trans-Lesion Synthesis Past Bulky Benzo[a]pyrene Diol Epoxide N²-dG and N⁶-dA Lesions Catalyzed by DNA Bypass Polymerases*

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The effectiveness of in vitro primer elongation reactions catalyzed by human bypass DNA polymerases κ (hDinB1), pol η (hRad30A), pol ι (hRad30B), and yeast pol ζ (Rev3 and Rev7) in site-specifically modified template oligonucleotide strands were studied in vitro. The templates contained single bulky lesions derived from the trans-addition of the mutagenic (+)- or (−)-enantiomers of r7,8-dihydroxy-r9,10-epoxy-7,8,9,10-tetrahydrobenzo[a]pyrene (a metabolite of the environmental carcinogen benzo[a]pyrene), to the exocyclic amino groups of guanine or adenine in oligonucleotide templates 33, or more, bases long. In “running start” primer extension reactions, pol κ effectively bypassed both the stereoisomeric (+)- and (−)-trans-guanine adducts but not the analogous adenine adducts. In sharp contrast, pol η, which exhibits considerable sequence homology with pol κ (both belong to the group Y family polymerases), is partially blocked by the guanine adducts and the (−)-trans-adenine adduct, although the stereoisomeric (+)-trans-adenine adduct is more successfully bypassed. Neither pol ι nor pol ζ, either alone or in combination, were effective in trans-lesion synthesis past the same adducts. In all cases, the fidelity of insertion is dependent on adduct stereochemistry and structure. Generally, error-free nucleotide insertion opposite the lesions tends to depend more on adduct stereochemistry than error-prone insertion. None of the polymerases tested are a universal bypass polymerase for the stereoisomeric bulky polycyclic aromatic hydrocarbon-DNA adducts derived from anti-BPDE.

Several new prokaryotic and eukaryotic DNA polymerases (pol)1 involved in trans-lesional synthesis have been recently identified (reviewed in Refs. 1–5). The functional characteristics of the UmuC/DinB/Rev1p/Rad30 DNA polymerases belong-

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‡ The abbreviations used are: pol, polymerase; BP, benzo[a]pyrene; anti-BPDE, r7,8-dihydroxy-r9,10-epoxy-7,8,9,10-tetrahydrobenzo[a]pyrene; 8-oxo-dG, 7,8-dihydroxy-8-oxo-2-deoxyguanosine; pol κ, human DNA pol κ (hDinB1); pol η, human DNA pol η (hRad30A); pol ι, human DNA pol ι (hRad30B); pol ζ, yeast DNA pol ζ (Rev3 and Rev7 proteins); [BP]-N²-dG or [BP]-N⁶-dA, adducts derived from the binding of anti-BPDE to the exocyclic amino groups of guanine and adenine, respectively.

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To Yeast pol ζ the catalytic subunit of the Rev3 and Rev7 proteins is also added, the mispairs opposite the thymine-thymine dimers are successfully extended to generate full bypass products.

The structural features of bulky lesions derived from the binding of (+)- or (−)-anti-BPDE to N²-dG or N⁶-dA (Scheme 1) have been extensively studied (37–39). Such lesions are strong blocks of DNA replication catalyzed by a variety of classical
trans-Lesion Synthesis Past [BP]-N²-dG and [BP]-N⁶-dA Lesions

Effectiveness of trans-Lesion Synthesis on Templates with [BP]-N²-dG Lesions—We employed “running start” assays under multiple hit conditions to evaluate trans-lesion bypass utilizing the template-primer complexes shown in Scheme 2. In the case of unmodified templates (Fig. 1A), pol κ was capable of extending the 17-mer primer strands to 31- and 32-mer rather than to fully extended 33-mer primer DNA concentrations were 10 nM in solutions of 25 mM potassium phosphate buffer (pH 7.0), 5 mM MgCl₂, 5 mM dithiothreitol, 100 µM/mL bovine serum albumin, 10% glycerol, 100 µM of each of the four dNTPs. The concentrations of DNA polymerases were, unless noted otherwise, 3.3 nM for pol κ, 5.5 nM for pol η, 1 nM in the case of pol ζ, and 20 nM in the case of pol μ. The reactions were quenched by 12 µl of denaturing loading buffer (95% formamide with 20 mM EDTA, 45 mM Tris borate, 0.1% bromophenol blue, and 0.1% xylene cyanol). The reaction products were resolved on a 20% polyacrylamide gel in the presence of 7M urea by guest on July 18, 2018http://www.jbc.org/Downloaded from

RESULTS

Effectiveness of trans-Lesion Synthesis on Templates with [BP]-N²-dG Lesions—We employed “running start” assays under multiple hit conditions to evaluate trans-lesion bypass utilizing the template-primer complexes shown in Scheme 2. In the case of unmodified templates (Fig. 1A), pol κ was capable of extending the 17-mer primer strands to 31- and 32-mer rather than to fully extended 33-mer primers, in agreement with the general observations of others (7, 9, 57–59). pol κ efficiently bypassed not only the bulky (+)-trans-[BP]-N²-dG adduct (12) but also the stereoisomeric (+)-trans-adducts, producing al-
most the same patterns of elongated primer strands as on the unmodified template. The polymerase stalled at the −1 site (Scheme 2) in both cases.

Human pol η is also a distributive DNA polymerase but is capable of elongating primer strands up to the end of the unmodified template (Fig. 1B), as was reported previously (6, 33). However, pol η was inhibited by both (+)-trans- and (−)-trans-[BP]-N²-dG adducts and slowly incorporated nucleotides opposite the lesion. Weak 30–33-mer primer bands were observed due to further extension beyond the (+)-trans- or (−)-trans-adducts.

As shown in Fig. 2A, in the case of the unmodified template, human pol ι extends the 17-mer primer by 7 bases and stalls opposite the T in the template at position 24 (Scheme 2). Extension by human pol ι is known to stop often at template T residues (19) with a preferential incorporation of a G base opposite a template T (19, 60). In the case of the BPDE-modified template, pol ι stalled at position −1, 1 base before the (+)-trans-adduct (Fig. 2A) or the (−)-trans-[BP]-N²-dG adduct (data not shown). Some weak base incorporation opposite the lesion as well as beyond the lesion was also observed (−2%). The primer strand was fully extended by yeast pol ζ in the case of unmodified DNA. However, pol ζ was almost completely blocked by the (+)-trans- (Fig. 2B) and the (−)-trans-[BP]-N²-dG adduct (data not shown). The combined activities of pol ι and pol ζ did not significantly enhance primer elongation on either the (+)-trans- (Fig. 2C) or the (−)-trans-[BP]-N²-dG adduct-modified templates (data not shown).

Base Incorporation Specificity Opposite (+)-trans- and (−)-trans-[BP]-N²-dG Lesion Sites—To examine the specificity of the incorporation opposite the lesion site, we employed a standing start assay based on the insertion, opposite the lesion, of a single nucleotide at a time (Fig. 3A) using a 19-mer primer strand (Scheme 2). pol κ incorporated mostly, although not exclusively, the correct C base opposite the (+)-trans- and the (−)-trans-[BP]-N²-dG adducts, as reported for the (−)-trans-adducts (12). The incorporation of C opposite both the (+)-trans- and the (−)-trans-[BP]-N²-dG lesion was dominant. Similar observations were reported for pol (κΔC), a truncated form of pol κ (29). pol ι is error-prone and preferentially incorporates G opposite (+)-trans-adduct, whereas both A and G are incorporated more weakly opposite the (−)-trans-[BP]-N²-dG lesion under identical experimental conditions (Fig. 3B). pol η is different from pol κ and pol ι because it tended to incorporate all 4 bases opposite both lesions in a rather indiscriminate manner (Fig. 3B). Furthermore, this polymerase is unusual because it added 1 additional base to the primer opposite the +1 template position (Fig. 3A). The detailed kinetics of single dNTP incorporation kinetics have been recently published for the [BP]-N²-dG adducts but in different sequence contexts (35). Although potential sequence-dependent differences will need to be explored, the efficiencies of incorporation of the different nucleotides opposite the two trans-[BP]-N²-dG adducts were found to be error-prone as well, and thus consistent with our results (Fig. 3).

Effectiveness of trans-Lesion Synthesis Past [BP]-N⁶-dA Lesions—Human pol κ was completely blocked by the (+)-trans- and (−)-trans-[BP]-N⁶-dA adducts (Fig. 4A). pol κ stopped just before the lesion site allowing for only a minor extent of base incorporation opposite the adduct after a 30-min reaction time. No further trans-lesion bypass of these adenine adducts was detected (Fig. 4A). In contrast, human pol η can successfully bypass the (+)-trans-[BP]-N⁶-dA adduct, although a prominent partial stall site was observed at the 0 template site, opposite the lesion (Fig. 4B). The extent of full bypass reached ~30% in the case of the (+)-trans-[BP]-N⁶-dA adduct after a 60-min reaction time. However, under the same reaction conditions, the primer was extended only up to the lesion site in the case of the stereoisomeric (−)-trans-[BP]-N⁶-dA adduct, and further extension beyond the lesion site was ineffective (Fig. 4B). Human pol ι extended the primer strands on unmodified templates by only a few bases (Fig. 5A). Furthermore, this polymerase stalled completely at the modified adenine base. In the case of the (+)-trans adduct, pol ι was strongly blocked just before the lesion site, and analysis of the PhosphorImager data (not shown) indicated that only ~10% of the primer was extended by the insertion of one dNTP opposite the lesion. In contrast, insertion opposite the lesion was more efficient (~75% under the same conditions) in the case of the (−)-trans-[BP]-N⁶-dA adduct. No further elongation was detected in the
case of either stereoisomeric adduct. However, it should be noted that pol \( \kappa \) also partially stalled opposite the same unmodified adenine on the template strand (Fig. 5A).

Yeast pol \( \zeta \) was fully blocked by both [BP]-N^6-da adducts. The combined activity of pol \( \kappa \) and pol \( \zeta \) did not enhance primer elongation past the lesion sites as was observed in the case of the (6-4)thymine-thymine photoproduct and AP site (18, 26, 28, 29, 41, 42). In contrast, pol \( \kappa \) was unable to insert efficiently any of the four nucleotides opposite the (−)-trans-[BP]-N^6-da lesion (Fig. 4). Thus, with respect to the [BP]-N^6-da adducts, pol \( \kappa \) behaves like some of the classical polymerases in terms of their lack of ability to bypass these lesions. Only pol \( \kappa \) and pol \( \zeta \) exhibit bypass of any of the bulky [BP]-DNA lesions studied. However, the efficiencies of trans-lesion bypass depended remarkably on adduct stereochemistry and on the BPDE-modified purine residue (guanine or adenine).

Although pol \( \kappa \) pauses at the −1 positions (Scheme 2), both the (+)-trans- and (−)-trans-adducts are bypassed predominantly, but not exclusively, in an error-free manner in the . . . CG\*C . . . sequence context. Pol \( \kappa \) is thus remarkably different from classical polymerases such as the Klenow fragment exo− of pol I from \( E. \) coli, T7 exo−, pol \( \alpha \), and pol \( \beta \). In contrast, pol \( \kappa \) is unable to insert efficiently any of the four nucleotides opposite the [BP]-N^6-da adducts, and trans-lesion bypass is thus severely hindered (Figs. 4 and 6). Thus, with respect to the [BP]-N^6-da adducts, pol \( \kappa \) behaves like some of the classical polymerases (22, 24, 40).

The activity of human pol \( \eta \) tends to be opposite to that of pol \( \kappa \); pol \( \eta \) is severely inhibited by both [BP]-N^2-dG adducts but is capable of bypassing the (+)-trans-[BP]-N^6-da but not the (−)-trans-[BP]-N^6-da lesions (Fig. 4). The insertion of the correct T opposite the (+)-trans-adduct is dominant, whereas in the case of the (+)-trans-[BP]-N^6-da lesion when all four dNTPs were present (Fig. 4B), these results suggested that the error-free insertion opposite the lesion favors full bypass, whereas extension from mismatched base pairs at the polymerase active site was inhibited. The correct insertion of T opposite the adduct allowed for full bypass of the (+)-trans-adduct, but the preferred insertion of the incorrect A opposite the (−)-trans-lesion did not (Fig. 4B).

Both pol \( \kappa \) and yeast pol \( \zeta \) rather indiscriminately inserted all four dNTPs opposite the lesions, although there was a rather strong preference for the insertion of a T opposite the (−)-trans-[BP]-N^6-da lesion (Fig. 6).

**DISCUSSION**

*The Processing of Lesions by Different Polymerases*—In general, classical DNA polymerases stall just before the bulky [BP]-DNA lesions and, in the case of some although not all cases, incorporate mostly an incorrect nucleotide opposite the adduct, whereas further extension is severely inhibited (21, 22, 24–26, 28, 40, 61, 62). For example, in the case of KF−, extension beyond the lesion is more than 1 order of magnitude less efficient than insertion of any of the four dNTPs opposite the lesion site (21, 27, 28). Human pol \( \kappa \), yeast pol \( \zeta \), or the combined activities of both polymerases are not able to fully bypass any of the stereoisomeric [BP]-N^2-dG or [BP]-N^6-da lesions studied and thus resemble some of the classical polymerases in terms of their lack of ability to bypass these lesions. Only pol \( \kappa \) and pol \( \zeta \) exhibit bypass of any of the bulky [BP]-DNA lesions studied. However, the efficiencies of trans-lesion bypass depend remarkably on adduct stereochemistry and on the BPDE-modified purine residue (guanine or adenine).
of the (−)-trans-[BP]-N⁰-dA adduct, the trans-lesion bypass is error-prone (Fig. 6). The striking differences in the effectiveness of bypass of the (+) and (−)-trans-[BP]-N⁰-dA adducts is possibly the most pronounced difference in trans-lesion synthesis exhibited by a pair of stereoisomeric bulky adducts observed to date.

Overall, we have found significant variabilities in the processing of identical lesions by the different polymerases studied. Particularly striking is the efficient processing of the two trans-[BP]-N²-dG lesions by pol κ but not by pol η or pol ζ. The bypass of one of the trans-[BP]-N²-dG adducts by pol η, whereas pol κ and pol ζ are completely blocked by both adenine adducts, is also remarkable. Similarly, [BP]-N²-dG and [BP]-N⁰-dA adduct-dependent trans-lesion synthesis efficiencies in vitro catalyzed by the SOS-induced E. coli Y-family polymerases pol IV and pol V were observed by Shen et al. (45). Structure-based sequence alignments indicate that there are significant homologies between Y family polymerases. Clearly, the differences in the processing of these [BP]-DNA and other lesions are rooted in the differences in the specific amino acid residues that are critical to lesion bypass. Without ternary crystal structures with BPDE-modified purines at the polymerase-active sites, it is impossible to draw firm conclusions about structure-function relationships indicated by these fascinating differences. Nevertheless, based on our limited knowledge, it is useful to examine some of the factors that may play a role in the observed phenomena.

trans-[BP]-N²-dG Adduct Stereochemistry and Conformation—The NMR solution structures of a portion of the template strand studied (Scheme 2), the 5'-AAGGCTAACCATCC fragment, hybridized with primer strands with the primer terminal base extending to the 3'-base flanking G² (47), or extending to the lesion site G² (48), have been studied. With reference to Scheme 2, these are designated as the −1- and 0-complexes, respectively. In the case of the (−)-trans-adduct, the BP residue appears to be oriented toward the duplex region of the template-primer complex because the 3 bp on the 3'-side of the lesion are significantly perturbed (47). It is reasonable to assume, although not known with certainty, that the BP residue in the (−)-trans-adducts in the −1-complex is located in the minor groove region on the 3'-side of G², as it is in a full duplex (44). In the case of the (−)-trans-G² adduct in the −1-complex, the hydrophobic [BP] residue is stacked plane-to-plane with the 3'-terminal guanine residue on the primer strand, whereas the modified guanine is in a syn rather than in the usual anti conformation (47). However, in the 0-complex, the BP residue is oriented within the nascent minor groove and points into the downstream 5'-side of the modified template strand as in the full duplex (46); the modified G² residue assumes a normal anti conformation as in B DNA (48), and the modified guanine residue G² is in a Watson-Crick base pair alignment with the 3'-terminal C of the primer. These two kinds of conformations are structurally related because of the nearly identical values of the torsion angles of the two bonds in the [BP](C10)-N²-dG adduct linking the [BP] and purine moieties. These two conformations, designated as base-carcinogen stacked and minor-groove structures, can interconvert from one to the other by an adjustment in the glycosidic torsion angle and the phosphodiester backbone (37), but the carcinogen-base stacked conformation is heavily favored in the −1-complex (47).

The slower bypass of the lesion, as indicated by the greater accumulation of the 20-mer primer (position 0) in the case of
the (+)-trans-[BP]-N²-dG relative to the isomeric (−)-trans-adduct (Fig. 1A), suggests that this stereochemical effect may arise from a difference in the adduct conformations. In the case of the (−)-trans-adduct, the bulky BP residue points into the 3′-direction of the template, and further progress of the polymerase is less obstructed than in the case of the (+)-trans-adduct, with its BP residue pointing into the 5′-direction (48).

Bypass of trans-[BP]-N²-dG Adducts and Structures of Polymerases—Whereas the crystal structure of human pol κ is not yet available, crystal structures of the N-terminal catalytic fragment (63) and the full-length (64) Sulfolobus solfataricus (P1) Dbh protein have been published. The crystal structure of a ternary DNA-protein complex of another DinB ortholog from S. solfataricus P2 has been investigated recently (65). The crystal structure of the full-length Dpo4 enzyme in a ternary complex with DNA and dNTP revealed mostly nonspecific interactions between protein and the sugar and phosphate moieties of the DNA substrate, except at or near the replicating base pair site. Whereas these DnP polymerases resemble the basic structures of the classical polymerases with catalytic core subdomains resembling a right-handed palm, thumb, and fingers, there are some striking differences as well. In the Y polymerases, the thumb and finger regions are considerably smaller than in classical polymerases. The replicating base pair in Dpo4 is less restrained by local interactions with amino acid residues and is more solvent-accessible than in classical polymerases. It has been suggested that in bypass polymerases this rather open, less sterically hindered active site allows for a local conformational flexibility that facilitates lesion bypass (65). We hypothesize that the active site in human pol κ might thus facilitate interconversion between [BP]-N²-dG adduct conformations that block bypass to conformations that are favorable for dCTP insertion opposite the lesion. In classical polymerases, e.g. T7, the poor lesion bypass suggests that blocking conformations are dominant. Interconversion from one adduct conformation to another is likely to be considerably more inhibited because of crowding from nearby amino acid residues. Such residues, especially those of the two “O” and “O1” helices in the finger region of the classical A family polymerases (66), are in contact with replicating base pair and serve as a fidelity check by an induced fit mechanism (reviewed in Ref. 67). Perlow and Brody (68) studied conformations of a (+)-trans-[BP]-N²-dG lesion at the active site of T7 polymerase by molecular modeling and dynamic simulation techniques. The [BP] residue is positioned in the major groove with the modified guanine in a syn conformation, capable of forming a hydrogen-bonded structure with dATP, but not with dCTP, in agreement with experimental observations.²

Bypasses of trans-[BP]-N²-dA Lesions—In double-stranded DNA with the base pairs in Watson-Crick type conformations, the BP residues are attached to guanine from the minor groove side and from the major groove in the case of the adenine adducts. The BP residues are intercalated between adjacent base pairs of the 5′-side in the case of the (−)-trans-[BP]-N²-dA adducts (39) and on the 3′-side in the case of the (+)-trans-adducts (38). The orientations at primer-template junctions are not known however. trans-Lesion synthesis beyond the adenine adducts is completely blocked in the case of pol λ. With the exception of the (−)-trans-[BP]-N²-dA adduct that favors insertion of the correct dTTP, the insertion of dNTPs opposite the lesion by pol λ is error-prone and practically independent of adduct stereochemistry. In the case of pol η and pol λ, a strong effect of adduct stereochemistry is observed only for the insertion of the correct dTTP (Fig. 6). However, the error-free insertion of dTTP is favored opposite the lesion for the (+)-trans-[BP]-N²-dA adduct by pol η and for the (−)-trans-adduct by pol κ. Thus, there is no apparent pattern favoring one or the other stereoisomeric adduct with either R or S absolute configuration at the linkage site.

In contrast to the trans-[BP]-N²-dG lesions, both the (+)- and (−)-trans-[BP]-N²-dA adducts cause pol κ to stall almost completely 1 base before the modified adenine residue. In the Dpo4 ternary crystals, the little finger domain fits tightly into the major groove and thus enhances the binding of the DNA to the polymerase. It is interesting to speculate that the existence of an analogous crowded major groove motif near the active site in pol κ could prevent a proper alignment of the modified adenine residue for proper pairing with the incoming dNTP and thus inhibit trans-lesion bypass. pol η, unlike pol κ, is unable to bypass any of the two trans-[BP]-N²-dG adducts. However, the (+)-trans-[BP]-N²-dA lesion, but not the isomeric (−)-trans-adducts, are bypassed by pol η, although both lesions completely block trans-lesion synthesis catalyzed by pol κ.

Analysis of the crystal structure of a pol η homolog (69) suggests, as in the case of pol κ, the existence of a more open active site and the absence of the “O” and “O1” helices that are found in the finger regions of the classical A family polymerases. Sequence alignment and comparisons of the primary structures of Dpo4, hpol η, and hpol κ point to partial homologies in the basic thumb-palm-finger motif of these different Y family polymerases. In Dpo4, amino acids in the finger domain are in contact with the replicating template-dNTP base pair and play an important role in determining the fidelity of replication and bypass (65). The amino acid composition in this domain is quite different in human pol η and pol κ raising the possibility that such differences could account for the observed differences in trans-lesion synthesis and lesion bypass fidelity.

Conclusions—None of the bypass polymerases studied here, including human pol κ and pol η, can bypass both the trans-[BP]-N²-dA and the trans-[BP]-N²-dG adducts. The intrinsic properties of the adducts, i.e. the nature of the modified purines, as well as the primary (and probably secondary) structures of these Y family polymerases contribute to the observed remarkable diversities of [BP]-DNA adduct trans-lesion bypass efficiencies and fidelities. The structural basis of the differences is of significant and fundamental interest for reaching a deeper understanding of the strategies utilized by this family of polymerases for bypassing bulky lesions.

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