Abasic Translesion Synthesis by DNA Polymerase β Violates the “A-rule”

NOVEL TYPES OF NUCLEOTIDE INCORPORATION BY HUMAN DNA POLYMERASE β AT AN ABASIC LESION IN DIFFERENT SEQUENCE CONTEXTS

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The “A-rule” reflects the preferred incorporation of dAMP opposite abasic lesions in Escherichia coli in vivo. DNA polymerases (pol) from procaryotic and eucaryotic organisms incorporate nucleotides opposite abasic lesions in accordance with the A-rule. However, recent in vivo data demonstrate that A is not preferentially incorporated opposite abasic lesions in eucaryotes. Purified human DNA polymerases β and α are used to measure the specificity of nucleotide incorporation at a site-directed tetrahydrofuran abasic lesion, in 8-sequence contexts, varying upstream and downstream bases adjacent to the lesion. Extension past the lesion is measured in 4 sequence contexts, varying the downstream template base. Pol α strongly favors incorporation of dAMP directly opposite the lesion. In marked contrast, pol β violates the A-rule for incorporation directly opposite the lesion. In addition to incorporation taking place directly opposite the lesion, we also analyze misalignment incorporation directed by a template base downstream from the lesion. Lesion bypass by pol β occurs predominantly by “skipping over” the lesion, by insertion of a nucleotide complementary to an adjacent downstream template site. Misalignment incorporation for pol β occurs by a novel “dNTP-stabilized” mechanism resulting in both deletion and base substitution errors.

In contrast, pol α shows no propensity for this type of synthesis. The misaligned DNA structures generated during dNTP-stabilized lesion bypass do not conform to misaligned structures reported previously.

Loss of purine and pyrimidine bases is a significant source of DNA damage in procaryotic and eucaryotic organisms. Abasic (apurinic and apyrimidinic) lesions occur spontaneously in DNA; in eucaryotes it has been estimated that about 10^4 depurination and 10^2 depyrimidation events occur per day (1). An equally important source of abasic DNA lesions results from the action of DNA glycosylases, most notably uracil glycosylase which excises uracil arising primarily from spontaneous deamination of cytosine (1, 2). A DNA template with an abasic lesion presents a strong block to DNA synthesis in Escherichia coli, which can be partially alleviated by induction of the SOS response (3). Although most abasic lesions are removed by a base excision repair pathway involving the combined action of various enzymes, e.g. N-glycosylase, endonuclease, phosphodeoxyribosyl phosphodiesterase, polymerase and ligase (4), a small fraction of lesions persist and are replicated leading to a high probability of a mutation occurring.

When an abasic lesion is copied in vivo in E. coli, A is predominantly incorporated opposite the lesion (3, 5).

Preferential incorporation of A opposite abasic lesions is referred to as the “A-rule” (5-8). In agreement with E. coli data (3), studies with purified DNA polymerases showed that incorporation of A opposite X occurs about 10-fold more frequently than G opposite the lesion and about 50-fold more frequently than either C or T (9, 10). Although neighboring sequence contexts have been observed to perturb relative nucleotide incorporation efficiencies (3, 9), the strong conclusion, until recently, remained that DNA polymerases and reverse transcriptases generally behave in accordance with the A-rule. A physically intuitive basis for the A-rule emanates from NMR studies showing that when A is opposite an abasic lesion it stacks in an intrahelical configuration causing virtually no distortion of the DNA helix (11, 12).

For the other bases, G may either be inter- or extrahelical depending on temperature, while the pyrimidine bases stack poorly and are likely to be extrahelical (13).

Recent studies in eucaryotes, however, suggest that the A-rule may not be a universal mechanism for preferential incorporation of A opposite noncoding template lesions. Sarasin and co-workers (14, 15) using a shuttle vector system, replicated in monkey COS7 cells, observed an essentially random incorporation of A opposite noncoding template lesions. A second site-activating point mutation was located at the 5′-site adjacent to the abasic lesion (18). Remarkably, in a shuttle vector system in yeast cells, Gibbs and Lawrence discovered a “C-rule” where incorporation of C occurred opposite a site-directed abasic lesion.

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The abbreviations used are: X, refers to a tetrahydrofuran abasic lesion on a DNA template strand; pol β and pol α, eucaryotic DNA polymerases β and α; dNTP, deoxyribonucleoside triphosphate; p/t DNA, primer-template DNA; nt, nucleotide.

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80% of the time (19). In the light of an accumulating body of evidence indicating significant differences in the processing of abasic lesions in procaryotic and eucaryotic cells, it is important to measure nucleotide incorporation specificities for eucaryotic DNA polymerases, especially the eucaryotic repair enzyme pol β, which has no known procaryotic counterpart.

In this paper, we use human pol β and pol α to measure deoxyribonucleotide incorporation and extension efficiencies at a site-directed tetrahydrofuran abasic site. The incorporation efficiencies were measured in the presence of different upstream and downstream nucleotides adjacent to the template abasic lesion. We distinguish between two types of incorporation mechanisms: “direct” incorporation and a novel form of “misalignment” incorporation. Direct incorporation refers to nucleotide insertions occurring directly opposite a lesion, and misalignment incorporation is defined by nucleotide insertions occurring opposite a normal template base downstream from the lesion, on a misaligned primer-template terminus. The novel form of misalignment incorporation, carried out by pol β, does not conform to misaligned p/t DNA structures reported previously (20–22). We use the term “dNTP-stabilized misalignment” to refer to our model for pol β-catalyzed misalignment incorporation at abasic lesions, which will be distinguished from a “standard misalignment” model presented previously (20–22). We are concerned primarily with two issues germane to translesion synthesis. First, we address the degree to which pol β and pol α incorporate nucleotides via direct incorporation in compliance with the A-rule. Second, we determine the extent to which the polymerases utilize direct and misalignment synthetic mechanisms to catalyze lesion bypass.

### EXPERIMENTAL PROCEDURES

#### Materials

**Enzymes**—Human pol β was purified as described (23). The turnover number for pol β is from 0.5–0.8 nucleotides s⁻¹ when assayed using poly(dA)-oligo(dT) at 20 °C, pH 7.4. Human pol α (2000 units/mg, see Ref. 24), a generous gift of Dr. T. S. F. Wang, refers to the large catalytic activities. 1 DNA polymerase unit is defined as the incorporation of 1 pmol deoxynucleotide into an acceptor DNA molecule in 1 min at 37 °C on poly(dA)-oligo(dT) at 20°C, pH 7.4. Human pol β (25) was purchased from U. S. Biochemicals.

**Nucleotides**—Nonradioactive nucleotides were purchased from Pharmacia Biotech Inc. (γ⁻³²P)ATP (4000 Ci/mmol) was purchased from ICN Radiochemicals.

**Synthesis of DNA Primer-Templates**—Abasic (1,4-anhydro-2-deoxy-β-rribitol) phosphoramidite was synthesized as described previously (25). 42-mer templates containing a single abasic site and control templates with no abasic site were synthesized on an Applied Biosystems DNA/RNA synthesizer. Oligodeoxynucleotides with a 5'-phosphate group used to form gaps for pol β were synthesized by Lynn Williams at the University of Southern California Comprehensive Cancer Center.

**DNA Substrates**—For studies of insertion opposite an abasic site by pol β, a 5-nucleotide gap with an abasic site in its center was formed by annealing a 42-mer template, 5'-TTTC TAG GTT TGC TAA CAT ACT TCN XMA TAA GGA GTC TTA ATC-3', where the X designates the tetrahydrofuran abasic lesion; N and M are, respectively, the downstream and upstream nearest-neighbor bases to be varied (Fig. 1, a and b). The upstream oligonucleotide primer was 5'-GAT TAA GAC TCC TTA-3', and the downstream oligonucleotides used to form a 5'-nucleotide gap for pol β was 5'-AAG TAT GTC AAA CCA CCT AAG-3'. The downstream oligodeoxyribonucleotide was phosphorylated at its 5'-end (Fig. 1). The 5'-phosphate group on the downstream oligonucleotide and a guanine base, as a function of the target dNTP substrate concentration results in a rectangular hyperbola whose slope in the linear region is given by apparent $V_m/K_m$. Apparent $K_m$ and relative $V_m$ values were obtained using a nonlinear least squares fit to the rectangular hyperbola. In p/t DNA sequence contexts where incorporation and extension were relatively inefficient, plots of $I_p/I_q$, versus dNTP concentration showed little or no curvature; in these cases, ap...
parent \( V_{\text{max}}/K_m \) values were obtained by a least squares fit of the data to a straight line. The same analysis was also used to calculate apparent kinetic constants for extension beyond an abasic site (following subsection). Based on a series of time course measurements, kinetic data were obtained using 30-min incubations. The data for both enzymes were in the linear range for incorporation and extension at the abasic lesions for each of the p/t DNA constructs, with the possible exception of dAMP incorporation opposite X by pol α. We note, however, that the relative efficiencies of incorporation of dAMP compared to dGMP opposite X are consistent with previous measurements using pol α to copy a tetrahydrofuran moiety (9, 10).

**Extension Past an Abasic Site**—Reactions were carried out as described above except that the abasic site was covered by the upstream primer with A, C, G, or T (see, e.g. Fig. 1c). These reactions were carried out using standing-start conditions. The reactions were run in the presence of only one nucleotide, either the one complementary to the first base downstream of X or to the second nucleotide downstream of the lesion. The concentration of dNTPs was varied between 10 and 500 μM, and the reactions were run for 30 min. The extension efficiency, \( V_{\text{max}}/K_m \), is proportional to \( (l_{i}X/IX-1) \times 1/t \), where \( l_{i}X/IX-1 \) is the integrated band intensity for band(s) extended beyond the abasic lesion, \( l_{i} \) is the integrated band intensity of the unextended primer bound opposite the lesion, and \( t \) is the reaction time. The proportionality constant is the same for incorporation of A, T, G, or C opposite X in all sequence contexts and so a comparison of the ratio \( l_{i}X/IX-1 \), for each nucleotide provides a direct measure of the relative extension efficiencies. In cases where \( l_{i} \) is not \( >l_{i}X-1 \), then \( l_{i} \) is replaced in the equation for \( l_{i}X/IX-1 \) by \( 0.5 l_{i}X-1 \), to correct for the change in concentration of input p/t DNA during the course of the reaction (30).

**Equilibrium Binding Constants for Pol β and Pol α to p/t DNA**—Apparent equilibrium dissociation constants were determined for binding of pol β or pol α to the p/t DNA used in the extension assays as described in Ref. 31.

**RESULTS**

To investigate the specificity of nucleotide incorporation opposite abasic template sites using eucaryotic pol β and pol α, template DNAs were synthesized with a site-directed model (tetrahydrofuran) abasic lesion (25) placed adjacent to each of the four different upstream and downstream nearest-neighbor bases (Fig. 1). The abasic lesion is denoted by the symbol X. Steady-state kinetic measurements were performed to determine nucleotide incorporation efficiencies opposite X (9, 32) and efficiencies of extension downstream from X (30, 32, 33).

**Nucleotide Incorporation Efficiencies at a Site-directed Abasic Lesion as a Function of Downstream Sequence Context**—Nucleotide incorporation kinetics for pol β and pol α were measured in running-start reactions, by varying the downstream base adjacent to the lesion. In the running-start reaction, two Ts are incorporated prior to encountering an abasic lesion, followed by incorporation of dNMPs A, C, G, or T, at different concentrations, opposite the lesion, X. Representative kinetic data are presented in which G is the base immediately downstream from the lesion (Fig. 2). The data illustrate that pol β incorporated all four nucleotides opposite X, favoring misalignment incorporation of dCMP which is complementary to the template base to the 5’-side of the lesion (Fig. 2a, dCTP lanes). In contrast, pol α strongly favored incorporation of dAMP and to a lesser extent dGMP (Fig. 2b). Pol α also catalyzed incorporation of dCMP, but with exceedingly low efficiency (Fig. 2b, dCTP lanes).

Two distinct mechanisms are responsible for the incorporation of C in this p/t configuration: (i) direct incorporation of dCMP opposite X and (ii) misalignment incorporation of dCMP opposite the downstream template G. Note that misalignment synthesis, where the incoming dNTP stabilizes a misaligned p/t structure, has not been reported previously. We refer to this novel type of synthesis as “dNTP-stabilized” misalignment synthesis to distinguish it from the standard “Streisinger” misalignment synthesis (20, 21, 34, 35) in which the p/t DNA forms a transiently misaligned structure (Fig. 3).

Time courses, covering a range from 2 to 60 min, were performed to measure nucleotide incorporation opposite X with the same p/t sequence used for the kinetic analysis shown in Fig. 2 (data not shown). The time course data were consistent with the kinetic data, i.e. the same polymerase-specific differences in the abilities of the two human polymerases to incorporate nucleotides opposite an abasic lesion were observed at each reaction time point. Pol α behaved in accordance with the A-rule since it readily incorporated dAMP, and less readily dGMP, opposite the lesion, whereas incorporation of pyrimidines opposite X was barely detectable, even following a lengthy (60 min) reaction period (data not shown). Pol β did not follow the A-rule since it incorporated all four dNMPs opposite X in a relatively impartial manner at each time point (data not shown).

Analysis of the data for each of the four downstream nearest-neighbors demonstrates pol β-catalyzed incorporations were most efficient when the downstream template base was complementary to the incoming dNTP substrate (Fig. 4a, Vₘₐₓ/Kₘ values indicated by arrows pointing downward along the diagonal). The large apparent Vₘₐₓ/Kₘ values along the diagonal suggest that a major fraction of misincorporations taking place opposite the lesion are actually “correct” incorporations in which an incoming dNTP is positioned opposite its complementary template base immediately downstream from the lesion. Except in the case of a downstream template A, misincorporations by pol β are dominated by this mechanism. Nucleotide misincorporations caused by p/t misalignments, as documented by Kunkel and co-workers (20, 21), contain a “looped out,” i.e. extrahelical base (in this case a looped out abasic moiety) stabilized by H-bonded base pairs surrounding the looped out
However, the misalignment incorporations found for pol β in running-start reactions (Fig. 4a) are novel because there is no base pair available downstream from the lesion to stabilize a putatively looped-out X. Instead, stabilization of the misaligned structure occurs when a dNTP complementary to the template base downstream from X binds in the active site of pol β.

The incorporation pattern for pol α (Fig. 4b) differs from that of pol β. The largest apparent \( \frac{V_{\text{max}}}{K_m} \) values for pol α correspond to incorporations of dAMP opposite X. Incorporations of dGMP opposite X were 5-12-fold lower in efficiency compared to dAMP. Incorporation of pyrimidine nucleotides opposite X occurred with lowest efficiencies. This trend is in accordance with A-rule behavior (8) for incorporation at abasic lesions in vitro (3, 9, 10). The lack of correlation between the magnitudes of \( \frac{V_{\text{max}}}{K_m} \) and the presence of a template base downstream from the lesion complementary to an incoming dNTP suggests that pol α acts principally by incorporating nucleotides directly opposite X, and not by dNTP-stabilized misalignment.

Comparison of Pol β and Pol α for Conformation to the A-rule for Synthesis at Abasic Lesions in the Direct Incorporation Mode—The 12 “off-diagonal” \( \frac{V_{\text{max}}}{K_m} \) values for pol β are dominated by direct incorporations occurring opposite the lesion (values that do not fall on the diagonal designated by the arrows, Fig. 4a). A synopsis of the 12 direct incorporation \( \frac{V_{\text{max}}}{K_m} \) values suggests that pol β incorporates nucleotides in a less biased manner, in comparison to pol α which favors incorporation of dAMP, and to a lesser extent dGMP. We conclude that, in relation to the incorporation of dAMP, pol β incorporates pyrimidine nucleotides in a more unbiased manner than pol α. The data further suggest that, when copying identical sequence contexts in the direct incorporation mode, pol β may incorporate pyrimidine deoxynucleotides with higher absolute efficiencies (\( \frac{V_{\text{max}}}{K_m} \) values) than pol α (Fig. 4), but this point has not been conclusively demonstrated.

The presence of an abasic lesion caused a strong reduction in incorporation efficiencies for both polymerases, typically by factors of between 10^1- and 10^5-fold compared to normal template sites. \( \frac{V_{\text{max}}}{K_m} \) values for dNMP incorporation at normal template sites (where X is replaced by a normal template base) were approximately 10^5 M^-1 to 10^6 M^-1 using pol β on gapped p/t DNA; pol β activity on ungapped p/t DNA was very low (data not shown). Corresponding measurements with pol α resulted in \( \frac{V_{\text{max}}}{K_m} \) values on the order of 10^5 M^-1, with activities roughly 2-fold higher on ungapped DNA (data not shown).

The reduction in incorporation and extension efficiencies at abasic template sites compared to normal sites reflects a strong kinetic block, i.e., an impediment in synthesis where reduction is not caused by the inability of pol β to bind in the vicinity of X; we found that the apparent equilibrium binding constants...
for both enzymes were not altered significantly in the presence of an abasic lesion (data not shown).

**Nucleotide Extension Efficiencies at a Site-directed Abasic Lesion as a Function of Sequence Context**—Nucleotide extension kinetics for pol β and pol α were measured in a standing-start reaction by varying the concentration of dNTP substrates targeted for incorporation opposite a complementary template base either one base downstream from the lesion site, corresponding to direct extension, or two bases downstream from X, corresponding to misalignment extension, in accordance with a standard misalignment structure (Fig. 3 and Fig. 5). Note that in the extension reaction, the primer-3'-terminal base covers the lesion (Fig. 1c).

Pol β extends past an abasic lesion using misaligned primer termini by incorporating the dNMP complementary to the template base on the 5'-side of X. The 12 apparent $V_{\text{max}}/K_m$ values off the diagonal, shown in panel a, correspond to incorporations catalyzed by pol β directly opposite X. Template sequences are shown to the left of the histograms.

A synopsis of extension efficiencies for direct and standard misalignment synthetic modes is shown in Fig. 6. Extensions past abasic lesions for pol β occurred almost exclusively from misaligned primer termini (Fig. 6a). Misalignment extension for pol α was strongly favored from dAMP$X$, dGMP$X$ primer termini (Fig. 6b). An important additional feature of pol α extension is that efficient direct extension also occurred for primers terminating in A while less efficient direct extension

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**FIG. 4.** Comparison of pol β and pol α nucleotide incorporation efficiencies opposite an abasic lesion, X, with different 5'-nearest neighbors. Incorporation efficiencies were calculated as described under “Experimental Procedures” for: a, pol β; and b, pol α. Each value shown is the average incorporation efficiency (apparent $V_{\text{max}}/K_m$) ± S.E. The four apparent $V_{\text{max}}/K_m$ values indicated by arrows pointing along the diagonal (left-hand panel) correspond to incorporations catalyzed by pol β from misaligned primer termini with dNTP substrates complementary to the template base on the 5'-side of X. The 12 apparent $V_{\text{max}}/K_m$ values off the diagonal, shown in panel a, correspond to incorporations catalyzed by pol β directly opposite X. Template sequences are shown to the left of the histograms.
Effects were observed for pol α, misalignment incorporation (Fig. 7a) from the lesion in the four p/t configurations, via dNTP-stabilized misalignment extension, using the structure indicated by a looped-out X, is strongly favored over direct extension for pol β when extending primers terminating with either C (panel a) or A (panel b). c, extension of a primer terminating with C is catalyzed with relatively low efficiency by pol α, with misalignment extension favored over direct extension; d, extension of a primer terminating with A is catalyzed with relatively high efficiency by pol α, with misalignment extension favored over direct extension. For misalignment extension, G(dGMP) is incorporated at a downstream template site adjacent to X. Reactions were run for 30 min. Template sequences are shown to the left of the gels.

For pol β, an unexpected finding was the remarkably strong effects caused by changing just one upstream base. Incorporation opposite X occurred much more efficiently on the template 3’-... ACXCC... compared to 3’-... ATXCC...; the incorporation efficiencies were greater by factors of 10-fold, 9-fold, 16-fold, and 2-fold for A, C, G, and T, respectively. We verified that there were no significant differences in primer utilization for any of the DNAs, ruling out the possibility that the large upstream nearest-neighbor effects on incorporation opposite the lesion could be caused by differential utilization of the four p/t DNAs (data not shown).

Lesion Bypass Occurs with a much Higher Efficiency When Pol β Incorporates a Nucleotide Opposite X and Then Extends It Compared to Extension of a Pre-existing Primer Terminating Opposite X—Pol β failed to incorporate the next correct nucleotide, dCMP opposite G (adjacent to X), when the 3’-primer terminus was placed directly opposite the abasic lesion (Fig. 5a, left-hand gel). In contrast, pol β was able to incorporate dCMP readily opposite the same neighboring template G in a running-start reaction (Fig. 5a, dCTP lanes). Thus, there appears to be a significant difference in lesion bypass efficiency when pol β initially incorporates a nucleotide opposite X in contrast to extension from a pre-existing primer terminating opposite X. We have made similar observations using other p/t DNA configurations (data not shown).

DNA Products Resulting from dNTP-stabilized Misalignment Synthesis by Pol β Lead to Both One Base Deletion and Base Substitution Errors—An analysis of product lengths in the gap-filling reaction for pol β reveals that one base deletions and base substitutions can result from lesion bypass caused by dNTP-stabilized misalignment. Extension of 32P-labeled primer bands by addition of 1–5 nt were observed as discrete gel bands when pol β copied the gapped templates, 3’-AACGC (Fig. 8a) and 3’-AAKCC (Fig. 8b). Lesion bypass occurred when either 4 or 5 nt were added during the extension reaction. The addition of 4 nt on a misaligned template (X out of the helical plane) would result in a 1-nt deletion, while the addition of 5 nt...
on a realigned template (X in the helical plane) would result in a base substitution.

Doublet bands were observed at the template G position when dGTP and dCTP were present in the reaction (Fig. 8a, lanes 2–4, lower two arrows). The less intense lower band of the doublet migrated in the same position as a primer band extended in the presence of dCTP but in the absence of dGTP (Fig. 8a, lane 1); thus, the lower doublet band (Fig. 8, lower arrow) was probably formed by the following steps: (i) incorporation dCMP opposite G on a misaligned template, (ii) realignment of the template strand (placing dCMP opposite X), (iii) incorporation of a second dCMP opposite G, (iv) dissociation of pol β, leaving a partially filled gap. The upper band of the doublet is much more intense than the lower band. This difference in intensities is consistent with the idea that a misaligned structure containing consecutive C:G and G:C base pairs downstream from the putative looped-out abasic moiety.

The origin of the primer extension bands for the 3'-AAXGC template can be inferred from the dNTP-stabilized misalignment model (Fig. 3). Formation of the relatively intense upper doublet band (Fig. 8, middle arrow) is likely to have occurred by incorporation of dCMP opposite G on a misaligned template (X looped out of the helix) followed by incorporation of dGMP opposite C using the same misaligned template. We suggest that the p/t structure responsible for generating the upper band would lead to a −1 nt deletion. This structure is stabilized by the presence of C:G and G:C base pairs downstream from the putative looped-out abasic moiety.

The lower doublet band (Fig. 8, lower arrow) was probably formed by the following steps: (i) incorporation dCMP opposite G on a misaligned template, (ii) realignment of the template strand (placing dCMP opposite X), (iii) incorporation of a second dCMP opposite G, (iv) dissociation of pol α, leaving a partially filled gap. The upper band of the doublet is much more intense than the lower band. This difference in intensities is consistent with the idea that a misaligned structure containing consecutive C:G and G:C base pairs, downstream from the looped-out lesion, should be considerably more stable than a realigned structure containing C opposite X followed by a single C:G base pair. NMR measurements showed that both C and X were located extrahelically when they were located opposite one another in double-stranded DNA (13).

The band running above the doublet opposite C (Fig. 8, upper arrow) corresponds to extension of the primer by 5 nt, to com-
pletely fill the gap. Therefore, template realignment can occur, allowing incorporation of dGMP opposite C, in spite of the reduced stability of the realigned structure relative to the misaligned structure. The presence of a band opposite C implies that base substitutions can result from dNTP-stabilized misalignment, although they would be much less likely to occur than base deletions (Fig. 8, middle arrow).

Further insight into dNTP-stabilized misalignment pathways can be arrived at by investigating the dependence of band intensities on dGTP concentration. As dGTP is increased relative to dCTP, the lower doublet band decreases in intensity (Fig. 8a, lanes 2–5). Incorporation of dCMP occurs efficiently opposite G with X out of the plane of the helix. As the concentration of dGTP is increased relative to dCTP, it becomes much easier to incorporate dGMP opposite C, from a misaligned template, than to incorporate a second dCMP opposite G from a realigned template. In other words, at high dGTP/dCTP ratios the template does not appear to have sufficient time to realign prior to incorporation of dGMP opposite C.

An analysis of primer extension products using a 3'-AAxXCC template demonstrates that template realignment can occur even if the misaligned structure is stabilized by two downstream G:C base pairs (Fig. 8b, lanes 1 and 2). Primer extension corresponding to the addition of dGMP opposite C (with X out of the helical plane), followed by addition of dGMP opposite C

Fig. 7. Comparison of pol β and pol α nucleotide incorporation efficiencies opposite an abasic lesion, X, with different 3′-nearest neighbors. Incorporation efficiencies for A, C, G, and T opposite X were calculated as described under “Experimental Procedures” for a, pol β, and b, pol α. Each value shown is the average incorporation efficiency (apparent $V_{\text{max}}/K_m$) ± S.E. Template sequences are shown to the left of the histograms. The designation n.d. refers to nucleotide incorporation reactions that were not detectable.
on the misaligned template, gives rise to a −1 base frameshift at low dGTP (100 μM) concentrations (Fig. 8b, lane 1). At a higher dGTP concentration (600 μM), both base substitutions and −1 frameshifts occur (Fig. 8b, lane 2). The ability to fill the 5-nt gap at high dGTP concentration suggests that the template can undergo realignment (placing C opposite X), even though template realignment is far less favorable than realignment of the misaligned template structure having two G:C base pairs downstream from a looped-out X. The ability to fill in the 5-nt gap completely, at a high dGTP concentration (Fig. 8b, lane 2), suggests that base substitutions can occur even though template realignment is a kinetically unfavorable process.

The data also show that pol β is processive in synthesizing past the lesion provided that the misaligned template does not realign. Pol β tends to dissociate following template realignment; a discrete extension band is observed opposite X (Fig. 8a, lanes 2–4). However, the band corresponding to insertion opposite X is absent (Fig. 8a, lanes 5 and 6) when the template is misaligned; pol β does not dissociate from the template when adding dCMP opposite G followed by dGMP opposite C in the dNTP-stabilized misalignment mode. Note, however, that two barely detectable doublet bands can be seen opposite X in lanes 5 and 6. These bands arise from inefficient incorporation of either dGMP or dCMP directly opposite the lesion, after which pol β dissociates.

**DISCUSSION**

It has been proposed that the A-rule (8), defined as the preferential incorporation of A at abasic DNA template lesions, serves as a DNA polymerase default mechanism allowing bypass of noncoding lesions encountered at a replication fork. Previous studies showed that polymerases purified from eucaryotic and procaryotic cells behave in accordance with the A-rule, preferentially incorporating A opposite abasic lesions with about a factor of 10 higher efficiency than G and with about a 50-fold greater efficiency than either C or T (9, 10).

However, recent in vivo experiments suggest that the A-rule may not be operative at abasic sites in eucaryotes. Data from eucaryotic systems demonstrate that incorporation can be random (14, 15), or that G (16, 17), T (18), and C (19) can be favored, depending on the particular system studied. The eucaryotic results raise the interesting possibility that perhaps one or more eucaryotic polymerases exhibit a broad specificity range for incorporation at abasic sites. We have tested this possibility by measuring the efficiency of nucleotide incorporation at abasic lesion sites using pol β, a repair polymerase found only in eucaryotic organisms.

Kinetic determinations of nucleotide incorporation efficiencies were performed at site-directed abasic lesions (X) placed next to four different downstream template bases, for each of the four dNTP substrates (Fig. 4). Nucleotide incorporation takes place directly opposite X in 12 of the 16 dNTP-p/Δ configurations. In the four configurations, where a dNTP substrate is complementary to a template base immediately downstream from the lesion, incorporation can occur either directly opposite X on a properly aligned primer, or opposite a downstream template base on a misaligned primer. Side-by-side comparisons were made for pol β and pol α using identical p/Δ DNA constructs.

Two definitive differences in the properties of pol β and pol α were found for nucleotide incorporation at abasic lesions. First, pol β does not exhibit A-rule behavior while pol α does (Figs. 2 and 4). Second, pol β incorporates nucleotides with higher efficiency, using misaligned primer-3′-termini (data indicated by arrows along the diagonal, Fig. 4a), while pol α does not (Fig. 4b). The misalignment mechanism for pol β is different from that previously observed (20–22, 36, 37) because it does not require the presence of H-bonded structures surrounding the extrahelical moiety as postulated for the standard misalignment model, shown in Fig. 3. Instead, the presence of an incoming dNTP complementary to the template base downstream from the lesion is sufficient to cause misalignment incorporation to take place; we refer to this novel type of misincorporation as “dNTP-stabilized misalignment” incorporation (Fig. 3). We have also shown that this novel mode of misalignment incorporation for pol β favors the occurrence of −1 frameshifts but can also give rise to base substitutions (Fig. 8). It has previously been shown that pol β catalyzes −1 frameshifts and base substitutions by the standard Streisinger slipped mechanism (20).

**Pol α Behaves in Accordance with the A-Rule, but Pol β Does Not**—The 12 apparent Vmax/Km values off the diagonal correspond to pol β incorporations occurring directly opposite the lesion, i.e. not via p/Δ DNA misalignment synthesis (Fig. 4a).

With A downstream from X, direct incorporation of G opposite X was favored slightly over A and C; with C downstream, A was favored by roughly 7-fold over C and T; with G downstream, A and T were almost equivalent and favored by about 2-fold over G; with T downstream, C and G are roughly equivalent and favored by about 2-fold over T. Thus, pol β appears to operate in a robust and less than partial manner than pol α with respect to direct incorporation opposite abasic template lesions. The properties of pol α are obviously different because A was favored over G for incorporation opposite X by factors ranging
from 5–12-fold, for all p/t configurations, while incorporation of the pyrimidine nucleotides was far less efficient.

A molecular basis to rationalize the A-rule stems from NMR studies showing that A is stacked within the plane of the DNA helix (11–13). G is intrahelical at low temperatures but is melted prior to denaturation of the helix, and that C and probably T are extrahelical (13). It is possible that base stacking interactions between incoming dNTPs and primer-3’-termini differ significantly for pol β in relation to other polymerases that have been investigated, e.g. pol α, human immunodeficiency virus-1 and avian myeloblastosis virus reverse transcriptase (32). The properties of pol β concerning untemplated incorporation at abasic template sites on physiologically relevant p/t configurations used here are also in contrast to the A-rule like results observed for untemplated end-addition reactions, noted both in solution (38) and in crystals of enzyme and blunt-ended DNA (39). In the end-addition reactions, base stacking interactions for the primer base and incoming base may be more important for stabilizing the transition state intermediate than in the case of abasic site untemplated synthesis on a primer-template. In the latter case, pol β-DNA interactions may impose a bend in the primer-template such that stacking between the primer base and incoming base is not critical to the free energy of stabilization of the transition state (40).

Lesion Bypass Occurs with Much Higher Efficiency in Running-start Compared to Standing-start Reactions—In a standing-start reaction, pol β appeared to have great difficulty adding a next correct dNTP onto pre-existing primers terminating opposite an abasic lesion (Fig. 5a). In contrast, in a running-start reaction, the enzyme had essentially no difficulty carrying out translesion synthesis by first incorporating a nucleotide using a misaligned primer-template and then using a realigned template to continue synthesis past the lesion by direct addition of the next correct dNMP (Fig. 2a, dCTP lanes). Although we have not attempted to carry out a systematic investigation using a large number of p/t DNA sequences, this observation does not appear to be restricted to specific sequence contexts.

Conceivably, pol β may assume different conformations depending on whether it binds at a pre-existing primer terminus opposite a lesion or is bound at the identical site just after having inserted a nucleotide opposite X. The difficulty of pol β to extend a pre-existing primer by addition of a dNMP complementary to the template base immediately downstream from the lesion, even after a 30-min incubation (Figs. 5a and b, left-hand gels), suggests that the enzyme is inhibited in forming a kinetically competent complex when bound opposite a lesion on a properly aligned primer terminus. However, when pol β was bound to a misaligned primer (indicated in Fig. 5a, and b, by the looped-out X shown out of the helical plane, right-hand gels), the enzyme was easily able to add the next correct dNMP (two bases downstream from the lesion. The underlying reasons for these interesting kinetic differences are not known and merit further study. These marked differences in standing-start compared to running-start kinetics can be exploited in delineating rate-limiting steps in the nucleotide incorporation pathway for pol β.

Another interesting aspect of the data shown in Fig. 5 is that pol β extends past an abasic lesion using misaligned primer termini by incorporating the dNMP complementary to the template site two bases downstream from X (Fig. 5a and b, right-hand gels). Following realignment of the template, dGMP which had been correctly incorporated opposite C, two bases downstream from X, is then relocated opposite G to form a dGMP-G mispair (Fig. 5a), or opposite T to form a dGMP-T mispair (Fig. 5b). This mechanism, strongly favored by pol β...
shown to alter the relative frequencies of mutations at cyclobutane dimers in vivo (51). However, there is as yet no evidence for the presence of UmuD C-like translesion bypass proteins in eucaryotic cells. The yeast REV 1 protein has recently been shown to alter the relative frequencies of mutations at cyclobutane dimers in a generally impartial manner. We show that the relaxed incorporation specificity of pol b copy abasic lesions in a generally impartial manner. We show that the relaxed incorporation specificity of pol b copy abasic lesions in a generally impartial manner. We show that the relaxed incorporation specificity of pol b copy abasic lesions in a generally impartial manner.

When allowance for each of these caveats is made, the salient point remains that pol β is the first example of an enzyme able to copy abasic lesions in a generally impartial manner. We suggest that the relaxed incorporation specificity of pol β provides a plausible biological mechanism to account for the lack of an A-rule in eucaryotes.

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