ø	ø/∗
P5/T5	ø/ø	ø/ø	ø/ø	ø/ø	+/ø	ø/ø
P6/T6	ø/§	ø/ø	ø/ø	ø/ø	ø/ø	ø/ø

[a] Symbols: + indicates H-bond; ø indicates no energetically important interaction; § indicates weak H-bond; ∗ indicates vdW contact. [b] BF is highly homologous to KF. [c] Data taken from ref *32*.

Table 4: Minor Groove Contacts Predicted from Functional and Structural Studies

	functional[a]		structural[a]	
P/T	pol β	T7	pol β	T7
dNTP/T0[b]	+/+	ø/ø	+/+	ø/∗
P1/T1	+/−	+/ø	+/ø	+/+
P2/T2	−/−	−/−	ø/ø	∗/ø
P3/T3	−/−	−/−	ø/ø	+/+
P4/T4	−/−	−/−	ø/ø	+/+

[a] Symbols: + indicates H-bond; ø indicates no energetically important interaction; ∗ indicates vdW contact; − indicates insufficient data from qualitative gel. [b] Data taken from ref *32*.

The enzymes polymerase α (pol α) and avian myeloblastosis virus reverse transcriptase require a single minor groove interaction at either the template base or the incoming triphosphate. Finally, human DNA polymerase β and Moloney murine leukemia virus reverse transcriptase appear to require minor groove interactions at both the template base and the incoming triphosphate for efficient DNA replication. Adding the present findings to this, it appears that there is little correlation between the number and strength of minor groove interactions for insertion and extension with a given polymerase. A summary of minor groove interactions identified functionally in the present studies compared with those found in X-ray structures of ternary complexes is given in Tables 3 and 4.

The present results for the extension step reveal varying abilities of polymerases (a) to maintain extension activity in the absence of minor groove H-bond acceptors and (b) to sense small changes in geometry. The first group, constituted by KF$^-$, Taq, and HIV-RT, efficiently extends base pairs containing analogue Q which provides a needed minor groove hydrogen bond acceptor. Taq and KF$^-$ clearly show this interaction at the primer strand of the first (terminal) base pair, displaying a 65−300-fold difference on extension of Q-T and Q-F versus Z-T and Z-F in the case of KF$^-$. This minor groove interaction is not important in the template strand. In the case of Taq, these observations partially agree with information obtained from the X-ray structure of a ternary complex (polymerase + dsDNA + nucleoside triphosphate) of this enzyme (*8*). Although the structure suggests H-bonding between Arg 573 and both terminal bases in the primer and the template strand (2.5 and 3.2 Å, respectively), only the minor groove interaction with the primer strand (which possesses the shorter distance in the crystal) seems to be energetically relevant for extension. In the published structure, other possible minor groove interactions are implicated further along the active site between Asn 583 and Lys 540 with bases in the primer strand located three to five bases below the active site. We cannot compare Z to Q in this case because Z is extended so poorly at the first step that no data are available further into the active site. Fortunately, however, the T-Z and F-Z cases can be compared. We find that although the initial extension of F-Z is inhibited, no further strong bands are seen, suggesting that these interactions are not energetically important in the later extensions further in the active site. Thus with Taq it appears that only the first extension requires a minor groove hydrogen bond and only with the base in the primer strand.

No structure of a ternary complex is available for KF$^-$, and no specific contacts in the proximity of the polymerase active site can be directly inferred from the binary complexes (polymerase + dsDNA), because the existing structures show DNA at the exonuclease site (*33*, *34*). However, there is a structure of a binary complex of a thermostable bacterial (*Bacillus stearothermophilus*) DNA polymerase I large fragment (BF) which shows strong structural (0.65 Å root-

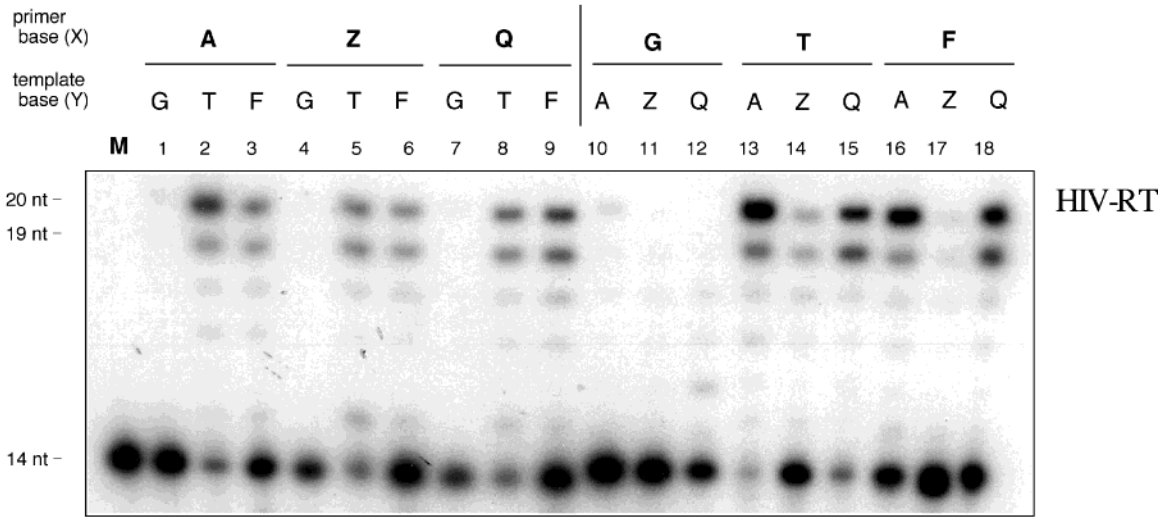

FIGURE 3: Autoradiogram of the denaturing PAGE gel showing minor groove interaction effects on extension of normal and modified base pairs. The data were taken at 37 °C with HIV-RT, 0.1 unit/μL, 150 nM primer-template duplex, 100 μM dNTP, and reaction time 1 h.

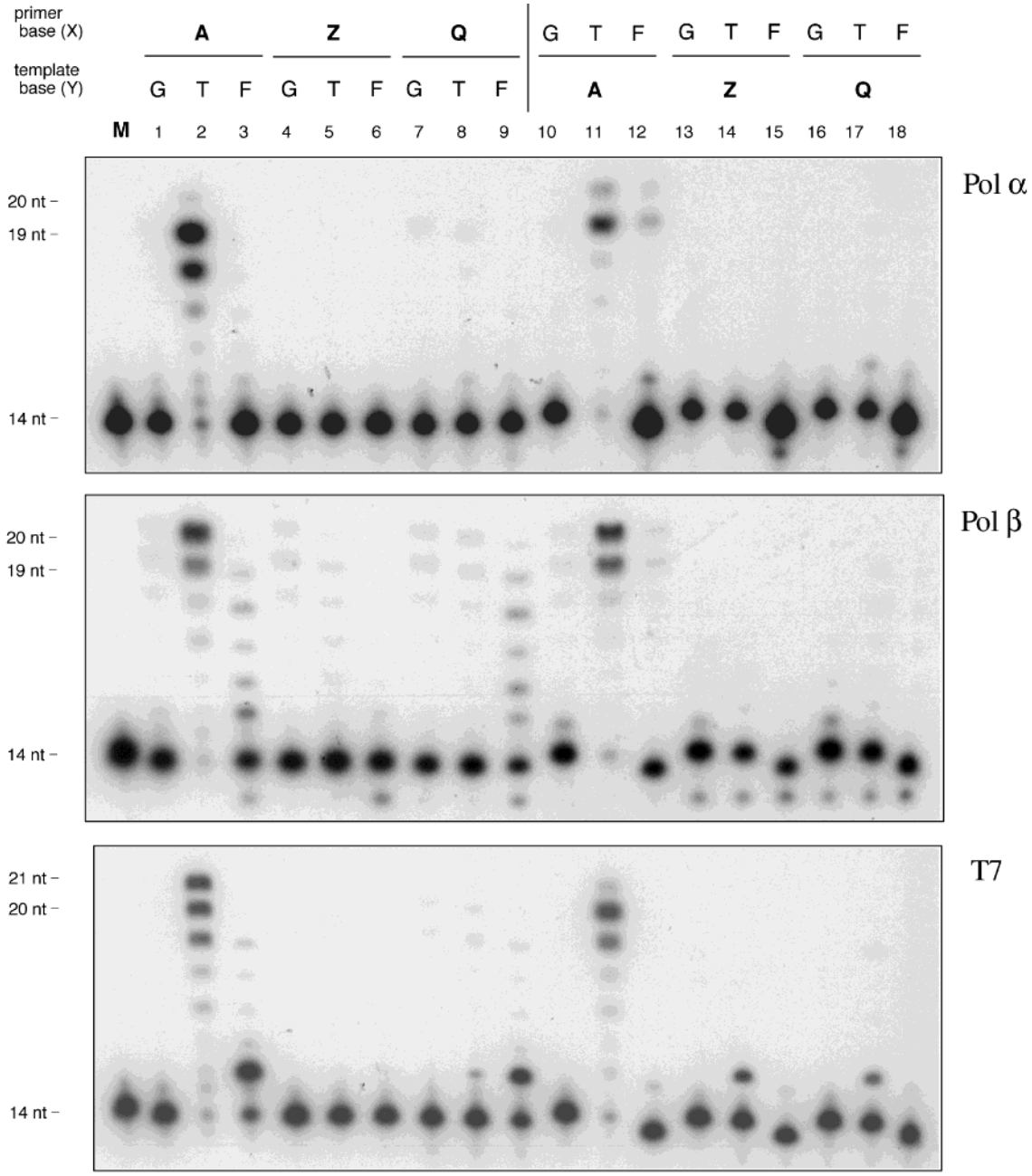

FIGURE 4: Autoradiogram of the denaturing PAGE gel showing minor groove interaction effects on extension of normal and modified base pairs at 37 °C. Conditions: pol α, 0.1 unit/μL, 150 nM primer-template duplex, 100 μM dNTP, and reaction time 3 h; pol β, 0.1 unit/μL, 150 nM primer-template duplex, 100 μM dNTP, and reaction time 3 h; T7$^-$, 0.1 unit/μL, 150 nM primer-template duplex, 20 μM dNTP, and reaction time 3 h.

mean-square deviation of Cα atoms) and sequence homology (49% identity) to KF$^-$ (*35*). On the basis of this homology, specific hydrogen bonds between highly conserved side chains with three base pairs below the insertion site are possible for KF$^-$ (see Table 3). This finding contrasts with our extension results for KF$^-$, which indicate that interactions in the minor groove of DNA are important only with the first base at the primer strand. We do note an apparent interaction with the template strand at the sixth position but not with any of the intermediate extension steps. This does not rule out such hydrogen bonds being formed in the KF$^-$—DNA complex, but it does suggest that they are not energetically important.

Mutagenesis studies of KF$^-$ have also examined the importance of these side chains for the insertion and extension steps in DNA replication (*11*, *12*, *15*). Alanine substitutions of several amino acids decrease the rate of extension of matched and mismatched terminal base pairs, but only mutation of Arg 668 diminishes mispair extension fidelity (*15*). This result is consistent with our findings since Arg 668 has been proposed to affect extension via an important hydrogen bond with the acceptor group at the primer strand of the terminal base. It is not yet clear from mutagenesis or structural studies which side chain, if any, could be interacting at the sixth base pair position.

The enzyme HIV-RT successfully extends base pairs containing the nonpolar base Q despite its slightly larger size relative to A. The data show that a crucial minor groove interaction seems to be needed with the first position in the template strand, because T-Q is completely extended under

our conditions whereas T-Z fails almost completely. This is in contrast with the results for KF^- and Taq, where an important H-bond interaction is needed on the other side of the groove, in the primer strand. A similar contact with the primer strand also seems to help HIV-RT to a lesser magnitude. Surprisingly, the recently reported ternary complex of HIV-RT shows only van der Waals contacts between Met 184, Pro 157, and Ile 94 with the four bases below the active site, and only Tyr 183 shows a hydrogen bond with the N3 of the second base in the primer strand (*7*). Our results are in discrepancy with the contacts observed in the X-ray structure, unless the Tyr 183 side chain can move and reach the minor groove area of the first base in the template strand, which seems unlikely. Another possible explanation is that the covalently attached DNA in the crystal structure could be slightly shifted in the structure relative to the active noncovalent complex, and therefore the H-bond contact of Tyr 183 with the first base at the template cannot be observed. Further studies are clearly needed before these results can be interpreted in a satisfactory way.

Although full extension is not successful, we do observe extension of nonpolar pairs by one base with $T7^-$. A hydrogen bond on position 1 in the primer strand seems to be energetically important since Q-F, T-Z, and T-Q are extended one base but no extension is observed for Z-F, F-Z, and F-Q. These results can be compared with the structural data from a complex of T7 with ds DNA and a deoxynucleotide (*6*). At position 1, two probable H-bonded contacts are observed in the structure between the minor groove acceptor groups at the template and the primer sites and side chains of Gln 615 and Arg 429, respectively. It is possible, then, that of the two contacts the one with Gln 615 is more functionally important.

The second group of polymerases, composed by pol α, pol β, and $T7^-$, largely failed to fully extend the DNA in the 14/20-mer sequence used. Several explanations can be invoked to explain the inefficient extension of the modified base pairs by this second group. One possibility is that minor groove interactions are needed at both the primer and template sides of the duplex in the first extension position. If this were the only requirement, however, then base pairs such as T-Q or Q-T should be able to be extended. A second possibility is that base pairs such as T-Q and Q-T are too large due to the extra size of the analogue Q, which is larger than A by ca. 0.7 Å. This might result in slight displacement of the 3′-OH of the primer terminus, moving it out of optimum position for phosphodiester bond formation. This is unlikely, since in $T7^-$ when a new G-C base pair has been synthesized (lanes 3, 9, 14, and 17 in Figure 4), the geometry will be the correct one, and yet we do not observe further extension. A third possibility is that these nonnatural pairs result in lower duplex stability, and the enzyme could be dissociating more rapidly from the less stable DNA, thereby decreasing extension efficiency. Our data show that DNA sequences containing nonpolar base pairs in a context resembling a primer-template duplex are less stable than matched base pairs by 0.4−0.65 kcal·mol^{-1}. This could be the reason for the poor extension of these nonpolar analogues since it is known that T7 and pol β have relatively weak binding affinity for small oligonucleotides. Dissociation rate constants of 0.2 and 1−2 s^{-1} have been reported for T7 and pol β, respectively, when measured on 25/36-mer and 25/45-mer oligonucleotides (*36*, *37*). In contrast, a value of 0.06 s^{-1} has been reported for KF^- when measured on a much shorter 13/20-mer oligonucleotide (*38*). This can provide an explanation why nonpolar base pairs that have the capacity of making minor groove interactions are still extended so poorly by the second group of DNA polymerases. Future studies with such analogues in longer DNAs should clarify this third possibility.

Interestingly, all of the nonnatural base pairs that can make the necessary H-bond in the primer strand, such as Q-F, Q-T, T-Z, and T-Q, are still extended by KF^- less efficiently than the natural base pairs A-T and T-A by a factor of 160−400-fold. The added size of nonpolar analogues Z and Q may well slightly misalign the 3′-OH primer terminus, accounting for some of this effect. However, the base pair A-F, which matches extremely closely the Watson−Crick geometry (*29*), is still 110-fold less efficiently extended than the natural base pair A-T. It is important to point out again that all of these base pairs are less stable than a matched base pair in a context similar to that used for these experiments by 0.4−0.65 kcal·mol^{-1}. It is possible that this difference in stability is enough to dissociate the double-stranded DNA or (in the case of proofreading enzymes) to allow it to be unwound and translocated to the exo domain to be edited. Previous studies have shown that the rapid proofreading of bases opposite F or Z residues at the duplex terminus seems to correlate well with the stability of these base pairs when placed in a context resembling a primer-template duplex (*39*).

The present DNA base analogues represent a quite conservative structural perturbation; the analogue F is virtually indistinguishable from T in a structural sense, and comparison of analogue Z with Q also represents a very small structural change. While providing complementary information, these replacements give a considerably smaller structural perturbation than might arise from protein mutagenesis (such as replacement of arginine with alanine). We anticipate that the same analogues will find utility in the study of other minor groove interactions as well.

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