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Substrate spectrum of human excinuclease: Repair of abasic sites, methylated bases, mismatches, and bulky adducts

(xeroderma pigmentosum/DNA damage/A·G mismatch)

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ABSTRACT **Nucleotide-excision repair is the repair system for removing bulky lesions from DNA. Humans deficient in this repair pathway suffer from xeroderma pigmentosum (XP), a disease characterized by photodermatoses, including skin cancers. At the cellular level, XP patients fail to remove cyclobutane pyrimidine dimers and pyrimidine(6–4)pyrimidone photoproducts induced by UV light, as well as other bulky DNA lesions caused by various genotoxic agents. XP cells are not particularly sensitive to ionizing radiation or to alkylating agents that cause mostly nonbulky DNA lesions. Therefore, it has generally been assumed that the human nucleotide-excision repair enzyme (excinuclease) is specific for bulky adducts. To determine the substrate range of human excinuclease we used the highly sensitive excision assay and tested bulky adducts, synthetic apurinic/apyrimidinic sites, N^6-methyladenine, O^6-methylguanine, and mismatches as potential substrates. We found that all of these "lesions" were removed by human excinuclease, although with vastly different efficiencies.**

Damage to DNA is repaired by three general mechanisms: direct repair, base-excision repair, and nucleotide-excision repair (1, 2). In addition to these repair systems, which act on modified (damaged) DNA, there are repair systems that correct mismatches generated by replication and recombination and employ base- and nucleotide-excision enzyme systems entirely different from those used in repairing damaged DNA (see ref. 3).

In nucleotide-excision repair, an excinuclease system made up of multiple subunits removes bulky adducts from DNA in an oligonucleotide. Cells from xeroderma pigmentosum (XP) patients that are deficient in nucleotide-excision repair are not particularly sensitive to methylating agents or ionizing radiation, which produce mostly nonbulky lesions. Similarly, nucleotide-excision repair mutants carry out normal mismatch correction by mismatch-specific or general mismatch-repair systems (3). Thus, nucleotide-excision repair has generally been assumed to play no role in the cellular response to ionizing radiation, damage by methylating agents, or removal of a mismatched nucleotide. However, a back-up role for nucleotide-excision repair in repairing these lesions has not been excluded. In fact, it has been found that the *Escherichia coli* excision-repair enzyme, the (A)BC excinuclease, removes O^6-methylguanine (O^6-mG) (4, 5), apurinic/apyrimidinic (AP) sites, and thymine glycols (6, 7) with moderate efficiency. In this study, we have investigated the susceptibility of various DNA lesions to human excinuclease to gain further insight into the damage-recognition mechanism of this enzyme and the role this repair mechanism might play in protecting cells from nonbulky lesions and in preventing mutations caused by replication errors.

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MATERIALS AND METHODS

Materials. The human cell lines HeLa S3 (wild type) and GM08437A (XP-F), and the Chinese hamster ovary cell line CHO-UV135 (XP-G) were obtained from the Lineberger Comprehensive Cancer Center (University of North Carolina), NIGMS Human Mutant Cell Repository (Coriell Institute, Camden, NJ), and the American Type Culture Collection repository, respectively. T4 DNA ligase and polynucleotide kinase were purchased from Boehringer Mannheim; [γ-^{32}P]ATP (7000 Ci/mmol; 1 Ci = 37 GBq) was obtained from DuPont/New England Nuclear. Dodecanucleotides containing 2-aminobutyl-1,3-propanediol (ABPD; "synthetic AP site"), O^6-mG, N^6-methyladenine (N^6-mA), or a uracil, as well as unmodified oligomers 14–64 nt in length, were purchased from Operon Technologies (Alameda, CA). The dodecamers containing thymine monoadduct of 4′-hydroxymethyl-4,5′,8-trimethylpsoralen (T-HMT) and cisplatin [*cis*-diamminedichloroplatinum (II)] 1,2-d(GpG) crosslink were from J. E. Hearst (University of California, Berkeley) and S. J. Lippard (Massachusetts Institute of Technology), respectively.

Substrates. The substrates were 156-bp duplexes obtained by ligating the terminally labeled dodecamer containing the lesion with seven other partially overlapping oligomers as described (8, 9). The sequence of the modified oligomer is 5′-G^1 A^2 A^3 G^4 C^5 T^6 A^7 C^8 G^9 A^{10} G^{11} C^{12}-3′. Derivatives of this oligomer contained the lesion T^6-HMT for psoralen monoadduct, O^6-mG at position 4, N^6-mA at position 7, ABPD instead of T^6 for "synthetic AP," as well as adenine at position 5 for A*·G mismatch, guanine at position 5 for G*·G mismatch, and guanine at position 6 for G*·A mismatch (asterisk indicates radiolabeling). The dodecamer containing the cisplatin-1,2-d(GpG) diadduct had the sequence TCTAGGCCTTCT (10).

Enzyme Systems. Cell-free extract (CFE) prepared by the method of Manley *et al.* (11) was the source of mammalian excinuclease. The UvrA, UvrB, and UvrC subunits of *E. coli* (A)BC excinuclease were prepared as described (12). In both systems the assays were conducted under saturating enzyme conditions.

Repair Assay. The "excision assay" (13) was used to measure the susceptibility of various substrates for human and *E. coli* excinucleases. The substrates, depending on the location of the lesion relative to the 5′ terminus of the modified dodecamer, contained ^{32}P label at the 4th, 5th, or 6th phosphodiester bond 5′ to the lesion. Thus, removal of the lesion by *E. coli* (A)BC excinuclease releases radiolabeled 12- to 13-nt-long oligomers, and the human excinuclease releases 27- to 29-nt-long oligomers. The reaction with

Abbreviations: XP, xeroderma pigmentosum; CFE, cell-free extract; AP, apurinic/apyrimidinic; T-HMT, thymine monoadduct of 4′-hydroxymethyl-4,5′,8-trimethylpsoralen; O^6-mG, O^6-methylguanine; N^6-mA, N^6-methyladenine; ABPD, 2-aminobutyl-1,3-propanediol.

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human excinuclease was done as follows. The reaction mixture (25 μl) contained 40 mM Hepes (pH 7.9), 80 mM KCl, 8 mM $MgCl_2$, 2 mM ATP, 20 μM of each dNTP, 1 mM dithiothreitol, 7% (vol/vol) glycerol, bovine serum albumin at 200 μg/ml, 50 μg of CFE, and 0.4–1.9 nM substrate, as indicated. The reaction was done at 30°C for 15–120 min as indicated. After deproteinization (13), the products were precipitated with ethanol and separated on 10% or 12% polyacrylamide sequencing gels. The (A)BC excinuclease reaction was conducted as follows. The reaction mixture (25 μl) contained 50 mM Tris·HCl (pH 7.5), 100 mM KCl, 10 mM $MgCl_2$, 2 mM ATP, 1 mM dithiothreitol, 5 nM UvrA, 80 nM UvrB, 40 nM UvrC, 0.07 nM pBR322, and 0.8 nM substrate. Mixtures were incubated at 37°C for 30 min. The reaction was stopped by adding EDTA to 100 mM, and the products were separated on 12% polyacrylamide. The level of excision was quantified by an AMBIS scanner (AMBIS Systems, San Diego). The region corresponding to 15–20 nt in *E. coli* assays and 35–40 nt in human excinuclease assays was taken as background. The fraction of radiolabel in these areas, which is assumed to represent nonspecific degradation, varied from 0.01 to 0.05%.

RESULTS

Substrates. DNA fragments containing a lesion 60 nt from the 5′ terminus and 44 nt from the 3′ terminus of the damaged strand are efficient substrates for the human excinuclease (9). Thus, our basic design for substrate consisted of ligating a dodecamer carrying a lesion (or a mismatch) with seven other oligomers to obtain a 156-bp duplex in which the lesion is 92 nt away from the 5′ end and 62 nt away from the 3′ end (Fig. 1). Four types of substrates known to be repaired primarily by one of the four major repair systems were tested: O^6-mG (direct repair), AP site (base-excision repair), T-HMT and cisplatin 1,2-d(GpG) diadduct (nucleotide-excision repair), and single-base mismatches G·A, A·G, and G·G (mismatch repair). In addition, N^6-mA was tested because, even though this is a natural base in *E. coli*, it is not a normal constituent of human DNA. The structures of these various "lesions" are shown in Fig. 2.

Excision of AP Site. Because an AP site is excised by the *E. coli* (A)BC excinuclease (6, 7) relatively efficiently, we initially tested the human excinuclease with an AP substrate generated by releasing a site-specifically incorporated uracil by uracil glycosylase. The AP site generated by this method was excised by human excinuclease in 27- to 29-mers (data not shown). However, because of the very potent AP endonuclease in the human CFE, the substrate was extensively degraded, generating high background superimposed over the relatively weak signal. We decided to use a "synthetic AP" that is less susceptible to AP endonucleases. We chose to use DNA containing ABPD. The propanediol part of this compound maintains the natural 3-carbon internucleotide phosphate distance like the deoxyribose in phosphodiester backbone, and the butyl side chain might be considered a ring-opened deoxyribose (14). When tested with human CFE, this structure was excised quite efficiently. To demonstrate that this excision was done by the human excinuclease, as defined by the UV-sensitive human and rodent mutant cell lines, we conducted complementation assays with CFE from

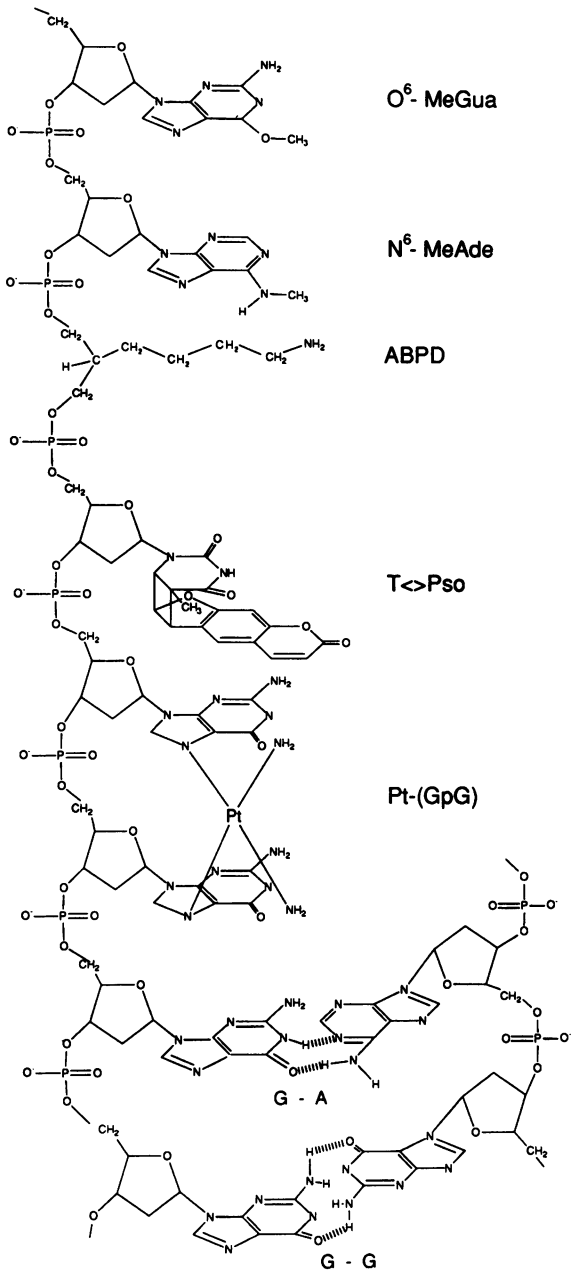

FIG. 2. Structures of DNA duplex alterations that constitute substrates for human excinuclease. T<>Pso, T-HMT.

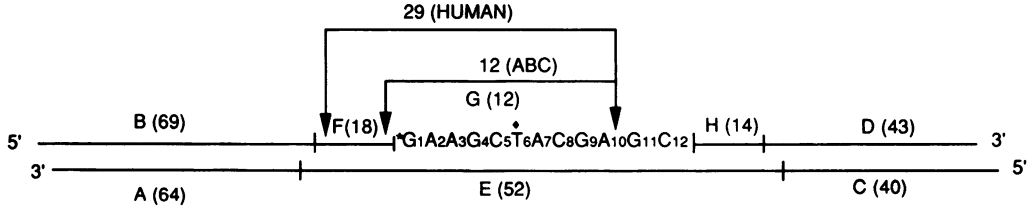

FIG. 1. Substrate construction. The central 12-nt oligomer (G), either unmodified or modified at positions 4, 5, or 6, was terminally labeled with ^{32}P and ligated with seven other oligomers (A, B, C, D, E, F, and H) to obtain the 156-bp duplex. The diamond indicates position of the T-HMT adduct or the "synthetic AP"; the asterisk indicates position of the radiolabel. The main incision sites of human and *E. coli* excinucleases relative to the modification at position 6 (diamond) and the sizes of the excised fragment are indicated by brackets.

different mutants (15). The result is shown in Fig. 3. CFE from cell lines with mutations in *XPF* or *XPG* genes did not excise 27- to 29-nt-long oligomers. However, mixing the two CFEs restored excision activity comparable to that seen with HeLa CFE (Fig. 3, lanes 3 and 4). Thus, we conclude that human excinuclease excises the "synthetic AP site" by the standard dual-incision mechanism.

To evaluate the relative affinity of human excinuclease for synthetic AP compared with the traditional nucleotide excision-repair substrates, we conducted excision assays with the synthetic AP duplex and with a substrate containing 1,2-d(GpG) cisplatin adduct, which is eliminated from DNA exclusively by excision repair at a rate comparable to cyclobutane pyrimidine dimer (9, 10). Fig. 4 shows that the two lesions are excised with comparable efficiencies with a rate of 75 fmol/min for synthetic AP and 30 fmol/min for cisplatin 1,2-d(GpG) crosslink under identical experimental conditions. Thus, synthetic AP appears to be a good substrate for

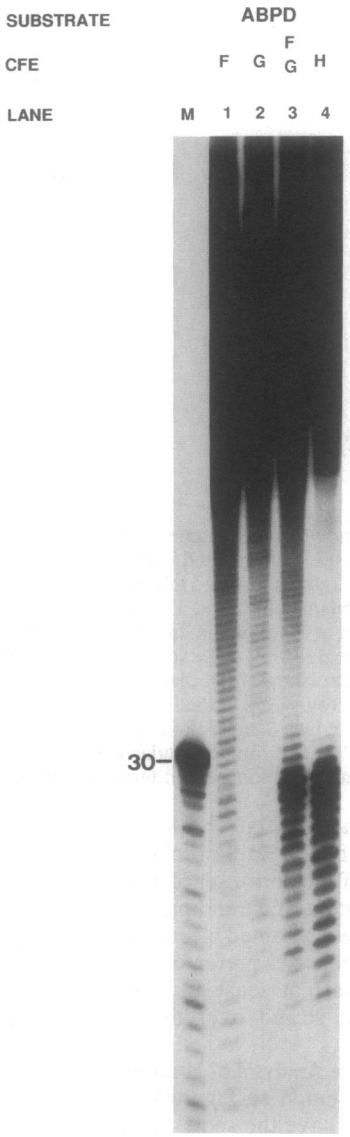

FIG. 3. Removal of synthetic AP (ABPD) requires functional excinuclease. The substrate (54 pM) with ABPD at position 6 of the central oligomer was incubated with CFE from XP-F (F) or XP-G (G) cell lines, a mixture of the two (FG), or HeLa (H) CFE. Reaction mixtures contained 100 μg of total protein, and incubation was for 1 hr at 30°C (M, 30-nt-long marker). The high nonspecific degradation seen in XP-F is uncharacteristic of this cell line but typical of this particular CFE.

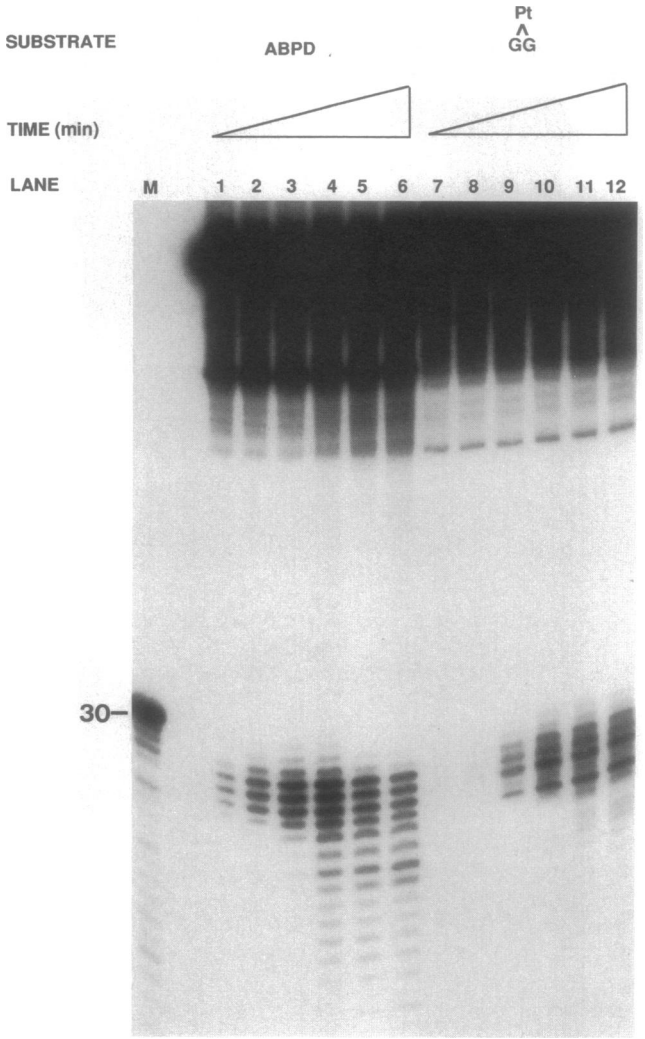

FIG. 4. Comparison of relative affinities of synthetic AP (ABPD) and cisplatin diadduct (Pt GG) to human excinuclease. Reaction mixtures containing 54 pM substrate and 100 μg of HeLa CFE, which were incubated for 5 (lanes 1 and 7), 10 (lanes 2 and 8), 20 (lanes 3 and 9), 40 (lanes 4 and 10), 60 (lanes 5 and 11), and 90 min (lanes 6 and 12). Maximum excision for ABPD was 3.9% at 40 min, and maximum excision for 1,-d(GpG) cisplatin crosslink was 3.3% at 90 min (M, 30-nt-long marker).

human excinuclease, and because of its ready availability, we used it as a reference substrate for other lesions tested.

Excision of Methylated Bases. O^6-mG is a mutagenic lesion that causes G·C → A·T transition (16). This lesion is eliminated from DNA by the suicide enzyme, O^6-mG-DNA methyltransferase. It has been reported that in Mer$^-$ or Mex$^-$ mammalian mutants lacking the enzyme (17, 18), the level of O^6-mG does not change detectably (19), suggesting that O^6-mG is not a substrate for human excinuclease. However, these experiments lack the resolution to detect a low level of removal by nucleotide-excision repair. Fig. 5 shows that O^6-mG is removed by the same enzyme system that removes bulky adducts both in *E. coli* (lane 3) and humans (lane 7). However, the extent of excision is low compared with the synthetic AP: ≈15% relative to synthetic AP in both systems. In contrast to O^6-mG, N^6-mA, which is found in prokaryotic but not eukaryotic DNA, is excised by the human excinuclease (lane 6) but not by the prokaryotic excinuclease (lane 2).

Excision of Mismatches. Do excision nucleases also act on mismatches? In initial screening of several mismatches, those

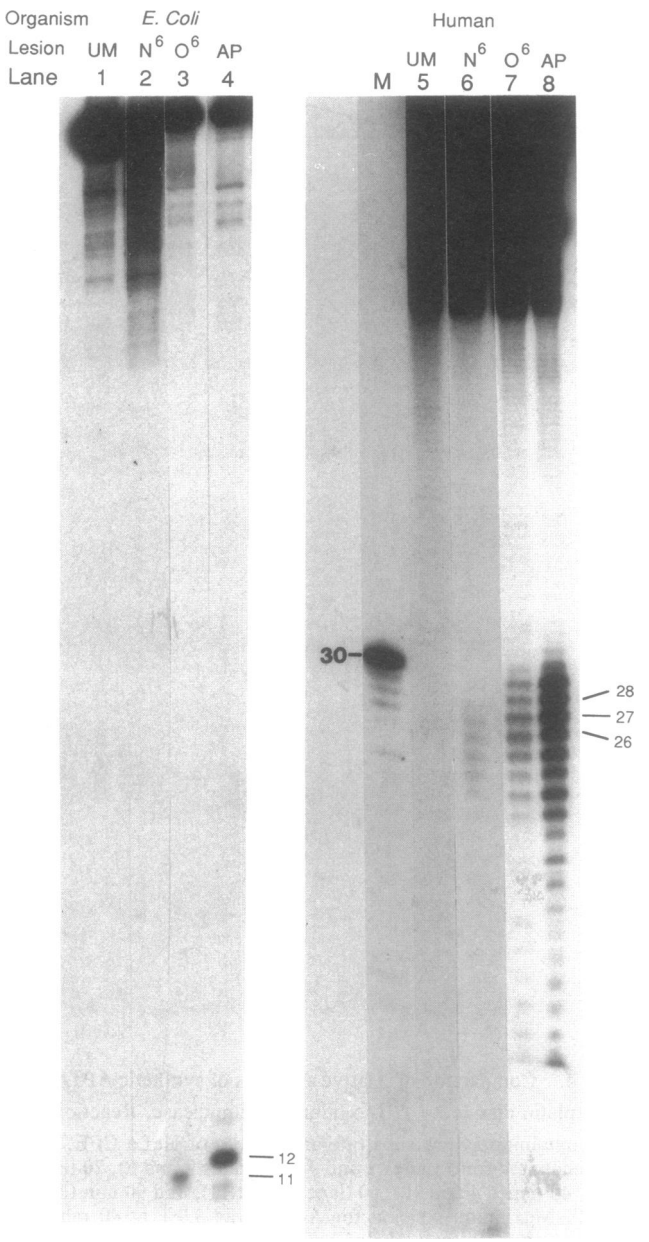

FIG. 5. Removal of methylated bases by *E. coli* and human excinucleases. Lanes: 1 and 5, unmodified DNA (UM); 2 and 6, N^6-mA (N^6); 3 and 7, O^6-mG (O^6); 4 and 8, synthetic AP; M, 30-nt-long size marker. DNA concentrations in all reactions were ≈0.5 nM, and reactions were for 30 min at 37°C for (A)BC excinuclease and for 1 hr at 30°C for HeLa CFE. Results from different gels were combined for presentation. Levels of excision were as follows: lanes 1, 2, and 5, nondetectable; lane 3, 1% (note O^6-mG is removed in 11-nt-long fragment in this sequence context); lane 4, 5%; lane 6, 0.06%; lane 7, 0.1%; lane 8, 1%.

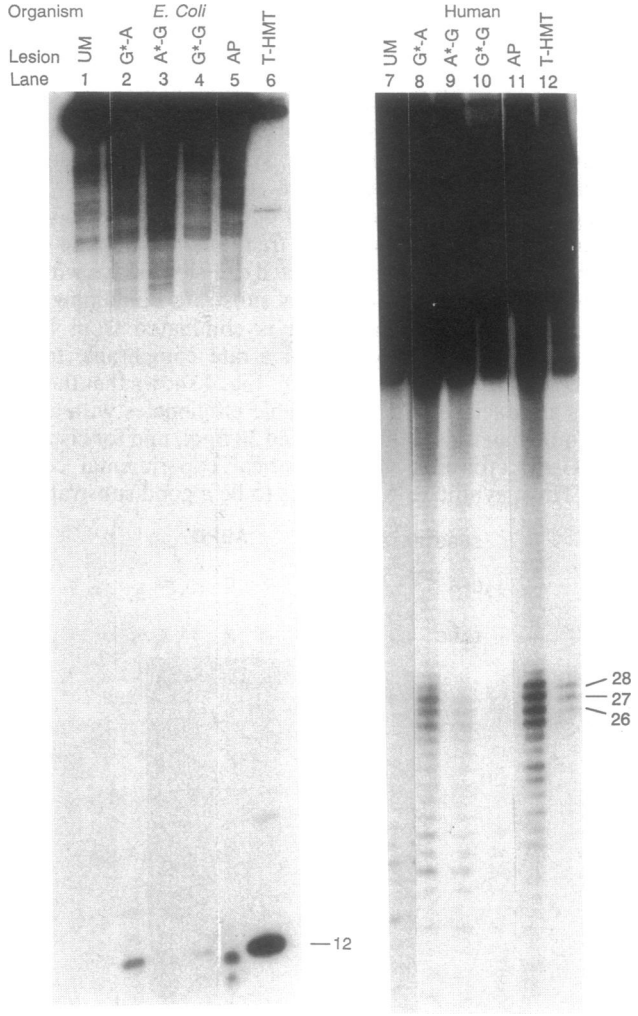

FIG. 6. Removal of mismatches, AP sites, and T-HMT adduct by excision nucleases. Lanes: 1 and 7, unmodified (UM) DNA; 2 and 8, G*·A mismatch (guanine strand labeled); 3 and 9, A*·G mismatch; 4 and 10, G*·G mismatch; 5 and 11, AP site; 6 and 12, T-HMT. All substrates were at ≈1.0 nM in 25-μl reaction mixtures. Incubation conditions were as for Fig. 5. Levels of excision were as follows: lane 1, not detectable; lane 2, 0.3%; lane 3, 0.05%; lane 4, 0.1%; lane 5, 2%; lane 6, 85% (only 10% of the DNA was loaded onto the gel); lane 7, not detectable; lane 8, 0.2%; lane 9, 0.1%; lane 10, 0.05%; lane 11, 5%; lane 12, 1%. The low-molecular-weight species seen in lanes 7–12 are generated by nonspecific nucleases in the CFE.

involving purine–purine mismatches yielded easily detectable signals; thus, we concentrated our studies on three mismatches. Fig. 6, lanes 2 and 8 show that the guanine in a G·A mismatch is excised by the excision-repair systems of both *E. coli* and humans. With human CFE and in this sequence context, the guanine strand of the G·A mismatch was excised ≈2-fold more than the adenine strand (Fig. 6, lanes 8 and 9) which in turn is "repaired" better than the mismatch in G·G (lanes 9 and 10). In *E. coli* also the guanine strand in the G·A mismatch was excised more efficiently than the adenine strand (lanes 2 and 3), and the G·G mismatch was also excised relatively efficiently (lane 4). All mismatches tested were repaired to a lesser extent than synthetic AP or psoralen monoadducts. The low extent of mismatch repair (0.05–0.3% of substrate) precluded a systematic analysis of repair rates of the entire set of mismatches and the effects of sequence context on strand preference. However, as data in Fig. 6 show, we obtained clear evidence that both *E. coli* and human excinucleases preferred mismatches over unmodified DNA and that, at least in one case, the excinucleases preferred one strand over the other in a duplex with a mismatch.

Comparative Analysis of Human and *E. coli* Excinucleases. The data presented so far indicate that human and *E. coli* excinucleases have the same range of substrates. However, their substrate preferences are quite different, as is seen most clearly in Fig. 6, which shows a direct comparison of substrate preference of these two enzyme systems. The *E. coli* excinuclease excises the T-HMT at ≈50-fold faster rate than the synthetic AP site (lanes 5 and 6). In contrast, the extent of repair of synthetic AP by human excinuclease is ≈10-fold higher than that for T-HMT (lanes 11 and 12). Thus, even though the two enzyme systems excise almost all modified

bases from DNA, they do so with vastly different preferences.

DISCUSSION

In both pro- and eukaryotes, nucleotide-excision repair is the repair system for bulky adducts (2, 20, 21). In *E. coli* this repair system was reported (2) to be active on all unnatural base modifications tested. Our study reveals that the same is also true for the human excinuclease system. How the same enzyme system recognizes base modifications ranging from N^6-mA to thymine dimer is an interesting question. A plausible answer is as follows. The recognition subunits of the enzyme bind to DNA nonspecifically with relatively high affinity. The binding induces certain conformational changes, such as kinking and unwinding at the binding site. If a given site is more prone to undergo such a conformational change because of the base modification, a higher affinity interaction ensues, and this long-lived complex then becomes a target for the nuclease subunits that bind to the recognition complex and induce the double incision (2). Implicit in this model is the hypothesis that excinucleases should, to a limited degree, also act on unmodified DNA. Indeed, evidence for such action in the case of *E. coli* (A)BC excinuclease has been obtained (22, 23). Although it is rather difficult to eliminate the possibility that the low level of excision seen with nominally undamaged DNA is, in fact, due to the low level of lesions resulting from handling and radiolabeling of the control substrate, long exposures of our autoradiographs of "undamaged DNA" treated with human excinuclease reveal low levels of 27- to 29-mers superimposed on the ladder, resulting from random degradation of DNA. In light of our current findings, we believe that low level of excision does occur, even on undamaged DNA.

The different substrate preferences of the human and *E. coli* excinucleases suggest substantially different recognition mechanisms. N^6-mA, which is found at a relatively high frequency in *E. coli*, is not a substrate for the *E. coli* enzyme but is excised by the human excinuclease. This result suggests that excinucleases have evolved to accommodate the base modifications found in those particular organisms as normal and others as a lesion. Indeed, expression of the Dam methylase in *Saccharomyces cerevisiae* (which normally does not contain N^6-mA) induced single-strand breaks that depended on an intact nucleotide-excision repair system (24), consistent with the hypothesis that the yeast excision-repair system, which resembles the human system (20), also excises N^6-mA.

The differential recognition mechanisms are also reflected in the substrate preference of *E. coli* and human excinucleases. There is a dramatic difference between the human and *E. coli* enzymes with regard to their effects on the synthetic AP substrate and the T-HMT, the human enzyme being more active on the former and the *E. coli* enzyme being more active on the latter. The differential substrate preference is not that surprising, considering the fact that the human and *E. coli* excision-repair subunits do not share homology. From a practical standpoint, these results suggest that caution must be exercised in extrapolating from prokaryotic model systems to humans with regard to repairability and, hence, mutagenic and cytotoxic effects of a given lesion.

The findings reported here also raise some questions regarding the role of excision repair in resistance to oxidative damage, methylating agent, and in correcting mismatches. Human XP cells (25) and their rodent counterparts (26) are exquisitely sensitive to UV light and not particularly sensitive to ionizing radiation or alkylating agents. We believe that excision repair plays mainly a back-up role for glycosylase and AP endonucleases (27) responsible for repairing the damage of the latter agents, consistent with reports that the contribution of excision repair can be seen only in an appropriate *E. coli* background (28).

Finally, mismatch "correction" by excinucleases is quite inefficient compared with the Mut(H)LS-dependent mismatch-correction system (29, 30); an earlier study using the less sensitive nicking assay failed to detect the effect of (A)BC excinuclease on mismatches (31). From a physiological standpoint, while the mismatch repair system can discriminate between correct and incorrect strands by the presence of a nick in the incorrect strand, no evidence suggests that the excinuclease has such a capability. Rather, the excinuclease appears to excise the mismatch from one or the other strand, as dictated by the structure of the particular mismatch. Thus, nucleotide-excision repair could cause mutation fixation by actually interfering with the mismatch correction of the Mut(H)LS system, rather than help in mutation avoidance. Whether this activity plays a role in spontaneous mutation rate *in vivo* remains to be seen.

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