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Recognition and Repair of Compound DNA Lesions (Base Damage and Mismatch) by Human Mismatch Repair and Excision Repair Systems

DAVID MU,[1] MIHRAY TURSUN,[1] DEREK R. DUCKETT,[2] JAMES T. DRUMMOND,[2] PAUL MODRICH,[2] AND AZIZ SANCAR[1]*

Department of Biochemistry and Biophysics, University of North Carolina School of Medicine, Chapel Hill, North Carolina 27599-7260,[1] and Howard Hughes Medical Institute and Department of Biochemistry, Duke University Medical Center, Durham, North Carolina 27710[2]

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Nucleotide excision repair and the long-patch mismatch repair systems correct abnormal DNA structures arising from DNA damage and replication errors, respectively. DNA synthesis past a damaged base (translesion replication) often causes misincorporation at the lesion site. In addition, mismatches are hot spots for DNA damage because of increased susceptibility of unpaired bases to chemical modification. We call such a DNA lesion, that is, a base damage superimposed on a mismatch, a compound lesion. To learn about the processing of compound lesions by human cells, synthetic compound lesions containing UV photoproducts or cisplatin 1,2-d(GpG) intrastrand cross-link and mismatch were tested for binding to the human mismatch recognition complex hMutSα and for excision by the human excision nuclease. No functional overlap between excision repair and mismatch repair was observed. The presence of a thymine dimer or a cisplatin diadduct in the context of a G-T mismatch reduced the affinity of hMutSα for the mismatch. In contrast, the damaged bases in these compound lesions were excised three- to fourfold faster than simple lesions by the human excision nuclease, regardless of the presence of hMutSα in the reaction. These results provide a new perspective on how excision repair, a cellular defense system for maintaining genomic integrity, can fix mutations under certain circumstances.

Mismatches in DNA resulting from replication errors, and base damage caused by physical (UV light) and chemical (polyaromatic hydrocarbons) agents, are responsible for the majority of human cancers (18). Although certain mismatches and base lesions can be eliminated from DNA by the base excision repair pathway initiated by glycosylases with narrow substrate ranges, in human cells there exists a general mismatch repair system and a general damage repair system of wide substrate range. The mismatch repair system removes the mismatched base as a nucleotide (44), and the excision repair system excises the damaged base(s) in an oligonucleotide (52, 67).

The general mismatch repair system (long-patch mismatch repair) corrects all eight single-base mismatches as well as small insertion sequence loops with comparable efficiencies (31, 44). The general nucleotide excision repair (excision repair) system (8) not only is the sole repair pathway for bulky lesions such as thymine dimers (T<>T) and cisplatin-guanine adducts but also repairs a wide variety of nonbulky lesions such as O^6-methylguanine (O^6-meG) at physiologically relevant rates (52). Thus, it appears that both repair systems recognize many dissimilar non-B DNA forms rather than a specific lesion structure.

Given the wide substrate ranges of both systems, it is not unreasonable to expect overlaps between the two substrate spectra, or that one repair system may facilitate the function of the other. Indeed, it has been shown that the excision repair system recognizes mismatches and removes the mismatched base in a manner identical to the removal of damaged bases (27). Since a DNA lesion, by definition, changes the hydrogen-bonding properties of the damaged base, all lesions can be considered mismatches and potential substrates for binding and processing by the general mismatch repair system. Furthermore, DNA lesions such as pyrimidine dimers are often miscoding during bypass DNA synthesis, giving rise to a lesion in one strand and a mismatch in the other. These damaged nucleotides superimposed on mismatches, which we call compound lesions, are generated at significant levels in cells exposed to DNA-damaging agents (18). Since compound lesions are formally substrates for both repair systems, they may cause the two systems either to act in concert or to compete in order to restore the integrity of DNA.

There are several in vivo studies pertinent to the issues of functional and mechanistic overlap between the two repair systems. It has been reported that *Escherichia coli uvrA* mutants have a normal mismatch correction function, and hence it was concluded the excision repair system did not play a role in mismatch correction carried out by the methyl-directed mismatch repair system (10). In contrast, several lines of evidence have implicated the long-patch mismatch repair in processing of UV damage both in *E. coli* and in humans. In *E. coli*, it was found that recombinogenic rescue of UV-irradiated lambda phage was dependent on *uvrA*, *-B*, and *-C* genes (excision repair) as well as on functional *mutL* and *mutS* genes (mismatch repair), suggesting that the two systems acted in a coordinated manner to generate a prerecombinogenic substrate (14, 15). Both in *E. coli* and in humans, mutations in *mutS* (h*MSH2* in humans) and *mutL* (h*PMS2* in humans) were found to render cells slightly sensitive to UV and to abolish the transcription repair coupling of the template strand (40, 41) in an actively transcribing gene. Finally, both *E. coli* (16) and human (11) mismatch repair mutants have been found to have

* Corresponding author. Mailing address: Department of Biochemistry and Biophysics, CB# 7260, University of North Carolina School of Medicine, Chapel Hill, NC 27599-7260. Phone: (919) 962-0115. Fax: (919) 966-2852.

TABLE 1. Oligonucleotides used to assemble the substrate DNAs

Oligonucleotide	Nucleotide sequence (5′ to 3′)
1	GGGACCTGAACACGTACGGAATTCGATATCCTCGAGCCAGATCTGCGCCAGCTGGCCACCCTGA
2	GTA**T**[6-4]**T**ATG
3	GTA**T**<>**T**ATG
4	GAGCGCCAAGCTTGGGCTGCAGCAGGTCGACTCTAGAGGATCCCGGGCGAGCTCGAATTCGCCC
5	GGGGCGAATTCGAGCTCGCCCGGGATCCTCTAGAGTCGACCTGCTGCAGCCCAAGCTTGGC
6	GCTCCATAATACTCAG
7	GGTGGCCAGCTGGCGCAGATCTGGCTCGAGGATATCGAATTCCGTACGTGTTCAGGTCC
8	GCTCGAGCTAAA**T**<>**T**CGTCAG
9	CGAATTTAGCTC
10	CGAGTTTAGCTC
11	CGAGGTTAGCTC
12	CGAGGGGAGCTC
13	CGGATTTAGCTC
14	GCTCGAGCTAAATTCGTCAG
15	GCTCCATAGTACTCAG
16	GCTAGCAAGCTGTCGATTCTAGAAATTCGGC
17	GCCGAATTTCTAGAATCGACAGCTTGCTAGC
18	TCTA**[G*G*]**CCTTCT[a]
19	GCTCAGAAGGCCTAGATCAG
20	GCTCAGAAGGCTTAGATCAG
21	TCTAGGCCTTCT

[a] Contains an intrastrand *cis*-platinated diadduct at the two guanidine [G*G*].

increased resistance to killing by cisplatin. These last findings suggest that in contrast to UV damage, the cisplatin lesions are recognized but cannot be eliminated by the mismatch repair system, leading to a futile and lethal cycle of excision and resynthesis which is known to occur with O^6meG-T lesions (4, 28). Indeed, it was found that the human mismatch recognition complex hMutSα (hMSH2-GTBP heterodimer) binds to G-T, O^6meG-T, and cisplatin-1,2-d(GpG) lesions with comparable efficiencies (13) and that hMSH2 binds to cisplatin-1,2-d (GpG) with high affinity (39).

While these are compelling data for functional overlap between excision repair and mismatch repair, there are several observations which are inconsistent with such a model. First, a study with strains of human mismatch repair mutants different from those used for strand-specific repair found that these mutants had normal UV resistance (2). Second, *Saccharomyces cerevisiae* has mismatch and excision repair systems which are structurally and functionally very similar to the human repair systems (18, 21, 31, 44). Interestingly, abolishing the yeast mismatch repair by mutations in *mutS* and *mutL* homologs had no detectable effect on UV survival or transcription-coupled excision repair of pyrimidine dimers in vivo (59), indicating that mismatch repair and excision repair are independent of each other. Finally, cell extracts (CEs) of *E. coli mutS* and *mutL* mutant strains carry out transcription-coupled excision repair in vitro, and the rate of transcription-stimulated repair is not affected by supplementing a *mutS* mutant CE with purified MutS protein (54).

We wished to address the issue of excision repair/mismatch repair connection in humans directly by conducting in vitro experiments. Using mutant CEs, the human excision repair system reconstituted from purified proteins, and the purified hMutSα protein, we investigated the processing of the two major UV photoproducts, the cyclobutane thymine dimer (T<>T) and (6-4) photoproduct (T[6-4]T), and the major adduct of the anticancer drug cisplatin, the 1,2-d(GpG) intrastrand cisplatin cross-link (34). The results show that the human long-patch mismatch repair system does not influence the repair of these lesions by human excision nuclease.

MATERIALS AND METHODS

Cell strains and repair factors. HeLa and HEC-1-A cell strains were from the stock of the Lineberger Cancer Center Tissue Culture Facility (University of North Carolina). The LoVo cell line (CCL-229) was purchased from American Type Culture Collection (Rockville, Md.). Whole-cell extracts were prepared by the method of Manley et al. (36), and nuclear extracts were prepared as described by Holmes et al. (24). The whole-cell extracts were used for the experiments reported unless indicated otherwise. The hMutSα heterodimer (12), hMutLα (33), and the six factors of human excision nuclease (45) were purified as described previously.

Incision/excision assays. Duplexes of 136 or 140 bp containing DNA damage, mismatches, or both were prepared from six partially complementary oligomers as described previously (26, 37). Table 1 shows the sequences of the 21 oligonucleotides used in this study. Oligonucleotides containing T<>T and T[6-4]T were prepared by the method of Smith and Taylor (56). The cisplatin-modified oligonucleotide was prepared as described by Zamble et al. (68). These modified oligomers, with the exception of oligomer 8 (provided by J.-S. Taylor), were a kind gift from X. Zhao. The unmodified oligonucleotides were purchased from Operon Biotechnology (Alameda, Calif.). To directly compare the incision and excision products of T<>T and T[6-4]T substrates, 136-mer duplex DNAs of the same sequence were constituted as shown in Fig. 1A. For determining the incision sites, oligonucleotide 1 was terminally labeled with [γ-^{32}P]-ATP (NEN-Dupont) and T4 polynucleotide kinase. For analyzing the excision products, the damage-containing oligomer was 5′-terminally labeled in the same manner. The labeled oligomers were ligated with the other five oligonucleotides to obtain either 5′-terminally labeled or internally labeled duplexes as described previously (26, 37). The full-length duplexes were separated from partially ligated products by sequential purifications through 8% denaturing and 5% nondenaturing polyacrylamide gels. The concentrations of substrates are given in terms of duplex DNA and were calculated from the specific activity (7,000 Ci/mmol) of [γ-^{32}P]ATP used in phosphorylating the appropriate oligomers.

The incision/excision assays were carried out as described previously (37, 45, 46). The reactions with the reconstituted human excision nuclease (45, 46) were done with mixtures contained 20 ng of XPA, 2 ng of TFIIH, 8 ng of XPC, 4 ng of XPF-ERCC1, 20 ng of XPG, 250 ng of replication protein A (RPA), and 25 fmol of substrate in 25 μl of excision buffer. The reaction times, unless indicated otherwise, were 2 h for the reconstituted excision nuclease and 1 h for reactions with CEs. The reaction products were separated on 8% denaturing polyacrylamide gels and visualized by autoradiography, and the excision products were quantified with a PhosphorImager (Molecular Dynamics, Inc.).

S1 nuclease digestion. Fifteen femtomoles of internally labeled substrate [T<>T(0), T<>T(1), T<>T(2), or T<>T(4)] was incubated at room temperature for 25 min with 0.5 U of S1 nuclease (Gibco BRL/Life Technologies) in 15 μl of reaction mixture containing 30 mM sodium acetate (pH 4.6), 1 mM zinc acetate, 150 mM NaCl, 5% glycerol, and ~1 nM substrate. The reaction was stopped by addition of formamide-dye, and the products were analyzed on an 8% denaturing polyacrylamide gel.

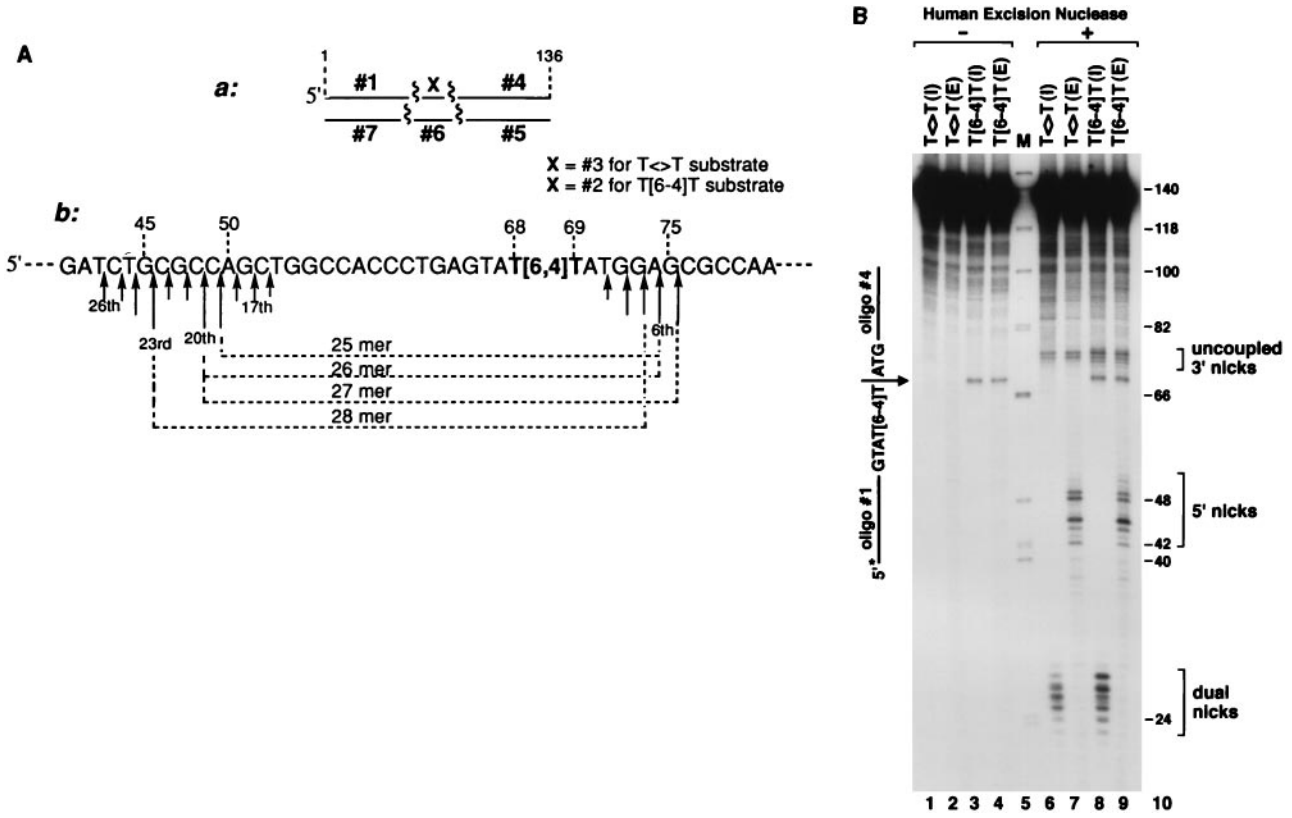

FIG. 1. Incision/excision of T<>T and T[6-4]T photoproducts by human excision repair nuclease. (A) Substrates and incision sites. a, the substrates were constructed by ligating the indicated six oligonucleotides. The resulting 136-bp duplexes were of the same sequence except for one containing T<>T and another containing T[6-4]T at positions 68 and 69. For incision assays 1 oligomer 1, and for excision assays oligomer 2 or 3, was 5′ phosphorylated with ^{32}P before annealing and ligation with the other oligomers. b, sequence around the photolesions and incision sites of human excision nuclease. The 5′ incision sites were determined from panel B and from other gels where the Maxam-Gilbert sequence ladder were run alongside the incision lanes. With 5′-labeled substrates, the 3′ incision sites can be seen only when they are uncoupled from the 5′ incisions, and the major uncoupled incisions may not be the same as the major 3′ coupled incisions. The major 5′ and 3′ (uncoupled) incisions were identical for the two photoproducts. The 23rd phosphodiester bond 5′ and the 6th phosphodiester bond were the major sites. There was very little incision at the 22nd and 21st phosphodiester bonds. The major excision products were 25 to 28 nt in length. Examples of how they can be generated by combination of 3′ and 5′ incisions are indicated. Note that the sequence of the T<>T-containing oligomer (oligomer 3) used in this experiment is different from that of oligomer 8 used for other experiments in this study. (B) Incision/excision assays. Internally labeled (I) or 5′-end-labeled (E) fragments were subjected to the indicated treatments and then separated on an 8% denaturing polyacrylamide gel. Lanes 1 to 4 contained substrates with no enzymatic treatment. Note that the T[6-4]T substrate contains a minor contaminant which terminates immediately 3′ to the photolesion, as evidenced by the treatment of this substrate with T4 DNA polymerase 3′ to 5′ exonuclease (which is blocked by the photolesion generating a fragment that marks the 3′ side of the lesion) (data not shown). Lanes 6 to 8, the indicated DNAs treated with reconstituted excision nuclease for 2 h at 30°C. Lane 5 contains size markers; the sizes are indicated in nucleotides in lane 10.

Gel retardation assay. The reaction conditions were adapted from those of Duckett et al. (13). Briefly, the binding reaction mixture contained (in 15 μl) 10 mM HEPES-KOH (pH 7.6), 100 mM KCl, 5 mM $MgCl_2$, 1 mM dithiothreitol, 50 μg of bovine serum albumin per ml, 200 fmol of unlabeled duplex 31-mer (made from oligonucleotides 16 and 17 [Table 1]), 8 fmol of internally labeled probe, and the indicated amounts of hMutSα. The mixture was incubated for 15 min at room temperature, then 3 μl of 80% glycerol was added, and the DNA-protein complexes were resolved on 5% nondenaturing polyacrylamide gels running in 20 mM Tris-acetate (pH 7.6)–1 mM $MgCl_2$ at 8 V/cm and room temperature with buffer circulation (1 liter/h). The retarded DNA was quantified with a PhosphorImager, assuming that all DNA migrating more slowly than the free DNA band in the control lane was retarded by the protein.

RESULTS

Substrates and incision/excision. We constructed 136- to 140-bp substrates by ligating partially overlapping oligonucleotides with a modified oligomer containing a DNA lesion in a predetermined position. Table 1 lists the oligonucleotides used in this study. The substrates were either terminally (5′) or internally (4th or 13th phosphate 5′ to the lesion) labeled for the purpose of analysis of the reaction products of human excision nuclease. Figure 1 shows the results of such an analysis with substrates containing T<>T and T[6-4]T photoproducts. Both lesions are good substrates for the reconstituted excision nuclease, and both are removed by dual incisions (25), mainly in 24- to 28-nucleotide (nt)-long oligomers (lanes 6 and 8), by incisions of phosphodiester bonds 16 to 26 (Fig. 1A and B, lanes 7 and 9) and phosphodiester bonds 3 to 7 3′ to the photodimer, with the incisions at the 19th and 20th and at the 23rd and 24th phosphodiester bonds 5′ and the 6th and 7th phosphodiester bonds 3′ to the lesion being the predominant ones. Quantitative analyses of the excision data in lanes 6 and 8 of Fig. 1B and of several experiments conducted under identical conditions reveal that the T[6-4]T photolesion is repaired threefold faster than the T<>T photoproduct, in agreement with the relative rates of removal of these lesions in vivo (42, 43). The excision rate and pattern of cisplatin 1,2-d(GpG) cross-link are comparable to those of T[6-4]T and have been reported previously (27, 68).

Binding of hMutSα to damage, mismatch, and compound lesions. According to current models for mismatch repair (31, 44), the binding of hMutSα to the mismatch is an early if not the earliest step in repair of simple single-base mismatches. Hence, to learn if there is a functional overlap between mis-

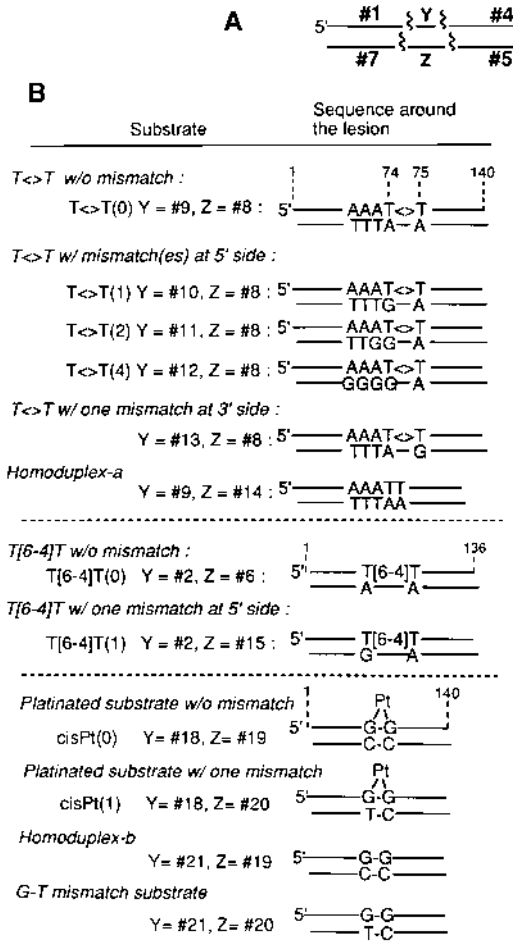

FIG. 2. Substrates for mismatch binding and excision experiments. (A) Substrate assembly strategy. All substrates were internally labeled by using Y oligomer that had been phosphorylated with [γ-^{32}P]ATP. (B) Schematic presentation of the various substrate and control duplexes used in the study.

match repair and damage repair, we constructed the substrates shown in Fig. 2 and tested the binding of hMutSα to UV photoproducts and cisplatin-1,2-d(GpG) intrastrand cross-link in the normal sequence context and in the form of compound lesions. Figure 3 shows that DNA containing T<>T or T[6-4]T has marginal affinity for hMutSα compared to the normal duplex. In contrast, cisplatin-1,2-d(GpG) cross-link binds to hMutSα with a higher affinity as reported previously (13). Of special significance, compound lesions of both UV photoproducts and the cisplatin diadduct in the context of a G-T mismatch were bound by hMutSα with about twofold-lower affinity than that of a simple G-T mismatch, suggesting that a G-T mismatch in a compound lesion is less susceptible to mismatch correction (Fig. 3C).

Effect of mismatch repair system on excision repair. The data presented so far indicate that DNA damage is recognized with various degrees of affinities by the mismatch repair system. In combination with the various in vivo reports on diminished damage repair in mismatch repair mutants (41), this finding raised the possibility that mismatch repair proteins directly participate in excision repair. To test for this, we conducted excision repair assays with mismatch mutant CEs, using DNA with damaged bases in the normal sequence context as well as in compound lesions. The results obtained with the

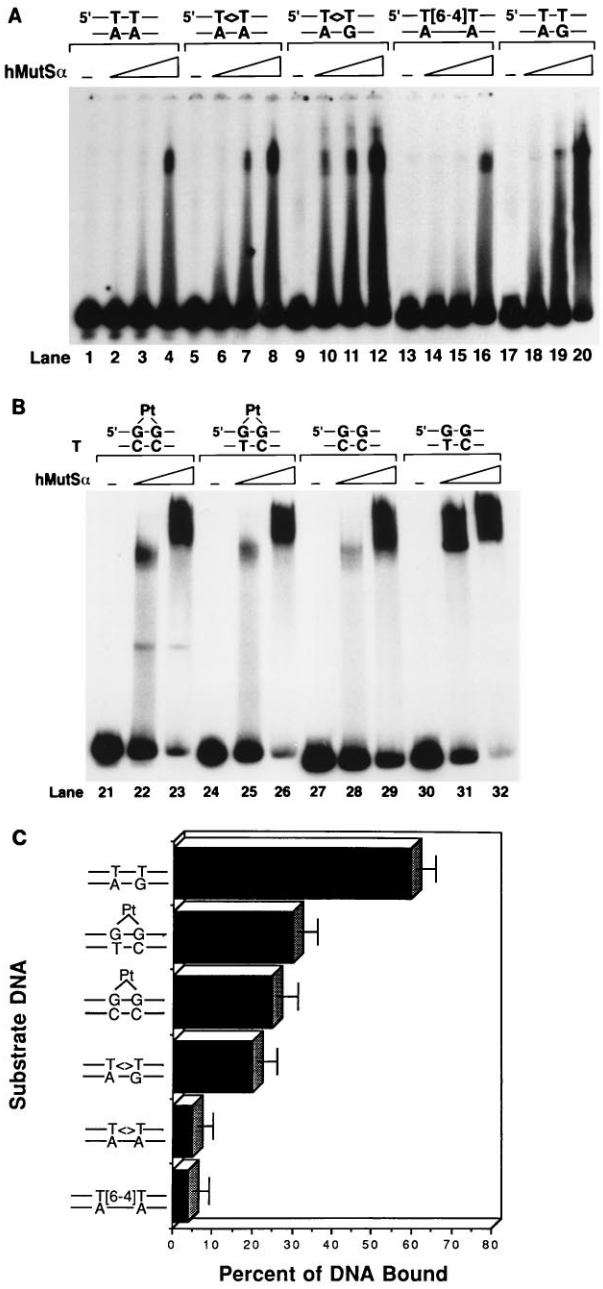

FIG. 3. Binding of hMutSα to DNA damages as simple or compound lesions, as tested by a gel retardation assay. (A) Binding to photoproducts. Reaction mixtures contained 1 nM the indicated substrate and 0, 6.3, 9.5, or 19 nM hMutSα. The G-T mismatch substrate used in lanes 17 to 20 was generated by photoreactivation of the compound lesion used in lanes 9 to 12, using *E. coli* photolyase. (B) Binding of hMutSα to cisplatin 1,2-d(GpG) simple and compound lesions. Reaction mixtures contained 1 nM the appropriate substrate and 0, 9.5, or 19 nM hMutSα. (C) Quantitative expression of the binding data. Binding data with 9.5 nM hMutSα from the experiments in panel A and two other experiments conducted under identical conditions and from panel B and a second experiment conducted under identical conditions are shown. All data have been normalized to the binding of the corresponding normal homoduplexes. Bars indicate standard errors. Note that the simple G-T mismatch substrate was generated by splitting the T<>T in the corresponding compound lesion.

T<>T substrate are shown in Fig. 4A. With a simple T<>T lesion, it appears that HeLa CE is more efficient than CE from either an hPMS2 mutant (MutL homolog) or an hMSH2 mutant (lanes 2 to 4). However, the excision efficiencies of extracts

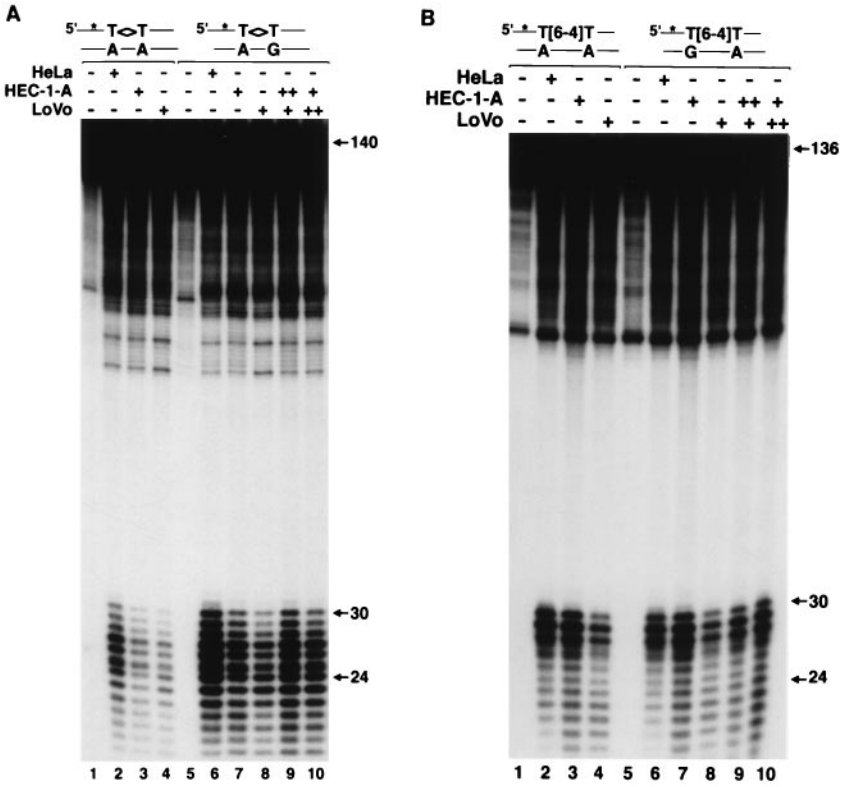

FIG. 4. Effect of the mismatch repair system on excision of UV photoproducts from simple and compound lesions. The excision reactions were carried out with CEs for 60 min at 30°C. The amounts of CEs used were 108 μg for HeLa, 56 μg for HEC-1-A (50), and 84 μg for LoVo (66). These amounts were empirically found to be optimal for individual CEs. In the complementation assays, in each panel, lane 9 contained 38 μg of HEC-1-A and 28 μg of LoVo CEs, and lane 10 contained 21 μg of HEC-1-A and 56 μg of LoVo CEs. (A) Excision assay with 140-bp duplexes containing T<>T. The levels of excision as percentages of the input substrates (25 fmol) were as follows: lane 1, not detectable; lane 2, 2.5; lane 3, 1.9; lane 4, 1.7; lane 5, not detectable; lane 6, 5.2; lane 7, 4.3; lane 8, 4.1; lane 9, 4.1; and lane 10, 3.8. (B) Excision assay with 136-bp duplexes (25 fmol) containing T[6-4]T. The levels (percentages) of excision were as follows: lane 1, not detectable; lane 2, 8.2; lane 3, 8.8; lane 4, 7.1; lane 5, not detectable; lane 6, 8.1; lane 7, 9.0; lane 8, 6.1; lane 9, 6.5; and lane 10, 6.9. Sizes are indicated in nucleotides.

are highly dependent on the cell lines (49), and hence this difference cannot be attributed simply to the difference in the mismatch repair status in the absence of complementary data.

Such data can be obtained by complementation experiments with mutant CE. We initially carried out these experiments with a simple T<>T lesion and saw no complementation (data not shown). However, since hMutSα binds only weakly to a simple T<>T but with higher affinity to a T<>T with a G-T mismatch, we reasoned that we would be more likely to see the stimulatory effect of mismatch repair system on excision repair with this compound lesion substrate. Figure 4A (lanes 5 to 10) shows the results of these experiments. Two points are noteworthy. First, the T<>T compound lesion was excised twofold more efficiently by HeLa CE than a T<>T simple lesion (compare lanes 2 and 6). This finding raised the possibility that the high-affinity binding of hMutSα to the T<>T compound lesion might contribute to more efficient repair by aiding in damage recognition. However, the same level of stimulation was observed with CEs from the hPMS2 mutant HEC-1-A (50) (compare lane 3 with lane 7) and the hMSH2 mutant LoVo (66) (compare lane 4 with lane 8), revealing that a T<>T compound lesion is a better substrate than a T<>T simple lesion regardless of the cell's mismatch repair status. Second, complementation assays with HEC-1-A and LoVo CEs (lanes 9 and 10) failed to improve the excision activity above that seen with HEC-1-A CE alone, again suggesting that the mismatch repair system plays no role in damage excision from either simple or compound T<>T lesions.

When the same experiments were performed with a T[6-4]T substrate, essentially the same results were obtained but with two important differences (Fig. 4B). First, under identical conditions, the T[6-4]T lesion was excised threefold more efficiently than a T<>T lesion across the board, in agreement with data in Fig. 1 showing that T[6-4]T is the better substrate. Second, in contrast with the drastic effect of a mismatch on the excision rate of a T<>T lesion, the rates of excision of T[6-4]T lesion from a normal duplex and a complex lesion are identical in CE (Fig. 4B) and in reconstituted excision nuclease (see Fig. 6). From the point of view of mismatch repair and excision repair overlap, however, the results with T[6-4]T completely parallel those obtained with T<>T; namely, CEs from mismatch repair mutants carry out excision repair efficiently, and mixing of CEs from two different complementation groups of mismatch repair did not improve the efficiency of damage excision (Fig. 4B; compare lanes 7 and 8 with lanes 9 and 10).

Effect of hMutSα and hMutLα on excision repair. Experiments with cisplatin-1,2-d(GpG) diadduct and CEs gave results more similar to those obtained with the T<>T substrate. However, in light of known affinity of hMutSα for the cisplatin adduct (13) (Fig. 3), we decided to conduct experiments by adding purified hMutSα or hMutLα to either mutant (LoVo and H6) CE or the excision nuclease reconstituted from purified proteins (45, 46). The results of the experiments with CEs are summarized in Fig. 5. Clearly, hMutSα does not affect the efficiency of excision of compound T<>T or cisplatin lesion by the LoVo CE (lanes 1 to 5). Likewise, hMutLα did not com-

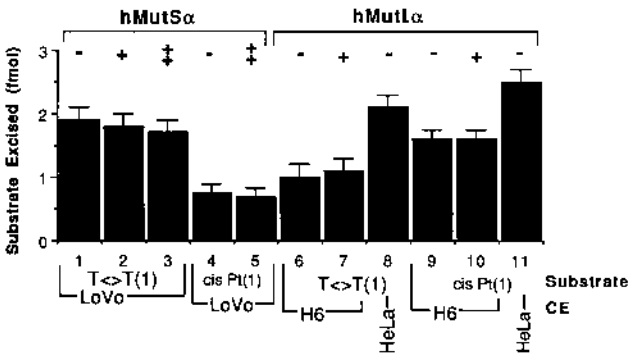

FIG. 5. Effects of hMutSα and hMutLα on excision of T<>T or cisplatin 1,2-d(GpG) [cis Pt(1)] from a compound lesion by CEs of mismatch repair mutants. The experiments were conducted as described for Fig. 4, and the level of excision was quantified with a PhosphorImager. Lane 2 contained 6.3 nM hMutSα; lanes 3 and 5 contained 19 nM hMutSα. Bars indicate standard errors. Fifty nanograms of hMutLα was present in lanes 7 and 10. Lanes 6, 7, 9, and 10, excision reactions with H6 CE only; lanes 8 and 11, reactions with HeLa CE only. The H6 nuclear extract was prepared by a procedure developed for measuring mismatch repair activity (24).

plement the excision nuclease activity in H6 CE, which is defective in the MLH1 mismatch repair factor (31, 44) (lanes 6, 7, 9, and 10). Furthermore, Fig. 5 (lanes 6 to 11) shows that the H6 extract is active in excision repair of both thymine dimer and cisplatin. Although the H6 CE seemed to be less active than HeLa CE, the excision activity of H6 CE was not affected by supplementing the extract with hMutLα, reinforcing the conclusion that in a cell-free system, the presence or lack of mismatch repair activity has no effect on excision repair.

To eliminate any uncertainty arising from our use of the rather ill-defined CE excision repair system, we repeated the experiments with the purified excision nuclease. The experiments were conducted with the compound lesion in the presence or absence of hMutSα and quantified. As shown in Fig. 6, like a T<>T and unlike a T[6-4]T compound, cisplatin 1,2-d(GpG) diadduct is removed with about twofold-higher efficiency than the simple lesion (compare lanes 1 and 3), regardless of the presence of hMutSα (lanes 2 and 4). Taken together with the data for CEs, our results suggest that mismatch repair proteins do not participate in or otherwise influence the damage removal carried out by human excision nuclease in vitro.

Effect of multiple mismatches on excision repair. The T[6-4]T lesion, which is the most efficiently excised damage of the three substrates tested in this study, breaks the hydrogen bonds of the two bases which make up the photoproduct (30, 62). In contrast, T<>T and cisplatin 1,2-d(GpG) distort the helix, but neither T<>T (29, 30, 63) nor cisplatin 1,2-d(GpG) intrastrand cross-link (61) disrupts the hydrogen bonds of the modified bases. Interestingly, breaking the hydrogen bonds of one base of the latter two lesions greatly stimulated their rates of removal, but introduction of a mismatch across from T[6-4]T had no effect on its rate of repair (Fig. 4B and 6). Since a bubble generated by the TFIIH helicase function of human excision nuclease is presumed to be an intermediate in the reaction pathway of this enzyme system (52, 67), we reasoned that introducing more mismatches around the lesion may facilitate the preincision complex formation and perhaps obviate the need for some of the excision nuclease factors or further increase the rate of excision.

We constructed T<>T substrates with one, two, or four mismatches 5′ to the T<>T as shown in Fig. 7. The mismatches were introduced on the 5′ side because the preincision bubble is hypothesized to extend to the 5′ incision site of the excision nuclease, which is 17 to 24 nt 5′ to the lesion site (Fig. 2). The substrates were tested for single strandedness by digestion with S1 nuclease (Fig. 7A). By this probe, the T<>T with two mismatches gave a weak signal (not visible in this reproduction); however, the lesion with four mismatches appeared to be single stranded over the entire length of the mismatch (Fig. 7A, lane 10). Attempts to accomplish excision with this substrate containing four mismatches [T<>T(4)] by omitting some of the repair factors failed. Indeed, even a further enlargement to a 10-nt bubble with T<>T(4) did not obviate the need of TFIIH for damage excision (46a). This is in contrast to the role of TFIIH in transcription, where a 12-bp mismatch upstream of the transcription initiation site eliminated the need for TFIIH in transcription by RNA polymerase II (47).

Even though a single mismatch improved the excision efficiency by a factor of 2 to 4 in CE and reconstituted excision nuclease (Fig. 7B; compare lanes 1 and 5 with lanes 2 and 6), two mismatches did not improve it any further. In fact, four mismatches reduced the repair signal slightly compared to the single mismatch (compare lanes 2 and 4 and lanes 6 and 8). The fact that similar trends of excision efficiency were observed with both the CE and the purified excision nuclease suggests that bubble-binding proteins which may be present in the CE do not affect excision repair. This is particularly relevant to the question of mismatch repair/excision repair overlap because of the presence in human cells of an hMutSβ (hMSH2-hMSH3) mismatch recognition complex with high affinity for loop structures (11a).

Excision of T<>T associated with either a 5′ or 3′ T-G mismatch. The results presented so far suggest that compound lesions are more mutagenic than simple lesions because they are removed more efficiently by excision repair and recognized less efficiently by the mismatch repair system. Most cisplatin-caused mutations at GG sequences are G-C to A-T transitions at the 5′ guanine (3, 6), consistent with our use of a G-T mismatch in the cisplatin 1,2-d(GpG) compound lesion (Fig. 2). However, most of the UV-induced mutations at TT sequences are T-A-to-C-G transitions at the 3′ T-A base pair (19, 32). Therefore, in addition to the 5′ T-G mismatches in our T<>T compound lesions, we wished to use 3′ T-G-associated compound lesions to ascertain that the preferential repair of compound T<>T lesions contributes to UV mutagenesis. Figure 8 shows the rates of T<>T removal from 5′ T-G and 3′ T-G compound lesions. As is apparent, the mismatch has the

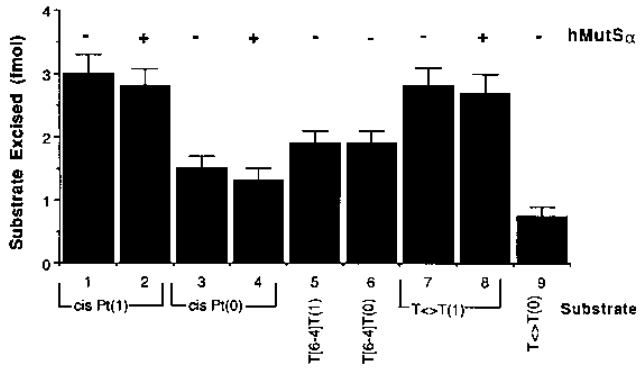

FIG. 6. Effect of hMutSα on the damage excision activity of reconstituted human excision repair nuclease. To the reaction mixture containing 1 nM the indicated substrate (25 fmol) and reconstituted human excision nuclease (46), 19 nM hMutSα was added (lanes 2, 4, and 8). cis Pt, cisplatin.

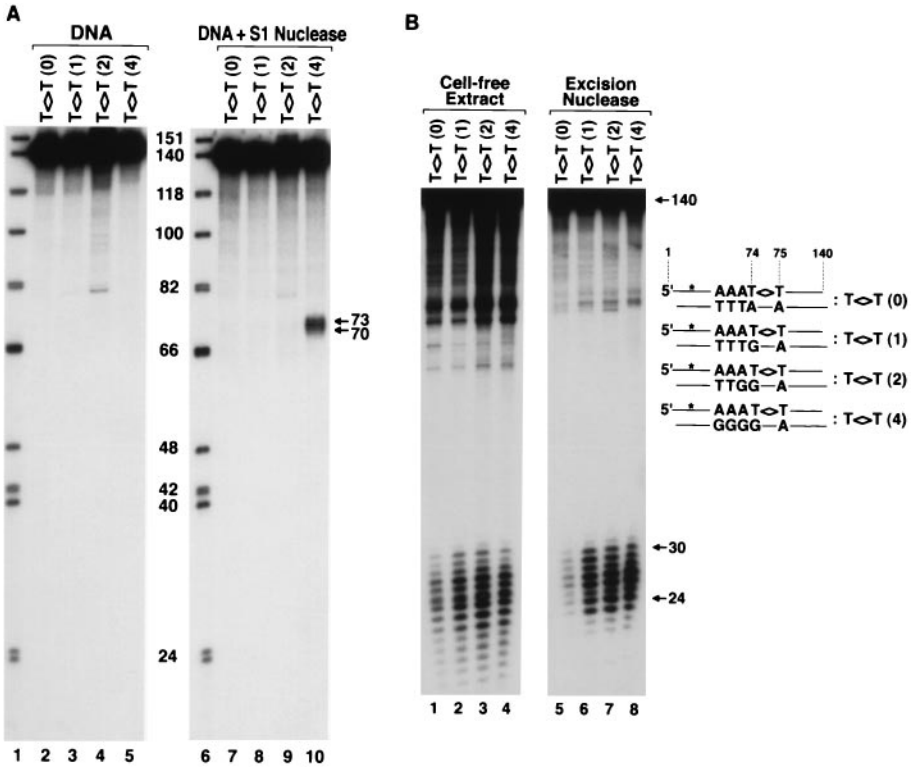

FIG. 7. Effect of the number of mismatches in a compound lesion on the removal efficiency of T<>T by human excision nuclease. (A) Probing for mismatch-induced single strandedness by S1 nuclease. Lanes 1 and 4 contained DNA size markers; lanes 2 to 5 and 7 to 10 contained substrates with the indicated number of mismatches without and with S1 nuclease digestion, respectively (see Fig. 2 for substrate constructs). (B) Excision of T<>T associated with zero to four mismatches by HeLa CE (lanes 1 to 4) and reconstituted human excision nuclease (lanes 5 to 8). The percentages of T<>T excised were as follows: lane 1, 1.9; lane 2, 4.2; lane 3, 4.4; lane 4, 4.0; lane 5, 2.3; lane 6, 9.4; lane 7, 9.6; and lane 8, 9.4. Sizes are indicated in nucleotides.

same stimulatory effect regardless of whether it is across from the 3′ T or the 5′ T of the photodimer. Thus, the most commonly occurring compound lesion, G-T mismatch across from the 3′ T of the T<>T, also stimulates the T<>T excision, and this rapid rate of damage removal may contribute to the differences in UV mutation spectra of repair-proficient and repair-deficient human cell lines.

DISCUSSION

Interfacing of mismatch and excision repair systems. There is no genetic evidence linking excision repair to mismatch correction; in contrast, several in vivo studies both in *E. coli* and in humans have implicated the mismatch repair system in DNA damage repair, in particular in the processing of bulky DNA lesions which can be removed from DNA only by nucleotide excision repair (44). Curiously, it appears that lack of mismatch repair has opposite effects on the cellular response to two DNA-damaging agents, UV and cisplatin. Mismatch repair-defective mutants are sensitive to UV (38, 40, 41) and are deficient in transcription-coupled excision repair of cyclobutane pyrimidine dimers (40, 41). In contrast, mutations in the *mutL* gene in *E. coli* (16) and the h*MLH1* (a *mutL* homolog) gene in humans confer increased resistance to cisplatin (1, 11).

These findings raise two questions: does mismatch repair eliminate bulky lesions from DNA, and why does the mismatch repair defect have opposite effects on lethalities of the two types of agents which cause bulky lesions? The answer to the first question is that the removal of both cyclobutane dimers (the major UV photoproduct) and of cisplatin 1,2-d(GpG) intrastrand cross-link (the major lesion of cisplatin) is absolutely dependent on excision repair (18), and human excision nuclease capable of removing these lesions at near-physiological rates has been reconstituted from purified proteins in the absence of any mismatch repair protein (45, 68). Hence, if the mismatch repair system plays any role in damage excision repair, it can exert a stimulatory effect only on the basal excision activity. However, we did not detect any effect of hMutSα on excision of T<>T or cisplatin in either sequence context by whole CE or excision nuclease reconstituted from purified proteins. Thus, our data do not support a model which involves joint actions of mismatch and excision repair systems to eliminate UV photoproducts from DNA.

Regarding the opposite effects of mismatch repair defect on cellular resistance to UV and cisplatin, it is of special relevance that hMutSα can bind to cisplatin 1,2-d(GpG) (13). Such binding may lead to death rather than survival. The discovery of genetic defects in the GTBP/p160 subunit of MutSα in cell lines selected for resistance to *N*-methyl-*N*′-nitro-*N*-nitrosoguanidine (4, 20, 28) has led to the suggestion that the mismatch repair system plays a role as a sensor of genetic damage in the general sense, that is, as a sensor of structural anomalies whether they arise from base mismatch or base damage (23, 28). Furthermore, it has been suggested that a signaling reaction initiated by such an interaction could lead to cell death by either a futile excision and resynthesis reaction provoked by the damage or by somehow activating an apoptotic response (1, 11). Whatever the mechanism, in this study we found no

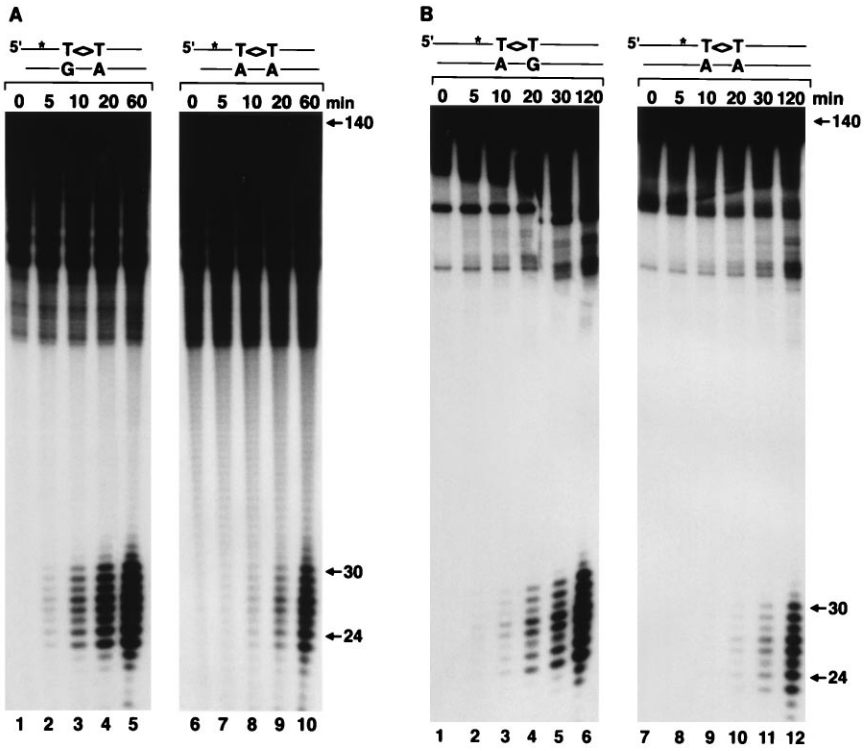

FIG. 8. Kinetics of excision of T<>T by reconstituted excision nuclease from a compound lesion with a 5′ or 3′ G-T mismatch. (A) 5′ G-T mismatch. Excision levels (as percentages of input substrate) were as follows: lane 1, not detectable; lane 2, 0.2; lane 3, 1.1; lane 4, 3.6; lane 5, 8.8; lanes 6 and 7, not detectable; lane 8, 0.1; lane 9, 0.6; and lane 10, 1.8. (B) 3′ G-T mismatch. The excision levels as percentages were as follows: lanes 1 and 2, not detectable; lane 3, 0.1; lane 4, 4.1; lane 5, 8.5; lane 6, 13; lanes 7 to 10, not detectable; lane 11, 1.1; and lane 12, 3.3. Sizes are indicated in nucleotides.

supporting evidence for the interfacing of mismatch repair and excision repair systems as a cause of cisplatin cytotoxicity.

It must be noted, however, that this study focused on excision repair in the absence of transcription. It has been reported that mutations in *mutL* and *mutS* in *E. coli* (40) and the human homologs of these genes (41) abolish transcription-coupled repair. Currently, there is no in vitro system for eucaryotic transcription-coupled excision repair, and hence this issue remains for future investigations (22). However, there are several observations which are relevant to the question. First, the *E. coli* transcription-coupled excision repair system has been reconstituted with purified proteins in vitro and is independent of mismatch repair proteins (53). In addition, CEs from *E. coli mutL* and *mutS* mutants are capable of performing transcription-coupled repair, and the addition of purified MutS protein to the *mutS* CE had no effect on the level of transcription-repair coupling (54). It has been found that in the yeast *S. cerevisiae*, whose mismatch repair and excision repair systems are structurally and functionally highly similar to the human repair systems (21, 31, 48), mutations in mismatch repair genes did not affect the preferential removal of T<>T from the transcribed strand (59). Thus, in light of currently available data, it is more likely that the mismatch repair system does not directly participate in coupling of excision repair to transcription. Perhaps the drastic effect of mismatch repair defect on DNA metabolism interferes with coupling by yet undiscovered mechanisms.

Mutagenic excision repair and mutation fixation. UV mutagenesis has been classified as type I or type II, depending on requirement for excision repair (35). Type I is dependent on replication and is presumed to result from translesion synthesis. Type II is dependent on excision repair. It can occur even in the absence of replication and is presumed to result from misincorporation by bypass synthesis during filling in of an excision gap in which the template strand contains a UV photoproduct. Recent in vitro studies have provided evidence that a significant fraction of UV-induced mutations may arise from type II mutagenesis (9, 65). Although this classification was originally made for UV-induced mutations in *E. coli*, evidence exists that damage-induced mutations may occur by similar mechanisms in humans (51). Here we show that excision repair of compound T<>T lesions may contribute to mutagenesis by these photoproducts.

Thymine cyclobutane dimer (T<>T), compared to T[6-4]T, is a notoriously poor substrate for excision repair (42, 58, 60). Unlike T[6-4]T, the cyclobutane dimer is also a poor replicational block (7, 57, 64), allowing translesion replication with occasional misincorporation (62). Even though the misincorporation frequency is low (19, 57), it is expected that compound lesions involving a T<>T and a mismatch would occur at a relatively high frequency (due to the abundance of T<>T). Our data show that such lesions are excised about fourfold faster than simple T<>T lesions by excision nuclease and are bound hMutSα (and presumably processed) with about twofold-lower affinity than a simple G-T mismatch. A possible outcome would be the removal of the damaged bases by excision nuclease before the removal of the mismatched base by the mismatch repair system, and hence mutation fixation by excision repair (Fig. 9).

In addition to UV-induced mutations, the putative mutagenesis pathway presented in Fig. 9 is expected to contribute to cisplatin-induced mutations as well, because a compound lesion of cisplatin 1,2-d(GpG) cross-link exhibits the same effect on excision and mismatch repair systems as the T<>T com-

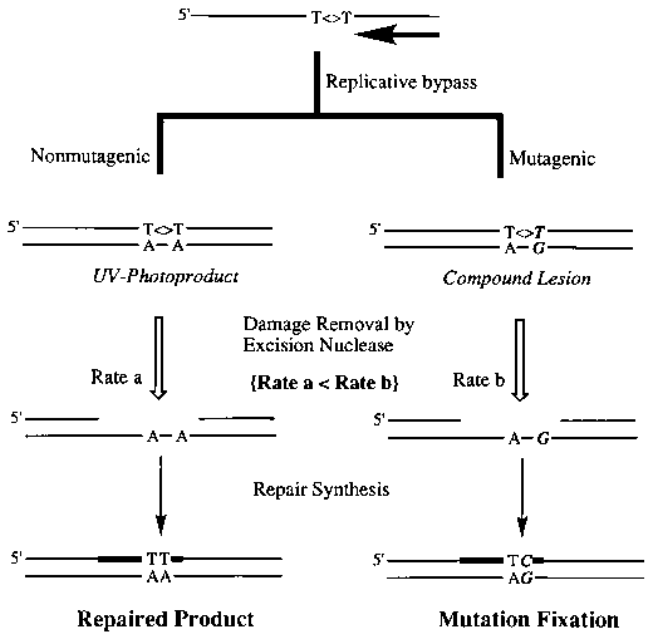

FIG. 9. Mutation fixation by excision repair. Misincorporation during translesion synthesis generates a compound lesion which is a poor substrate for binding of hMutS family proteins. Hence, the G-T pair in the compound lesion escapes mismatch correction. In contrast, T<>T [or cisplatin 1,2-d(GpG)] in compound lesions is removed about fourfold faster, leading to generation of a homoduplex by gap filling and hence fixation of the mutation before it can be corrected. A second possible biological source of compound lesions (which is not discussed here) is the high susceptibility of mispaired bases to spontaneous hydrolysis (17), attack by metabolites (5), and environmental carcinogens (55).

pound lesion. On the contrary, since T[6-4]T in a compound lesion is not a better substrate than a simple T[6-4]T lesion, and since T[6-4]T is a strong block to replication, the proposed mutagenesis is not expected to play an important role in mutations induced by T[6-4]T. The model in Fig. 9 predicts that mutations at TT sequences which arise almost exclusively from T<>T constitute a higher fraction of mutations in repair-proficient cells than in repair-deficient cells. Indeed, a recent study with a UV-irradiated shuttle vector found that wild-type cells had a 12-fold increase in mutations at A-T pairs compared to an xeroderma pigmentosum (XP) group A mutant, and a literature survey of p53 mutations found in XP and non-XP cancers revealed the same tendency (32). However, these conclusions are based on a relatively small data set. More studies of this type are needed to solidify the proposed model.

Finally, the finding that compound lesions which destabilize the helix are excised faster than the same lesions in an otherwise base-paired duplex suggests that helix unwinding is on the reaction path of human excision repair nuclease. Further research using probes for DNA melting on DNA-protein complexes formed with subassemblies of the excision nuclease is needed to provide more definitive data on the structure of DNA in the pre- and postincision excision nuclease complexes.

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