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Strand-specific mismatch correction in nuclear extracts of human and *Drosophila melanogaster* cell lines

(DNA repair/gene conversion/base-pair mismatch)

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ABSTRACT **Nuclear extracts derived from HeLa and *Drosophila melanogaster* K_C cell lines have been found to correct single base-base mispairs within open circular DNA heteroduplexes containing a strand-specific, site-specific incision located 808 base pairs from the mismatch. Correction in both extract systems is strand specific, being highly biased to the incised DNA strand. Different mispairs within a homologous set of heteroduplexes were processed with different efficiencies (G·T > G·G ≅ A·C > C·C), and correction was accompanied by mismatch-dependent DNA synthesis localized to the region spanning the mispair and the strand break, thus demonstrating that mismatch recognition is associated with the repair reaction. Correction of each of these heteroduplexes was abolished by aphidicolin but was relatively insensitive to the presence of high concentrations of ddTTP, indicating probable involvement of α and/or δ class DNA polymerase(s). These findings suggest that higher eukaryotic cells possess a general, strand-specific mismatch repair system analogous to the *Escherichia coli mutHLS* and the *Streptococcus pneumoniae hexAB* pathways, systems that contribute in a major way to the genetic stability of these bacterial species.**

DNA mismatch correction is best understood in *Escherichia coli*, where the process has been addressed by both biological and biochemical methods. This organism possesses several systems for mismatch correction, with the most extensively studied being the MutHLS-dependent, methyl-directed pathway that serves to enhance the accuracy of chromosome replication and ensures the fidelity of genetic exchange (1–4). This system, which is capable of processing a variety of mispairs, catalyzes a strand-specific reaction in which repair is targeted to a DNA strand that lacks d(GATC) methylation or contains a persistent strand break (5, 6).

Much less is known about the mechanisms and functions of mismatch repair in higher cells. Mutations in yeast *PMS1*, *PMS2*, and *PMS3* loci confer a mutator phenotype, a high frequency of postmeiotic segregation, and a defect in mismatch correction (7–9). Although the mispair specificity of the *PMS*-dependent pathway is similar to that of the *E. coli* methyl-directed system and while the *PMS1* gene product has been shown to be homologous to the bacterial MutL protein (9), no evidence for strand specificity of the yeast pathway has been presented. Compelling support for the occurrence of mismatch correction in several higher eukaryotic systems has also been reported. Evidence to this effect has been based on the demonstration that different mispairs are subject to differential processing when introduced into mammalian cells by heteroduplex transfection (10). As in the case of yeast, however, it is not clear whether higher eukaryotes possess a strand-specific correction pathway that is capable of processing a set of distinct mispairs in a manner analogous to the bacterial methyl-directed system. Although the transfection results of Hare and Taylor (11) have suggested that DNA strand breaks or methylation might serve to determine strand specificity of correction in mammalian cells, the mismatch rectification events observed in their experiments were not shown to be provoked by the mispairs tested.

In this paper, we report nuclear extract systems derived from *Drosophila* K_C and HeLa cell lines that support efficient, strand-specific mismatch correction *in vitro*. Efficiency of correction in both systems is dependent on the nature of the mispair, indicating that mismatch recognition is associated with repair.

MATERIALS AND METHODS

DNA Heteroduplex Preparation. Circular heteroduplex DNAs containing a single base-base mispair and a site-specific single-strand break (Fig. 1) were prepared as described (12) using f1MR phage DNAs (13), except that ligation and CsCl centrifugation steps were omitted. After heteroduplex annealing and removal of excess circular, single-stranded DNA by hydroxylapatite chromatography (12), the resulting mixture of open circular heteroduplex and linear homoduplex DNAs was treated with *Micrococcus luteus* exonuclease V (United States Biochemical) to hydrolyze the latter species. After incubation with this enzyme (0.25 unit per μg of DNA), which was performed according to the manufacturer's recommendations except that $MgCl_2$ was present at 5 mM, the reaction mixture was extracted with phenol, extracted with ether, and precipitated with ethanol. DNA was dissolved in 0.01 M Tris·HCl, pH 7.6/0.3 M NaCl/1 mM EDTA, and the open circular heteroduplex was purified by Sephacryl S-300 chromatography in 0.01 M Tris·HCl, pH 7.6/0.3 M NaCl/1 mM EDTA. Purified DNA was precipitated with ethanol and then dissolved in 0.01 M Tris·HCl, pH 7.6/1 mM EDTA. Control homoduplexes lacking a mismatch were prepared in an identical manner.

Preparation of *Drosophila* and HeLa Cell Extracts. HeLa S_3 cells were grown at 37°C in Joklik's modified medium containing 5% fetal calf serum to a density of 4–7 × 10^5 cells per ml. *Drosophila* K_C cells were grown according to Price *et al.* (14). Subsequent steps were performed at 0°C–4°C, and cited phenylmethylsulfonyl fluoride (PMSF) concentrations are expressed as volume percent based on addition of an isopropanol solution saturated at room temperature with the protease inhibitor. Harvesting of HeLa cells and preparation of nuclei were performed according to Challberg and Kelly (15) except that all buffers contained 0.1% PMSF, and nuclei were not frozen. *Drosophila* nuclei were prepared in a similar manner except that cells were harvested at 4000 × *g* for 5 min and nuclei were obtained from lysed cells by centrifugation at 9000 × *g* for 5 min. Subsequent steps were identical for *Drosophila* and HeLa extracts. Nuclear pellets were resuspended in 0.05 M Hepes, pH 7.5/0.5 mM dithiothreitol/0.1% PMSF/10%

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Abbreviation: PMSF, phenylmethylsulfonyl fluoride.

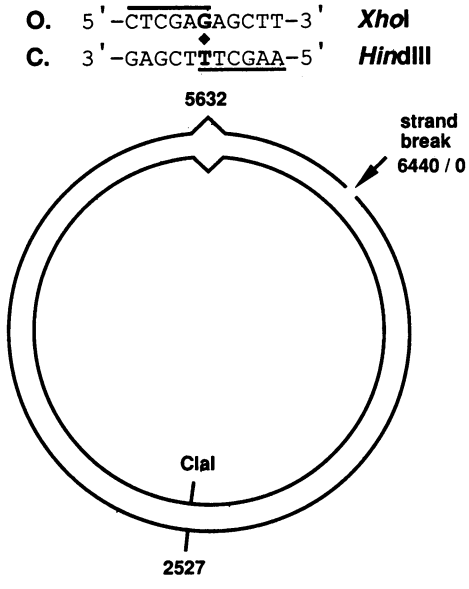

FIG. 1. Heteroduplex substrates for mismatch correction. Each 6440-base-pair heteroduplex contained a strand-specific break at position 6440 and a single base-base mispair at position 5632. The presence of the mismatch within overlapping recognition sites for two restriction endonucleases renders the DNA resistant to hydrolysis by either enzyme, with correction conferring sensitivity to one endonuclease or the other depending on the strand that is repaired. O and C, orientation of the mispair relative to open and covalently closed strands, respectively, of the heteroduplex.

(wt/vol) sucrose (2.5 ml per liter of cell culture). After addition of 0.031 vol of 5 M NaCl, the nuclear suspension was mixed on a Lab Quake shaker (Labindustries) for 1 hr. Nuclei were centrifuged at 15,000 × *g* for 20 min, and the resulting supernatant was concentrated by ammonium sulfate precipitation (0.42 g/ml). The ammonium sulfate pellet was collected, dissolved, and dialyzed as described (12) except that buffers were supplemented with 0.1% PMSF. After clarification by centrifugation at 14,000 × *g* for 15 min, this fraction (20–50 mg of protein per ml as determined by the Bradford assay) was frozen in small aliquots in liquid N_2 and stored at −70°C.

Mismatch Repair and Endonuclease Assays. Mismatch correction in HeLa and *Drosophila* nuclear extracts was determined in a manner similar to that described for *E. coli* methyl-directed mismatch correction (13). Reaction mixtures (10 μl) contained 0.02 M Tris·HCl (pH 7.6), 0.04–0.08 M KCl (extracts were titrated with KCl to determine the optimal concentration), 5 mM $MgCl_2$, 1 mM glutathione, bovine serum albumin (50 μg/ml), 0.1 mM each dNTP, 1.5 mM ATP, 0.1 μg (24 fmol) of heteroduplex DNA, and nuclear extract (4–18 mg/ml). After incubation for 60 min at 30°C (*Drosophila*) or 15 min at 37°C (HeLa), reactions were terminated by the addition of 30 μl of 25 mM EDTA, 0.67% SDS followed by treatment with proteinase K (50 μg/ml) for 15 min at 37°C. After phenol extraction and ethanol precipitation (12), extent of mismatch correction was quantitated by determination of sensitivity to appropriate restriction enzymes (13). Endonuclease activity in HeLa nuclear extracts was determined as for mismatch repair reactions except that supercoiled, homoduplex f1MR3 DNA (13) was used as substrate, and DNA was not subject to restriction endonuclease digestion prior to electrophoresis.

RESULTS

Mismatch Correction in Nuclear Extracts Is Directed by a DNA Strand Break. The methylation state of d(GATC) sequences determines the strand specificity of mismatch correction by the *E. coli* methyl-directed pathway, but it has been shown that a persistent strand break can bypass the requirements for both d(GATC) sequences and MutH, the protein that recognizes such sites (5, 6, 16). A DNA terminus is also thought to be the natural signal governing strand specificity of mismatch correction in *Streptococcus pneumoniae* (17), and the transfection results of Hare and Taylor (11) suggest that a strand break may suffice to determine strand specificity of mismatch repair in mammalian cells. We have therefore tested a set of circular heteroduplex molecules that contained a site-specific, strand-specific break as substrates for mismatch correction in nuclear extracts of *Drosophila* and human cells. As shown in Fig. 1, each heteroduplex contained a base-base mismatch located 808 base pairs from the single strand break. The placement of the mismatch within overlapping recognition sites for two restriction endonucleases permits assessment of strand specificity of repair, and since the location and the immediate sequence environment of each mismatch is identical from heteroduplex to heteroduplex, any differential sensitivity to repair can be attributed to differences in the efficiency of recognition of the different mispairs (13).

Fig. 2 demonstrates that concentrated nuclear extracts derived from *Drosophila* K_C or HeLa cell lines process G·T, A·C, G·G, and C·C open circular heteroduplexes in a strand-specific manner, with repair in each case being highly biased to the incised DNA strand (Table 1). Both systems were effective in mediating *in vitro* repair, with more than 50% of the input G·T heteroduplex being corrected in HeLa nuclear extracts during an hour of incubation (Table 1), an efficiency comparable to that observed for the methyl-directed reaction in *E. coli* extracts at similar protein concentrations (12, 13). No detectable correction of a covalently closed circular heteroduplex was observed with *Drosophila* nuclear extracts, nor was correction detected on the covalently continuous strand of open circular molecules with such extracts. In contrast, the covalently closed form of the G·T heteroduplex was subject to significant levels of repair by HeLa nuclear extracts, but this reaction displayed little strand bias (Fig. 1, Table 1). This finding is similar to that of Brown and Jiricny (10), who observed that correction of covalently closed circular heteroduplexes occurred with limited strand bias upon transfection into mammalian cells. Although the

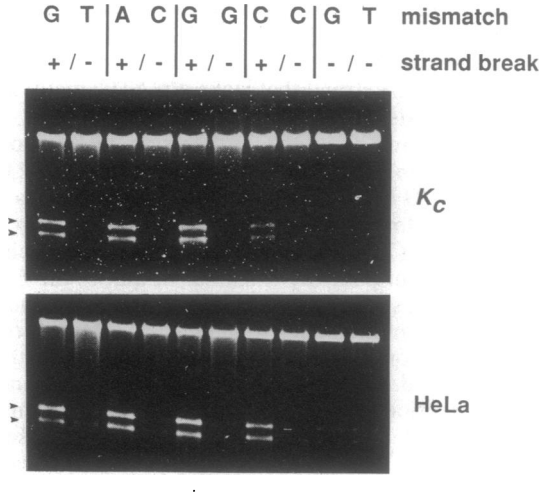

FIG. 2. Strand-specific repair of heteroduplex DNAs in *Drosophila* K_C and HeLa nuclear extracts. Repair reactions, with nuclear extract from *Drosophila* K_C (12 mg/ml) or HeLa (16 mg/ml) cell lines, were performed as described in *Materials and Methods*. DNA products were digested with *Cla* I and the appropriate restriction endonucleases to score mismatch correction occurring on each DNA strand. Arrowheads indicate the 3.34- and 3.10-kilobase fragments diagnostic of repair. The presence or absence of a strand-specific break is indicated by + or −.

Table 1. Efficiency of mismatch repair in *Drosophila* K_C and HeLa nuclear extracts

Mismatch	Correction, fmol			
5'-TCGAXAGCT	*Drosophila* K_C		HeLa	
3'-AGCTYTCGA	Open strand	Closed strand	Open strand	Closed strand
Open/closed				
G·T	7.4 (7.9–10)	<0.5	9.2 (14–17)	0.9
G·G	5.5 (5.2)	<0.5	9.5 (8.6)	1.3
A·C	6.7 (3.6)	<0.5	8.9 (8.1)	1.1
C·C	2.6 (1.7)	<0.5	5.9 (2.8)	1.3
G·T (ccc)	—	<0.5/<0.5	—	2.9/3.1

Other than the exceptions noted below, all mismatch repair reactions (10 μl, 24 fmol of open circular heteroduplex) were performed as described in *Materials and Methods*. Results shown in parentheses were obtained in a similar manner except that each reaction mixture (20 μl) contained 24 fmol of open circular G·T substrate as well as 24 fmol of A·C, G·G, or C·C heteroduplex, and incubation was at 30°C (*Drosophila*) or 37°C (HeLa) for 60 min. Parenthetic values for A·C, G·G, and C·C thus reflect strand-specific correction of these molecules occurring in competition with G·T repair. Conversely, the range of values shown in parentheses for the G·T heteroduplex indicates the level of G·T repair in the presence of A·C, G·G, or C·C substrates. O and C, open and covalently closed strands of open circular heteroduplex DNAs, respectively, used in these experiments. Thus, correction occurring on the open strand of the G·T heteroduplex yields an A·T base pair. The last entry illustrates the substrate activity of the covalently closed circular (ccc) form of the G·T heteroduplex, which was prepared as described (13). Repair values for the individual DNA strands are separated by a slash.

covalently continuous strand of open circular heteroduplexes was also subject to detectable correction by HeLa extracts, the presence of a preexisting strand break was sufficient to direct repair to the incised DNA strand, resulting in a correction strand bias of 4.5:1 (C·C) to 10:1 (G·T) as compared to only 0.9:1 for the covalently closed form of the G·T substrate (Table 1). A strand break can thus target mismatch repair occurring in human nuclear extracts. We attribute correction events on strands that were originally continuous to random strand incision by endonucleolytic activities, which were demonstrable in HeLa nuclear extracts (data not shown).

Mismatch Specificity of Repair Events. The heteroduplex substrates used in the experiments summarized above are highly homologous. With the exception of the mismatch, G·G and C·C heteroduplexes are identical, as are G·T and A·C substrates (13). The only difference, other than identities of the mispairs, between these two sets of heteroduplexes is the presence of a G·C base pair at coordinate 5626 in G·G and C·C DNAs and a T·A at this position in G·T and A·C heteroduplexes. Nevertheless, these heteroduplexes were subject to differential repair in both *Drosophila* and human nuclear extracts. Analysis of individual heteroduplexes suggested that G·T, G·G, and A·C are better substrates than C·C for correction by *Drosophila* and HeLa extracts (Table 1). The substrate specificities of both systems were examined in a more rigorous manner by a competition assay in which the A·C, G·G, or C·C heteroduplex was incubated with nuclear lysate in the presence of an equimolar concentration of the G·T substrate. These experiments (Table 1, parenthetic values) showed that each of the heteroduplexes is processed with a characteristic efficiency. Given the highly homologous nature of this set of substrates, their differential repair implies that mismatch recognition is associated with the correction events observed in both *Drosophila* and human extracts.

Table 2. Nucleotide cofactor requirements for mismatch repair in nuclear extracts

Omission	Mismatch repair, fmol	
	Drosophila K_C	HeLa
Complete	7.9	7.8
− ATP	<0.5	<0.5
− dTTP	<0.5	<0.5
− dGTP	<0.5	<0.5
− dATP and dCTP	<0.5	<0.5

Mismatch repair assays were performed as described in *Materials and Methods* using a G·T heteroduplex with nucleotide omissions as indicated.

Requirements for Correction in Nuclear Extracts and Involvement of an Aphidicolin-Sensitive DNA Polymerase. As observed for methyl-directed mismatch correction in *E. coli* extracts, the only exogenous cofactors required for *in vitro* repair by HeLa or *Drosophila* cell-free systems were ATP and the four dNTPs. As shown in Table 2, correction was abolished upon omission of ATP or one or more of the dNTPs. *In vitro* repair was also extremely sensitive to inhibition by aphidicolin. As shown in Fig. 3, correction of the G·T heteroduplex by HeLa nuclear extracts was inhibited by aphidicolin at 25 μg/ml but was only slightly reduced in the presence of ddTTP at a concentration 10 times that of dTTP. Inhibition by aphidicolin and relative insensitivity to the presence of ddTTP was also characteristic of A·C, G·G, and C·C repair in HeLa nuclear extracts, and identical results were obtained with the G·T substrate in nuclear extracts from *Drosophila* K_C cells (data not shown). Aphidicolin is a

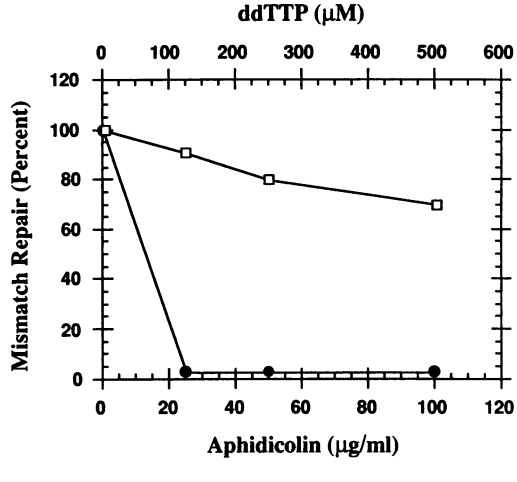

FIG. 3. Effects of DNA polymerase inhibitors on mismatch repair in HeLa nuclear extracts. Mismatch correction of the open circular G·T heteroduplex (see Fig. 1) was assayed as described in *Materials and Methods* except that ddTTP or aphidicolin was present as indicated, reaction mixtures were incubated 60 min at 37°C, and the concentration of dTTP was reduced to 50 μM in reaction mixtures containing the dideoxynucleotide. ●, Aphidicolin; □, ddTTP.

specific inhibitor of the α and δ classes of DNA polymerase (18), while dideoxynucleotides have been shown to be potent inhibitors of the β DNA polymerase under the conditions tested (19, 20).

Mismatch-Provoked DNA Synthesis Is Associated with Correction. Mismatch repair by the *E. coli* methyl-directed pathway is associated with nonrandom repair DNA synthesis (21). A similar effect is shown in Fig. 4 for correction of the G·T heteroduplex in HeLa nuclear extract. The pattern of synthesis supported by the G·C control homoduplex is that expected for random repair incorporation, indicating that presence of a strand break was insufficient to promote localized synthesis. In contrast, localized DNA synthesis spanning the shorter distance between the mispair and the strand break was observed with the G·T substrate (Fig. 4*A*, fragment Aa). Moreover, cleavage of fragment Aa with *Hin*dIII, which only hydrolyzes the G·T → A·T repaired species, demonstrated an even greater enrichment for sequences that had participated in DNA synthesis (Fig. 4*B*). Since G·C and G·T substrates used in these experiments are identical except for the mispair, these observations prove that the localized synthesis observed in the G·T heteroduplex was provoked by the mismatch.

DISCUSSION

The results summarized above have led us to conclude that both *Drosophila* and human cells are capable of recognizing and processing base-pair mismatches in a strand-specific manner, with a DNA terminus being sufficient to provide directionality in both systems. Since G·T, A·C, G·G, and C·C heteroduplexes responded in the same manner to a strand break and displayed similar sensitivities to DNA polymerase inhibitors, it also seems probable that the repair events described here are mediated by a single pathway. The conclusion that mismatch recognition is involved in provocation of these events is based on two findings. First, members of a homologous set of heteroduplexes were subject to differential correction when present in the same extract. Second, repair was accompanied by differential DNA synthesis that occurred only on molecules containing a mispair and was localized to the region containing the strand break and the mismatch. It therefore seems highly unlikely that the correction events described above are a consequence of nonspecific exonuclease attack, commencing at the strand break and followed by repair of the ensuing random gaps.

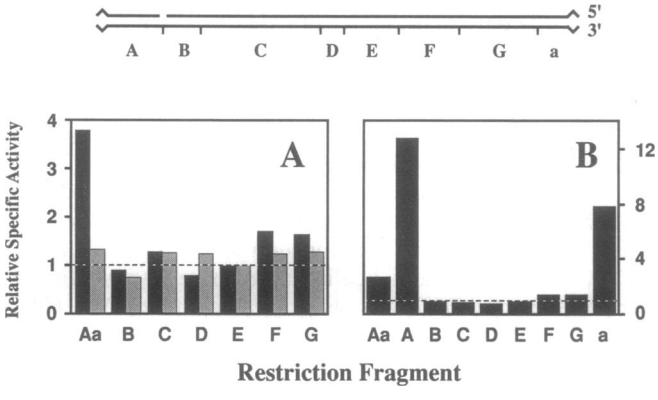

FIG. 4. Mismatch-provoked DNA synthesis. Mismatch repair in HeLa nuclear extract was performed as described in *Materials and Methods* except that reaction mixtures were scaled up to 0.14 ml, and [α-^{32}P]dTTP (2.9–7.3 × 10^4 cpm/pmol) was present at 50–100 μM. DNA substrates (1.4 μg) used were the G·T heteroduplex (see Fig. 1) or an otherwise identical G·C homoduplex (see *Materials and Methods*). After quench and phenol extraction, DNA was separated from unincorporated nucleotide by filtration through Sephacryl-300, and samples were hydrolyzed with restriction endonucleases (see below). Products were separated by electrophoresis on both 1.2% agarose gels and 5% polyacrylamide gels. DNA bands were visualized and excised, and labeled material was extracted from the gel matrix by using Protosol (polyacrylamide) or by heating in 1 M HCl (agarose). ^{32}P was determined by liquid scintillation counting, and specific radioactivity was calculated as the quotient of cpm and the number of A·T base pairs present in a given restriction fragment. Specific activities shown are normalized to those of fragment E, which was resolved from other DNA species in both gel systems. Results are averages of two experiments. (*A*) G·T heteroduplex (solid bars) and G·C homoduplex (stippled bars) were hydrolyzed with *Hga* I and *Hin*cII after isolation from the nuclear extract. (*B*) G·T heteroduplex was treated as in *A* except that hydrolysis also included *Hin*dIII. This endonuclease cleaves restriction fragment Aa into A and a in G·T → A·T corrected molecules. Repair was 28–30% of input DNA, and specific radioactivities shown for fragments Aa, A, and a in *B* are corrected for the fact that they are present in reduced molar yield relative to other hydrolytic products (0.7 mol/mol for Aa and 0.3 mol/mol for A and a).

The features of the repair systems described here differ from previously identified mismatch repair activities in extracts of eukaryotic cells. Muster-Nassal and Kolodner (22) have described mismatch-specific correction of covalently closed circular heteroduplexes in lysates of mitotic *Saccharomyces cerevisiae*. However, these experiments did not address signals that might govern strand specificity of repair other than the natural asymmetry of a mismatch or its sequence environment. In higher eukaryotes Glazer *et al.* (23) used an *E. coli* transfection assay to monitor heteroduplex correction in HeLa cell lysates, while Brooks *et al.* (24) used restriction enzyme cleavage to score mismatch repair in *Xenopus* egg extracts. The G·T and A·C heteroduplexes examined in each of these studies were corrected *in vitro*, but efficiency of repair was low (1% of input DNA in HeLa extracts, 6% in the *Xenopus* system), and neither mismatch specificity nor strand specificity was demonstrated. The terminus-directed reaction reported here is also distinct from that identified in HeLa nuclear extracts by Wiebauer and Jiricny (25), who used a synthetic oligonucleotide substrate. The G·T mispair within the oligonucleotide heteroduplex was specifically processed to a G·C base pair, possibly via action of a G·T-specific thymine glycosylase (25). Since this reaction is thought to function in the processing of G·T mismatches resulting from deamination of 5-methylcytosine in a G·m^{5}C base pair (25), it may represent an analogue of *E. coli* very short patch repair (26).

While the G·T reaction identified by Wiebauer and Jiricny appears similar to bacterial very short patch correction, the terminus-directed repair reaction that we have identified resembles the general, strand-specific repair pathways characterized in bacteria. As in the case of the methyl-directed system of *E. coli* (1–3) and the *hex* pathway of *S. pneumoniae* (17), HeLa and *Drosophila* nuclear extracts mediate strand-specific correction and can process distinct classes of mispairs, with C·C being the weakest substrate. This parallel may also extend to the DNA polymerase requirement. *E. coli* methyl-directed correction is dependent on DNA polymerase III holoenzyme (6), an activity that is required for replication (27) and may also participate in repair events (28) but is independent of DNA polymerase I, the conventional repair activity (29). Aphidicolin sensitivity of mismatch correction in nuclear extracts is consistent with the involvement of α and/or δ classes of DNA polymerase, activities that have been implicated in eukaryotic replication and repair (reviewed in refs. 28, 30, and 31), while insensitivity to dideoxynucleotide inhibition excludes a major role for DNA polymerase β, an enzyme that is generally attributed a repair function (28, 32). Although identification of the eukaryotic DNA polymerase(s) involved in the strand-specific mismatch correction will require their isolation, it is noteworthy that a δ activity, which may be distinct from that involved in

replication, has been implicated in repair of UV damage in permeabilized human cells (33).

Occurrence of strand-specific mismatch repair in higher cells suggests that like bacteria (1–4), higher eukaryotes may rely on such systems to ensure genetic stability. Strand specificity is a prerequisite for such a function, and we have shown that DNA termini can suffice in this respect. Although it is not clear that DNA ends represent the natural signal for strand targeting in human or *Drosophila* cells, DNA termini are in principle sufficient to target the postreplication correction of replication errors since growing DNA chains possess at least one end. Strand discrimination by the *hex* pathway of pneumococcus is thought to be determined in this manner (17), and under certain conditions a DNA terminus can determine strand specificity of repair by the *E. coli* methyl-directed system (5, 6). In fact, the function of the *E. coli* protein responsible for strand discrimination in the methyl-directed pathway is to generate a DNA end (6, 16).

The possible existence in higher eukaryotes of a mismatch repair system similar to the methyl-directed and *hex*-dependent bacterial pathways is also consistent with the recent identification of human and mouse genes encoding polypeptides that are highly homologous to the bacterial MutS (34, 35), the protein responsible for mismatch recognition in the methyl-directed pathway (13, 36). In addition, mutations at the *mei-9* locus of *Drosophila* result in elevated postmeiotic segregation frequencies, a finding that has been interpreted in terms of a role for this locus in mismatch repair (37). The *in vitro* assay described here should facilitate analysis of the involvement of such proteins in mismatch correction, the identification of other components involved in the reaction, and testing of the relationship of the eukaryotic reaction to bacterial strand-specific systems.

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