Proc. Natl. Acad. Sci. USA
Vol. 92, pp. 6329–6333, July 1995
Biochemistry

Recognition by viral and cellular DNA polymerases of nucleosides bearing bases with nonstandard hydrogen bonding patterns

[human immunodeficiency virus type 1 reverse transcriptase/DNA polymerases α, β, and ε/2′-deoxy-N^1-methyloxoformycin B/2′-deoxyxanthosine/2,4-diamino-5-(β-D-2′-deoxyribofuranosyl)pyrimidine]

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Communicated by Peter B. Dervan, California Institute of Technology, Pasadena, CA, April 3, 1995 (received for review September 22, 1994)

ABSTRACT **The ability of DNA polymerases (pols) to catalyze the template-directed synthesis of duplex oligonucleotides containing a nonstandard Watson–Crick base pair between a nucleotide bearing a 5-(2,4-diaminopyrimidine) heterocycle (dκ) and a nucleotide bearing either deoxyxanthosine (dX) or N^1-methyloxoformycin B (π) has been investigated. The κ-X and κ-π base pairs are joined by a hydrogen bonding pattern different from and exclusive of those joining the AT and GC base pairs. Reverse transcriptase from human immunodeficiency virus type 1 (HIV-1) incorporates dXTP into an oligonucleotide opposite dκ in a template with good fidelity. With lower efficiency and fidelity, HIV-1 reverse transcriptase also incorporates dκTP opposite dX in the template. With dπ in the template, no incorporation of dκTP was observed with HIV reverse transcriptase. The Klenow fragment of DNA pol I from *Escherichia coli* does not incorporate dκTP opposite dX in a template but does incorporate dXTP opposite dκ. Bovine DNA pols α, β, and ε accept neither dXTP opposite dκ nor dκTP opposite dπ. DNA pols α and ε (but not β) incorporate dκTP opposite dX in a template but discontinue elongation after incorporating a single additional base. These results are discussed in light of the crystal structure for pol β and general considerations of how polymerases must interact with an incoming base pair to faithfully copy genetic information.**

Recognition between base pairs in complementary oligonucleotide strands is mediated by two rules of complementarity: size complementarity (large purines pair with small pyrimidines) and hydrogen bonding complementarity (hydrogen bond donors are matched with acceptors). Hydrophobicity and planarity in the bases are also believed to be important for the stability of the double helical structure. Hydrophobicity allows transfer of the bases from water to the core of the double helix to make an energetic contribution to duplex formation. Planarity allows sequence-independent stacking of the bases to form an aperiodic crystalline structure necessary for duplex stability.

Even with these constraints, considerable structural flexibility remains within the Watson–Crick base pair. For example, Rich (1) pointed out in 1962 that isocytosine, which presents a hydrogen bond acceptor–acceptor–donor pattern to the complementary strand, might fit the Watson–Crick geometry when paired with isoguanine (Fig. 1).‡ Zubay (2) proposed a base pair joined by a nonstandard hydrogen bonding pattern, although in a ring system that was not planar and therefore presumably not able to stack.

Some time ago, we noted (3) that the Watson–Crick formalism could readily be extended to include 12 independently

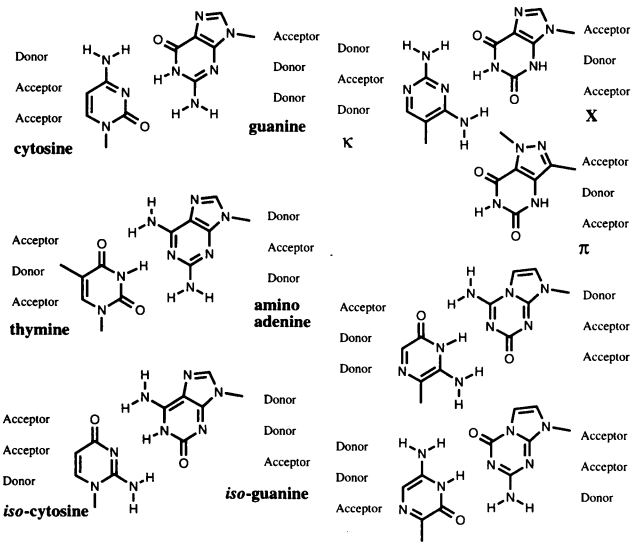

FIG. 1. Six base pairs that meet the constraints imposed by the Watson–Crick base pairing geometry.

replicatable bases joined in 6 base pairs by mutually independent hydrogen bonding patterns, provided that some bases were joined to the sugar via a carbon—carbon bond (a "C glycoside") (Fig. 1). We then asked whether natural DNA and RNA polymerases (pols) could catalyze the template-directed incorporation of these pairs into duplex DNA. If so, this could increase the number of amino acids that might be encoded by a mRNA (4), provide nonstandard sequence "tags" on oligonucleotides used in diagnostic applications, expand the versatility of experiments for the *in vitro* evolution of nucleic acids (5–7), and assist efforts to obtain self-replicating oligonucleotides as models for the origin of life (3).

Previous work has shown that pols can accept the isocytosine-isoguanine base pair (8–10), where both the purine and the pyrimidine analogs are joined to the sugar ring by a carbon—nitrogen bond. The Klenow fragment of DNA pol I also incorporates deoxyxanthosine (dX) (11), a purine presenting a hydrogen bond acceptor–donor–acceptor pattern to a complementary strand, opposite 2,4-diamino-5-(β-D-2′-deoxyribofuranosyl)pyrimidine (dκ), a pyrimidine presenting

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Abbreviations: pol, DNA or RNA polymerase; HIV-1, human immunodeficiency virus type 1; BSA, bovine serum albumin; DTT, dithiothreitol; M, marker; P, primer.

‡In dκ, the heterocycle is joined to the sugar via a C—C bond. A crystal structure of ribo-κ as a hydrochloride (not shown) (49) shows that the sugar ring of ribo-κ exists in a pronounced C2′-endo conformation, as in DNA. This suggests that the C—C bond need not by itself exclude dκ as a substrate; other studies suggest that C-glycoside linkages need not be intrinsically problematic for a pol (12).

the complementary donor–acceptor–donor hydrogen bonding pattern. However, pols that accept triphosphates of C glycosides have proven difficult to find. Recent work in these (11–13) and other (14) laboratories has suggested that C nucleosides might generally form weaker base pairs and might be generally poor substrates for pols. We therefore undertook a more systematic study of the enzymology of base pairing between 2,4-diamino-5-(β-D-2′-deoxyribofuranosyl)pyrimidine (dκ, a C glycoside) and either deoxyxanthosine (dX) or 2′-deoxy-1-methyloxoformycin B (dπ) (Fig. 1), focusing on viral and cellular DNA pols (15).

EXPERIMENTAL

Synthesis of Nucleosides Bearing Nonstandard Bases. N^1-Methyloxoformycin B (π) was prepared by the procedure of Ugakar *et al.* (16). 2,4-Diamino-5-(β-D-ribofuranosyl)pyrimidine (κ) was synthesized by the route of Chu *et al.* (17). These were converted to 2′-deoxygenated nucleoside analogs as described by Piccirilli (18) and to 5′-dimethoxytritylated β-cyanoethyl phosphoramidites by standard methods (19). 5′-Dimethoxytrityl-2′-deoxyxanthosine with both heterocyclic ring oxygens protected as *p*-nitrophenylethyl ethers was prepared following Van Aerschot *et al.* (20) and converted to the phosphoramidite. The triphosphates dκTP and dXTP were prepared following literature procedures (21, 22). Standard dNTPs were from Pharmacia. Oligonucleotides were prepared by solid-phase synthesis (Applied Biosystems) from β-cyanoethyl-protected phosphoramidites, purified by the "trityl on" procedure, deprotected, and purified again by HPLC (23). Primer (18 bases, 5′-TAATACGACTCACTATAG-3′) was labeled at the 5′ end with [γ-^{33}P]ATP (Amersham) using T4 polynucleotide kinase (24).

Enzymes, Proteins, and Chemicals. Human immunodeficiency type 1 (HIV-1) reverse transcriptase overexpressed using the plasmid pJS3.7 in *Escherichia coli* was purified by a published procedure (25). Calf thymus DNA pols α, β, and ε were purified according to the methods of Podust *et al.* (26), Chang (27), and Weiser *et al.* (28), respectively. The Klenow fragment of pol I (sequencing grade) was from Boehringer Mannheim. DNase-free bovine serum albumin (BSA) was from New England Biolabs. Other reagents were of analytical grade from Fluka, Merck, or Aldrich.

Nucleic Acids. The template (5′-AGCCQGGCGCTAT-AGTGAGTCGTATTA, 27 bases, Q is a nonstandard nucleotide) was annealed with primer in 750 μl of total buffer [50 pmol of template and 15 pmol of labeled primer, in 1.8 mM Tris·HCl (pH 7), containing 0.5 mM $MgCl_2$ and 23 mM NaCl] by cooling from 75°C to the assay temperature over a period of 1 hr.

DNA Pol Assays. Pol assays were performed in a final volume of 20 μl containing the following: for pol α, 20 mM potassium phosphate (pH 7.2), 0.1 mM EDTA, 4 mM dithiothreitol (DTT), 0.25 mg of BSA per ml, and 10 mM $MgCl_2$; for pol β, 40 mM Tris·HCl (pH 7.5), 1 mM DTT, 0.2 mg of BSA per ml, and 10 mM $MgCl_2$; for pol ε, 20 mM Tris (pH 7.2), 0.5 mM DTT, 0.1 mg of BSA per ml, 10 mM $MgCl_2$, and 10 mM $MgCl_2$; for HIV-1 reverse transcriptase, 50 mM Tris·HCl (pH 7.2), 5 mM $MgCl_2$, 80 mM KCl, 1 mM DTT, and 0.5 mM EDTA; for Klenow fragment of pol I, 50 mM Tris·HCl (pH 7.5), 10 mM $MgCl_2$, 0.1 mg of BSA per ml, and 1 mM DTT. The amount of enzyme used and the concentrations of templates, primers, and nucleoside triphosphates are specified in the figure legends. One unit of pol is defined as the amount of enzyme that incorporates 1 nmol of total dNTP per hour at 37°C.

The reactions were quenched by adding a formamide (3–5 μl) containing xylene cyanol (0.3%), bromphenol blue (0.3%), and EDTA (0.37%), the samples were heated for 10 min at 75°C, and aliquots (3–5 μl) were loaded onto a 13% polyacrylamide gel (16 cm × 42 cm × 0.6 mm) containing 7 M urea. Following electrophoresis (50°C at 1500–1800 V), the gels were fixed, dried, and autoradiographed.

RESULTS

Klenow Fragment of DNA Pol I from *E. coli*. When the Klenow fragment of pol I was incubated at 23°C with templates containing the nonstandard bases using enough pol to elongate all of the primer in 20 min, no incorporation of either dκTP or dXTP opposite to its counterpart in a template could be detected (Fig. 2*a*). Elongation of the primer stops just before incorporation opposite the nonstandard base. With a 40-fold

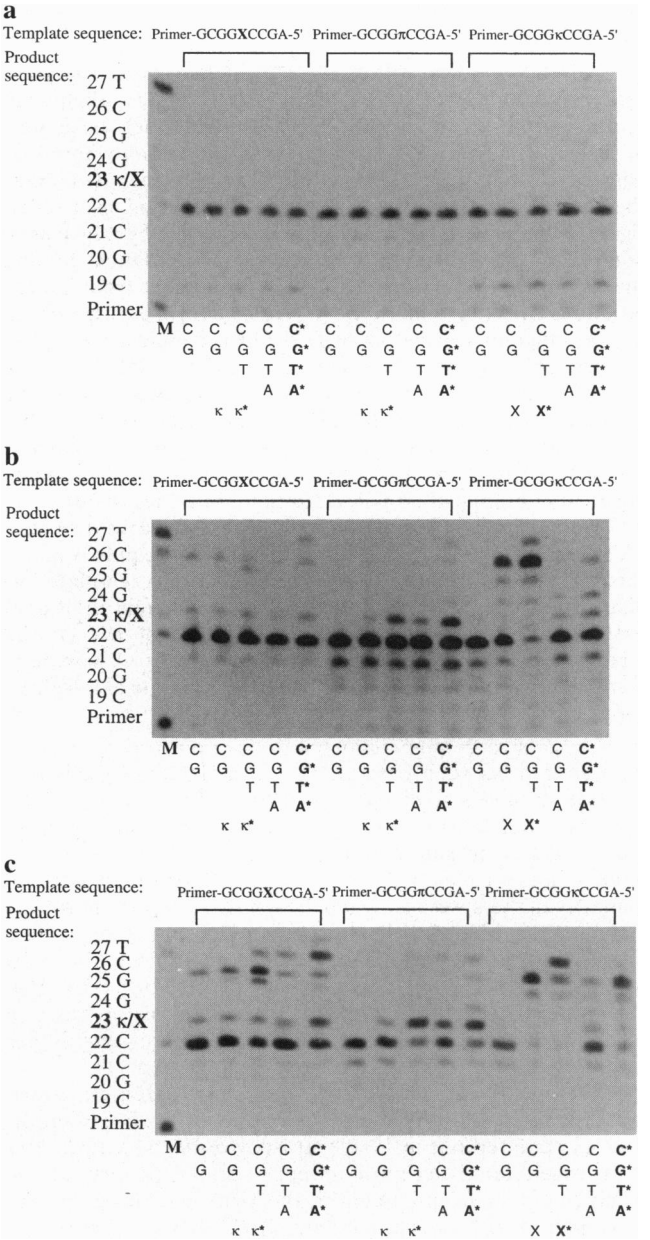

FIG. 2. Primer extension by the Klenow fragment of DNA pol I from *E. coli*. Triphosphates present are indicated below. M indicates markers (primer, full-length product). (*a*) Deoxynucleoside triphosphates (2 μM; 50 μM when indicated by *) incubated at 23°C for 20 min with primer/template (as 3′-OH primer end, 0.12 pmol) and 0.15 unit of the Klenow fragment of pol I in a final volume of 20 μl. (*b*) As in *a* but with 0.8 unit of the Klenow fragment of pol I and incubation for 30 min. (*c*) As in *a* but with 6.0 units of the Klenow fragment of pol I and incubation for 30 min.

increase in pol concentration, no incorporation of dκTP was observed opposite π in the template (Fig. 2*c*); nor could convincing evidence be found for the incorporation of dκTP opposite dX. In both cases, incorporation of dκTP and further elongation were observed, but control experiments suggested that these might reflect misincorporation of a standard base opposite dX. An additional band (Fig. 2*c*) obtained with very high concentrations of dκTP and enzyme suggested that dκTP might be very inefficiently incorporated.

The Klenow fragment did, however, incorporate dXTP opposite dκ in a template with increasing enzyme concentrations (Fig. 2 *a–c*), and increasing dXTP concentrations (to 50 μM). Incorporation of dXTP is selective, as similar elongation was not observed in the absence of dXTP with the four standard bases. A substantial amount of product missing the final base was also observed. This is frequently seen with fill-in experiments using the Klenow fragment. These results confirm and extend results obtained by Piccirilli *et al.* (11).

HIV-1 Reverse Transcriptase. HIV-1 reverse transcriptase incorporated dXTP opposite dκ in a template at low concentrations of enzyme and low concentrations of dXTP (Fig. 3*a*). A product with a single base incorporated opposite dκ and a smaller amount of fully extendible product were seen. No misincorporation of the standard dNTPs could be detected under similar conditions. At 25-fold higher concentrations of dXTP, primer elongated by addition of dXTP was the major product, with substantial amounts of full-length product also observed. Higher concentrations of standard dNTPs yielded only a trace of misincorporation. Thus, HIV-1 reverse transcriptase incorporates dXTP opposite dκ in a template with good yields and high selectivity. However, the pol still clearly recognizes dXTP as foreign, as considerable chain termination occurred immediately before incorporation of dXTP and, at concentrations of dXTP where pausing is eliminated, after incorporation of the dX-dκ base pair into the duplex product.

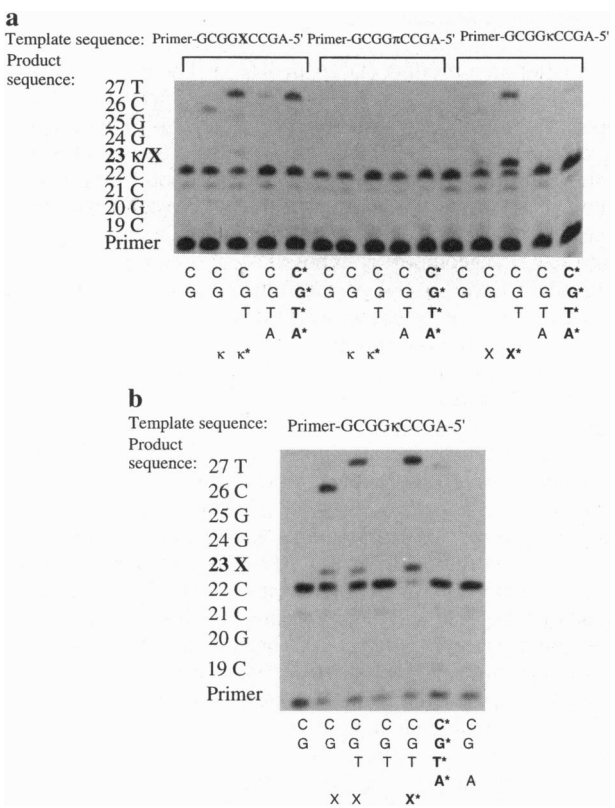

FIG. 3. Primer extension by HIV-1 reverse transcriptase. Triphosphates present are indicated below. (*a*) Deoxynucleoside triphosphates (2 μM; 50 μM when indicated by *) incubated at 37°C for 30 min with primer/template (as 3′-OH primer end, 0.13 pmol) and 0.12 unit of HIV-1 reverse transcriptase in a final volume of 20 μl. (*b*) Deoxynucleoside triphosphates (2 μM; 25 μM when indicated by *) incubated at 37°C for 45 min with primer/template (as 3′-OH primer end, 0.08 pmol) and 0.37 unit of HIV-1 reverse transcriptase in a total volume of 20 μl.

With templates containing dX, a small amount of fully extended product is observed at low concentrations (2 μM) of dκTP. Substantial amounts of full-length product are observed at 50 μM dκTP when the standard dNTPs remained at low concentration (2 μM). Misincorporation of the standard dNTPs (50 μM) also yielded full-length products (Fig. 3). Thus, the discrimination between dκ and the standard dNTPs opposite dX in a template is not particularly good. However, it is interesting to note that HIV-1 reverse transcriptase does not pause significantly after incorporation of dκTP opposite dX in the template, in contrast to the pause observed after the incorporation of dXTP opposite dκ. Thus, although dκTP is incorporated with lower selectivity than dXTP by HIV-1 reverse transcriptase, it is incorporated with higher overall efficiency.

Experiments at higher concentrations of enzyme showed the selectivity of incorporation of dXTP opposite dκ in the template. With dGTP and dCTP alone, primer extension stops just before the dκ in the template without substantial misincorporation. Further, neither dT nor dA was misincorporated in the absence of dX at 2 μM. Even at high (25 μM) concentrations of each of the four standard dNTPs, only a trace of full-length product was observed.

Calf Thymus DNA Pols α, β, and ε. These three pols all did not incorporate dXTP opposite dκ in a template (Fig. 4 *b* and *d*). Furthermore, no misincorporation was observed. Similarly, neither incorporation of dκTP nor misincorporation was observed opposite π (Fig. 4*a*). With templates containing dX, the pols behaved differently. No incorporation of dκTP was detectable with pol β, even at 50 μM concentrations. Pol α incorporated dκTP opposite dX in a template (Fig. 4*c*). Primer extension stops, however, after incorporation of a single additional base following dκ, with substantial amounts of pausing. In the presence of dCTP and dGTP alone with pol α, primer extension was terminated at the nonstandard base. A small but significant amount of misincorporation opposite dX was observed only at high concentrations of standard dNTPs (50 μM each). With pol ε (29–32), a similar but not identical behavior was observed (Fig. 4*d*). Incubation in the presence of dκTP, dCTP, and dGTP showed incorporation of a base opposite dX in the template and continued elongation by only a single additional base. Unlike with pol α, however, less pausing was observed after dκTP was incorporated. The amount of dκ incorporated increased at higher concentrations of dκTP.

DISCUSSION

The ability of pols to accept the dκ-dX nonstandard base pair is remarkably variable, especially considering that all pols appear to be homologous (judging by the common three-dimensional fold in those studied). In some cases, dκTP was not incorporated. In others, difficulties in polymerization were encountered only after the incorporation of dκTP, as if the pol rejected dκ in the primer. dXTP was easily incorporated by some polymerases and firmly rejected by others. During the divergent evolution of the polymerase superfamily, features in the active site that make contact with the bases in the template, primer, and triphosphate must have diverged considerably, and these interactions (rather than an intrinsic property of the nonstandard nucleosides themselves) must be the primary determinant of the outcome of a replication experiment with a nonstandard base.

The ability of pols to accept a nonstandard base does not correlate cleanly with overall specificity. Pol β is the least selective of the pols examined here. Indeed, pol β is so efficient

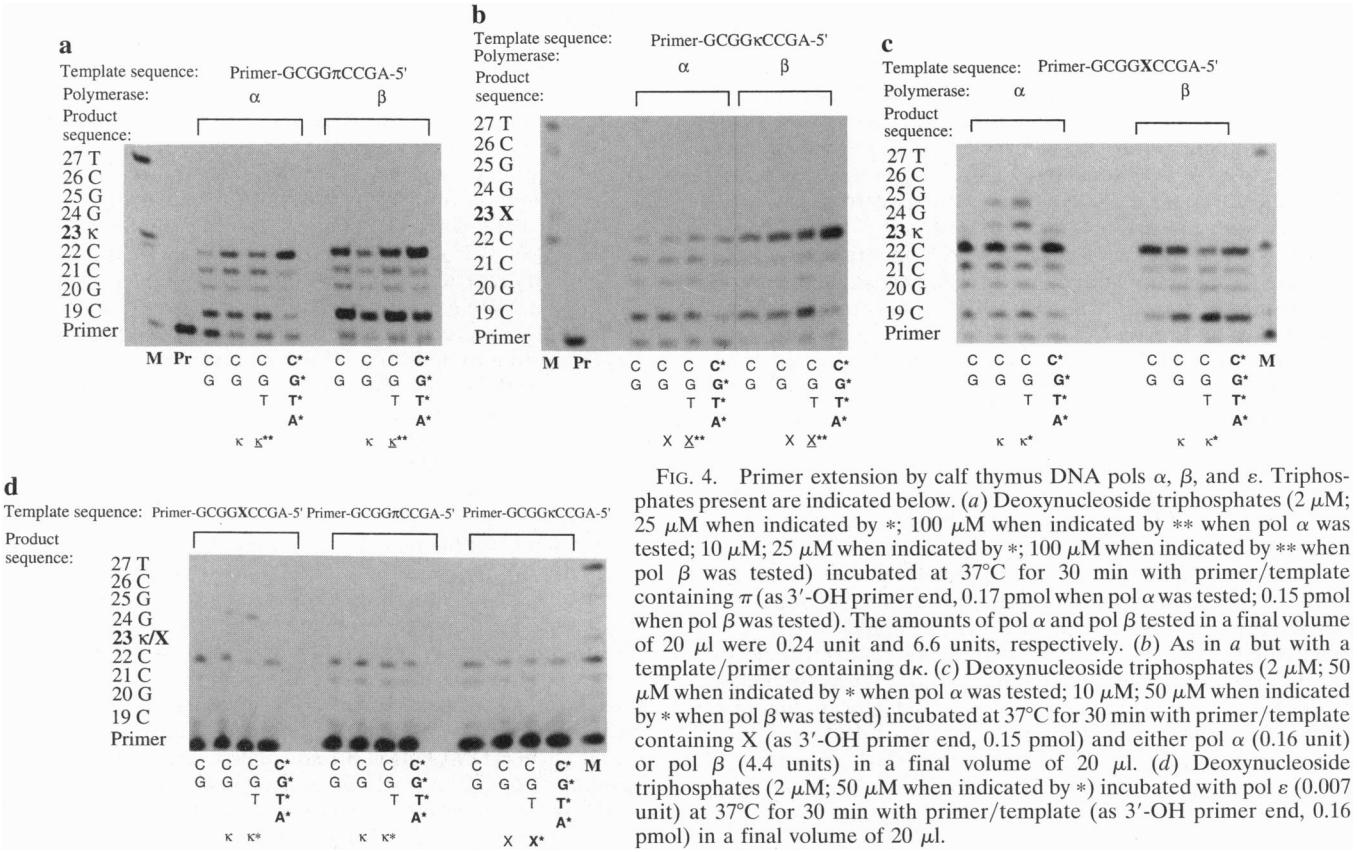

FIG. 4. Primer extension by calf thymus DNA pols α, β, and ε. Triphosphates present are indicated below. (*a*) Deoxynucleoside triphosphates (2 μM; 25 μM when indicated by *; 100 μM when indicated by ** when pol α was tested; 10 μM; 25 μM when indicated by *; 100 μM when indicated by ** when pol β was tested) incubated at 37°C for 30 min with primer/template containing π (as 3′-OH primer end, 0.17 pmol when pol α was tested; 0.15 pmol when pol β was tested). The amounts of pol α and pol β tested in a final volume of 20 μl were 0.24 unit and 6.6 units, respectively. (*b*) As in *a* but with a template/primer containing dκ. (*c*) Deoxynucleoside triphosphates (2 μM; 50 μM when indicated by * when pol α was tested; 10 μM; 50 μM when indicated by * when pol β was tested) incubated at 37°C for 30 min with primer/template containing X (as 3′-OH primer end, 0.15 pmol) and either pol α (0.16 unit) or pol β (4.4 units) in a final volume of 20 μl. (*d*) Deoxynucleoside triphosphates (2 μM; 50 μM when indicated by *) incubated with pol ε (0.007 unit) at 37°C for 30 min with primer/template (as 3′-OH primer end, 0.16 pmol) in a final volume of 20 μl.

at incorporating mismatches into duplex DNA that early speculation suggested that it required no template (33), a speculation reinforced by its homology with terminal deoxynucleotidyltransferase. Yet pol β is extremely discriminating with the nonstandard dκ-dX base pair. This discrimination cannot be explained by proofreading (34–36), as pol β does not have a proofreading 3′ → 5′ exonuclease activity. The discrimination must occur during the elongation reaction itself.

Direct contacts between the bases and the side chains of residues in pol β may be invoked to explain the extreme specificity of the enzyme. Pelletier *et al.* (37) report that Lys-234 in pol β forms a hydrogen bond to the lone pair of electrons on oxygen-2 of a cytosine in the template, Tyr-271 forms a hydrogen bond to the O-2 lone pair of a cytosine in the primer, and Arg-283 forms a hydrogen bond to a lone pair on N-4 of a guanine in the template. These lone pairs of electrons are significant, as they are the only structural feature presented in common to the grooves of duplex DNA by all four base pairs (AT, TA, GC, and CG). This lone pair is replaced by an N—H bond in both dκ and dX. While Tyr-271 might be ambivalent (accepting both hydrogen bond donor and acceptor groups) at this position in the oligonucleotide, neither Lys-234 nor Arg-283 should tolerate this replacement. Accordingly, site-directed mutagenesis experiments targeted against these two residues might yield mutant forms of pol β that accept dκ and dX.

Crystal structures for other pols are available (38–40), but without a full complement of bound substrates. Thus, specific residue–substrate interactions that might explain specificity in these enzymes are difficult to assign, especially as conformational changes evidently occur upon formation of the active complex (41–44). For these pols, site-directed mutagenesis (45, 46) should, in combination with studies using nonstandard bases, prove valuable in interpreting crystallographic information regarding these interactions.

The idiosyncracies displayed by pols are also surprising in light of the physiological role that a pol must play. Pols must faithfully convert information in a template strand (DNA or RNA) into information in a product (DNA or RNA). The pol must choose the appropriate sugar, enforce a Watson–Crick geometry (as opposed to a wobble geometry, for example) (47), and provide most of the selective binding to the pentacoordinated phosphate in the transition state to catalyze the reaction. However, the template, not the pol, should choose the pattern of hydrogen bond donor and acceptor groups in the incoming base once the Watson–Crick geometry is imposed. Any preference that a pol itself might have for one of the four natural base pairs would decrease its overall efficiency and fidelity.

In this view, the pol should make contacts with the bases at only those positions where the standard bases have similar structural features, for example, with the lone pairs of electrons on O-2 of pyrimidines and N-3 of purines. The crystal structure of pol β suggests that these contacts, *and no others*, are made between the enzyme and the bases (37).

This view cannot, however, be general. The Klenow fragment accepts X as a triphosphate and κ as a template. Both replace the common lone pair (O-2 of pyrimidines, N-3 of purines) by an NH group. It is hard to see what Klenow might be identifying that allows it to discriminate against dκ (but not dX) as a triphosphate, or against dX (but not dκ) in the template, without creating an interaction that will disrupt the neutrality of the pol with respect to the four standard base pairs. This becomes doubly problematic in light of recent results that show that Klenow accepts isocytosine in the template and as a triphosphate, although with some bias against isocytosine (8). Klenow cannot be searching rigorously for the O-2 lone pair (for pyrimidines) or the N-3 lone pair (for purines).

A final point must be made concerning HIV-1 reverse transcriptase, which accepts a wide range of substrates, including nucleoside triphosphates modified on the sugar (48). There is no reason *a priori* why tolerance of modified sugars should foreshadow tolerance of nonstandard base pairs. That HIV tolerates variation in both is consistent, however, with the common view that the active site in HIV-1 reverse transcriptase is "looser" than

in other pols. The implications of this for the design of inhibitors for HIV reverse transcriptase are evident.

We thank A. Müller for the help with nucleoside synthesis and M. Haugg for discussion. This work was supported by the Swiss National Science Foundation (31-28592.90 to U.H.; 31-36713.92 to M.H.). M.H. is a recipient of a Swiss National Science Foundation grant for the M.D.–Ph.D. program.

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