

Hypermutable and Mismatch Repair Deficiency in RER⁺ Tumor Cells

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Summary

A subset of sporadic colorectal tumors and most tumors developing in hereditary nonpolyposis colorectal cancer patients display frequent alterations in microsatellite sequences. Such tumors have been thought to manifest replication errors (RER⁺), but the basis for the alterations has remained conjectural. We demonstrate that the mutation rate of (CA)_n repeats in RER⁺ tumor cells is at least 100-fold that in RER⁻ tumor cells and show by in vitro assay that increased mutability of RER⁺ cells is associated with a profound defect in strand-specific mismatch repair. This deficiency was observed with microsatellite heteroduplexes as well as with heteroduplexes containing single base-base mismatches and affected an early step in the repair pathway. Thus, a true mutator phenotype exists in a subset of tumor cells, the responsible defect is likely to cause transitions and transversions in addition to microsatellite alterations, and a biochemical basis for this phenotype has been identified.

Introduction

A genetic basis for cancer development is now widely accepted, with etiology of the disease attributed to somatic and inherited genetic changes that lead to alterations in cell growth control. Although progressive somatic genetic alterations are associated with development of several human cancers (Weinberg, 1989; Fearon and Vogelstein, 1990; Stanbridge, 1990), the molecular origins of such changes are poorly understood, and the contributions of exogenous versus spontaneous mutagenesis are uncertain. It has been postulated that an early step in carcinogenesis may confer a mutator phenotype that leads to intrinsic genetic instability (Harwood et al., 1991; Loeb, 1991). This proposal is consistent with recent findings that a discrete subset of sporadic colorectal tumors (Ionov et

al., 1993; Thibodeau et al., 1993) and most tumors developing in hereditary nonpolyposis colorectal cancer (HNPCC) patients (Aaltonen et al., 1993) contain frequent mutations within (CA)_n and other simple repeated sequences. Such tumors have been dubbed RER⁺ (for replication error), and the widespread alterations in them have been hypothesized to result from a heritable genetic destabilization that is manifested in tumor cells (Aaltonen et al., 1993; Ionov et al., 1993).

The high prevalence of alterations in simple repeated sequences in RER⁺ tumors could be due to a variety of factors. It could be caused by a higher rate of mutation of these sequences in RER⁺ compared with RER⁻ tumors or by selection bias in the absence of a difference in mutation rate. The mutations could be the result of a few cataclysmic cell divisions in which numerous alterations appeared simultaneously or due to a stable defect that continuously generates mutations as the tumor expands. The tandem nature of elemental units within simple repeats is thought to render such sequences prone to slipped-strand mispairing and hence particularly prone to insertion or deletion mutagenesis during replication (Levinson and Gutman, 1987b; Kunkel, 1993). We demonstrate here that (CA)_n repeats are unstable in RER⁺ tumor cells, confirming the mutator hypothesis as a contributory factor in this malignancy. Furthermore, we show that such cells have a biochemical defect at an early step in strand-specific mismatch repair, a process that is known to stabilize repetitive sequence elements in *Escherichia coli* and *Saccharomyces cerevisiae* (Levinson and Gutman, 1987a; Strand et al., 1993). Interestingly, the accompanying manuscript (Leach et al., 1993 [this issue of *Cell*]) shows that a gene responsible for HNPCC is likely to encode a homolog of bacterial MutS, the protein responsible for mismatch recognition in methyl-directed mismatch repair (Su and Modrich, 1986; Su et al., 1988).

The reduction in mutability afforded by the *E. coli* methyl-directed system has been attributed to its role in the strand-specific elimination of DNA biosynthetic errors (Meselson, 1988; Modrich, 1991) and to its function as a barrier to illegitimate crossovers occurring between quasihomologous sequences (Rayssiguier et al., 1989; Petit et al., 1991). This system has been extensively studied, and its specificity and mechanism are reasonably well understood (Modrich, 1991). The biochemical experiments described here are based on the demonstration that human cells possess a homolog of the bacterial pathway as judged by conservation of substrate specificity and mechanism (Holmes et al., 1990; Thomas et al., 1991; Fang et al., 1993; Fang and Modrich, 1993) and function in genetic stability (Kat et al., 1993). As in the case of the bacterial pathway (Lahue et al., 1989), a DNA strand break can provide the requisite strand targeting in the human reaction (Holmes et al., 1990; Thomas et al., 1991), and both systems share an unusual bidirectional excision capability. Mismatch-provoked excision by either system removes that portion of the incised strand spanning the strand break

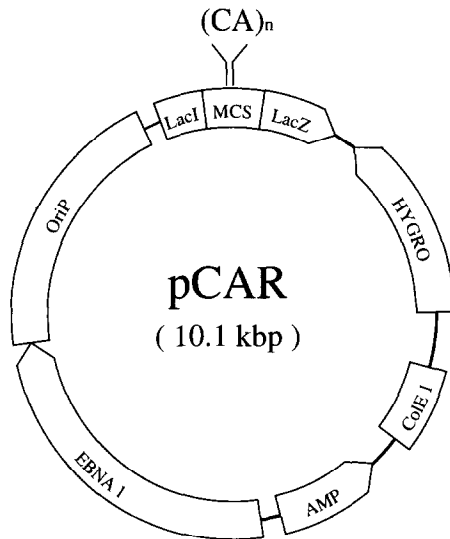


Figure 1. Shuttle Vectors for (CA)_n Mutability Assay

Abbreviations: OriP, EBV origin for episomal replication; EBNA 1, EBV nuclear antigen 1 gene required for episomal replication; LacI, *lac* repressor; MCS, multicloning site, containing one or two copies of (CA)_n in pCAR1 and pCAR2, respectively; LacZ, β -galactosidase gene fragment conferring α -complementation; HYGR0, hygromycin resistance gene; ColE1, plasmid origin for bacterial replication; AMP, β -lactamase gene conferring ampicillin resistance.

and the mispair, irrespective of placement of the incision 3' or 5' to the mismatch (Fang et al., 1993; Fang and Modrich, 1993; Grilley et al., 1993).

Results

Hypermutable in RER⁺ Cells

To determine whether simple repeated sequences were unstable in RER⁺ tumor cells, we transfected cells with shuttle vectors containing a (CA)_n tract within the coding sequences of a β -galactosidase reporter gene (Figure 1). The vector contained the Epstein-Barr virus (EBV) origin of replication to allow episomal replication and a hygromycin resistance element. Insertions or deletions in the (CA)_n tract during replication of the vector in recipient cells could therefore result in restoration of frame, restoring β -galactosidase activity to the reporter. Similar reporters

have been used to assess (CA)_n tract instability in *E. coli* and yeast cells (Levinson and Gutman, 1987a; Strand et al., 1993). The pCAR1 vector contained a (CA)₁₄ insert while pCAR2 contained two contiguous copies of the (CA)₁₄ insert in opposite orientations.

Two colorectal cancer cell lines were used for these experiments. H6 cells were derived from an RER⁺ tumor with typical clinical and cytological characteristics of those occurring in HNPCC patients (cecal cancer, near euploid, surgically curable [Ionov et al., 1989; Aaltonen et al., 1993; Thibodeau et al., 1993]). SO cells are derived from the more common type of colorectal cancer, which is RER⁻, aneuploid, and often metastatic. The pCAR1 and pCAR2 vectors were transfected into these lines, and clones were selected after 17–21 days of hygromycin selection. Episomal DNA was recovered from pools of 50–100 clones, digested with DpnI to remove any unreplicated vector, and used to transform *E. coli* cells to detect β -galactosidase activity. The results in Table 1 show that a much higher fraction of the H6 episomes had β -galactosidase activity than SO episomes. With the pCAR1 vector, β -galactosidase activity was restored in an average of 0.97% of the H6 episomes but in only 0.094% of the SO episomes. The pCAR2 vector, containing twice the number of CA repeats as in pCAR1, was twice as mutable as pCAR1 in H6 cells and slightly more unstable in SO cells (Table 1).

To determine the nature of the sequence changes resulting in restored β -galactosidase activity, blue colonies were picked and plasmid DNA from the colonies sequenced. Each of 15 such colonies from H6 cells was found to contain a deletion of a single CA dinucleotide from the (CA)_n tract, resulting in restoration of the β -galactosidase reading frame. In contrast, no mutations were observed in the CA tracts of plasmids recovered from six blue colonies derived from SO cells. Instead, these plasmids had deletions or insertions outside of the (CA)_n tract that restored the β -galactosidase reading frame. The results of sequencing, together with the frequencies noted in Table 1, show that the rate of development of (CA)_n deletions was at least two orders of magnitude higher in H6 than in SO.

To examine instability in these cells with another method, we performed *in situ* analysis. A vector similar to pCAR1 was constructed, substituting a eukaryotic promoter derived from cytomegalovirus (Grogger et al., 1989) for the prokaryotic promoter controlling β -galactosidase

Table 1. Hypermutable of Exogenous Sequences

Vector	H6				SO			
	pCAR1		pCAR2		pCAR1		pCAR2	
	Percent ^a	Colonies Tested ^b	Percent ^a	Colonies Tested ^b	Percent ^a	Colonies Tested ^b	Percent ^a	Colonies Tested ^b
Experiment 1	1.1	744	1.2	256	0.0	1,488	0.14	1,392
Experiment 2	1.0	896	2.7	800	0.17	7,888	0.13	11,056
Experiment 3	0.42	944	3.2	252	0.071	1,400	0.18	1,136
Experiment 4	1.6	620	1.7	892	0.058	10,384	0.082	9,712
Total	0.97	3,204	2.2	2,200	0.094	21,160	0.12	23,296

^a Percent β -galactosidase-positive colonies.

^b Total colonies tested.

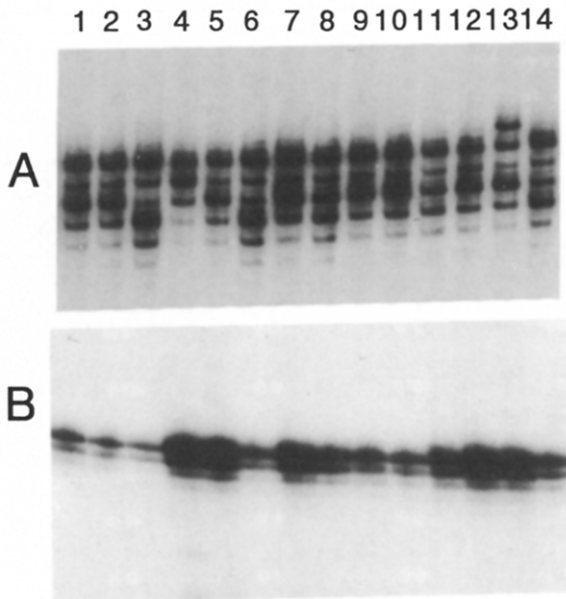


Figure 2. Microsatellite Alterations in Subclones

DNA from subclones of H6 cells (A) or SO cells (B) was used as template for polymerase chain reaction with primers specific for the *AFM164xe3* marker. The polymerase chain reaction products were subjected to electrophoresis, and representative autoradiographs of the dried gels are shown. In (A), the clones in lanes 1, 2, 5, and 7–12 contained only the parental alleles, while the other clones contained new alleles. In (B), all clones exhibited the same pattern of alleles, identical to that found in the parental clone.

in pCAR1 and including the complete coding region of β -galactosidase instead of the smaller α -complementing fragment present in pCAR1. This vector was transfected into H6 and SO cells, and after 2 weeks of hygromycin selection, 50 colonies from each of two flasks were chosen randomly for staining with X-Gal (5-bromo-4-chloro-3-indolyl- β -D-galactoside) to detect β -galactosidase activity. A median of 50 and 38 cells was present in the H6 and SO colonies, respectively. Of the 100 H6 colonies, 46 contained at least one blue cell, while only 1 of the 100 SO colonies contained any blue cells. In the 46 H6 clones, the average fraction of cells staining blue was $12.4 \pm 11.8\%$ (mean \pm one SD), while in the single positive SO clone, only 1 of the 53 cells was blue. Altogether, 439 blue cells were observed among 7833 H6 cells, while 1 blue cell was observed among 4819 SO cells.

Hypermutability of Chromosomal Sequences

The results obtained with the pCAR vectors show that exogenous microsatellite sequences that replicate extra-chromosomally are unstable in RER⁺ cells. To determine whether the rate of mutation of endogenous (CA)_n is elevated in RER⁺ cells, we examined genomic (CA)_n-containing markers in subclones of H6 and SO cells. H6 and SO cells were plated at limiting dilution, and two clones from each line were grown for approximately 22 generations, at which time 1.8×10^6 to 5.0×10^6 cells were present in each clone. The cells from each of these four clones were in

Table 2. Mutability of Endogenous Sequences

Cell Line	Clone	Number of Subclones Analyzed	Percent Subclones with Variants ^a	
			<i>AFM164xe3</i> Marker	<i>AFM212xg5</i> Marker
H6	A	113	4.4	7.1
H6	B	154	11	8.4
SO	A	100	0	0
SO	B	40	0	0

^a Variants had new alleles (not found in the parental clone) constituting at least 25% of the total alleles observed in the subclone.

turn seeded at limiting dilutions and grown for an additional 15 generations, when 5.0×10^3 to 5.0×10^4 cells were present in each subclone. DNA was then purified from 40–154 such subclones and analyzed for microsatellite alterations within (CA)_n-containing markers (Weissenbach et al., 1992). The results, shown in Figure 2, demonstrate that there was a striking difference between H6 and SO subclones in the patterns observed with the *AFM164xe3* and *AFM212xg5* markers. Many different alleles were observed in the various H6 subclones, while all of the 140 SO subclones exhibited only the alleles found in the parent SO cells. As documented in Table 2, 4.4%–11% of the two H6-derived subclones contained major variant alleles, i.e., those accounting for at least 25% of the total alleles in the subclone. Several additional H6 subclones exhibited minor alleles, present in only a minority of the cells. The low frequency of variants in the SO cells is consistent with the previously measured rate of (CA)_n repeat alterations in normal mitotic and meiotic cells (Weber and Wong, 1993) and was at least 20-fold lower than that observed in H6 cells.

HNPCC Cell Lines Are Deficient in Repair of Slipped-Strand Mismatches

Heteroduplexes containing a base–base mismatch and a single-strand break are repaired in nuclear and whole-cell extracts derived from several human cell lines, with repair occurring via a mismatch-provoked excision reaction that is restricted to the incised strand (Holmes et al., 1990; Thomas et al., 1991; Fang and Modrich, 1993; Kat et al., 1993). Since repair of the eight base–base mispairs is inhibited by aphidicolin, and since extracts derived from the MT1 mutator human cell line are deficient in repair of the base–base mismatches (Kat et al., 1993), a single pathway is apparently responsible for correction of this set of mispairs.

Repair of insertion/deletion mismatches of the type expected to result from slipped-strand mispairing has not been described in human cells. In view of the role of mismatch repair in stabilizing repeat sequences in *E. coli* and yeast (Levinson and Gutman, 1987a; Strand et al., 1993), we have constructed heteroduplex substrates containing di-, tri-, and tetranucleotide heterologies to compare rectification of slipped-strand mismatches in nuclear extracts of SO and H6 cells. Five of these constructs are based on placement of the insertion/deletion mispair within over-

Table 3. H6 Cells Are Deficient in Repair of Slipped-Strand Mismatches

Mismatch	Restriction Markers	Repair (fmol)			
		Sau96I Nick		gpII Nick	
		SO	H6	SO	H6
5'-CCAGCCTG-TGTGGC 3'-GGTCGGAC-ACACCG AC	BglII-XcmI	9.1	<0.3	13	<0.3
5'-CCAGCCTG-TGTGGC 3'-GGTCGGAC-ACACCG TG	XcmI-BglII	7.0	1.4	6.4	0.3
5'-CCAGCCTG-CTGGGC 3'-GGTCGGAC-GACCCG GAC	BglII-XcmI	13	<0.3	9.6	<0.3
5'-CCAGCCTG-CTGGGC 3'-GGTCGGAC-GACCCG CTG	XcmI-BglII	13	3.6	2.2 ^a	<0.3
5'-CCAGCCTG--TGGC 3'-GGTCGGAC-ACCG ACAC	PfI-MI-XcmI	12	0.8	7.6	<0.3
5'-AAGCTTG (CA) ₂₀ GTCTAGA 3'-TTCGAAC (GT) ₁₉ CAGATCT	—	9.2	<0.3	7.9	1.0
5'-AAGCTTG (CA) ₁₉ GTCTAGA 3'-TTCGAAC (GT) ₂₀ CAGATCT	—	5.6	0.5	ND	ND

Mismatch repair was determined as described in Experimental Procedures using 50 μg of nuclear extract and 24 fmol of circular heteroduplex DNA containing the indicated slipped-strand mismatch. Substrates contained an incision at the Sau96I site in the complementary DNA strand or the gpII site in the viral strand (see Figure 3). Restriction endonuclease recognition elements are shown in bold. Correction of (CA)₁₉·(GT)₂₀ and (CA)₂₀·(GT)₁₉ heteroduplexes was determined by the method of Figure 5. ND, not determined.

^a This heteroduplex is also weakly processed by HeLa extracts.

lapping recognition sites for two restriction endonucleases with interrupted recognition sequences (Table 3; see Table 5; Figure 3). Presence of a heterology within the interior of such recognition sites renders the heteroduplex resistant to cleavage by both enzymes, with strand-specific repair conferring sensitivity to one endonuclease or the other. As shown in the first five entries of Table 3, each of these open circular substrates was repaired in nuclear extracts derived from SO RER⁻ cells. In each case, repair was highly biased to the incised strand, with correction on the continuous strand being less than 10% (and typically less than 5%) of that on the open strand (data not shown). As demonstrated previously for base-base mismatch correction in HeLa nuclear extracts (Holmes et al., 1990; Thomas et al., 1991; Fang and Modrich, 1993), repair in SO nuclear fractions was directed by a site-specific incision in either strand and was abolished by aphidicolin (data not shown). In contrast with the high levels of repair observed in SO nuclear extracts, heteroduplex correction in RER⁺ H6 nuclear extracts was limited and in some cases undetectable (Table 3), regardless of placement of the site-specific incision in the viral or complementary strand. This defect is not due to presence of a diffusible inhibitor in the H6 line since little if any inhibition of repair was observed upon mixing of SO and H6 extracts (Figure 4, lanes 4–6).

While convenient for scoring repair of small heterologies, the number of di- and trinucleotide repeat elements that can be placed within the heteroduplexes described above is limited by the nature of the restriction endonuclease recognition sites used (see Table 5). Since natural microsatellite tandem repeats are typically larger than those that can be placed in interrupted endonuclease recognition sequences, we have also constructed f1MR phage containing (CA)₁₉ and (CA)₂₀ repeats and have used these molecules to prepare (CA)₁₉·(GT)₂₀ and (CA)₂₀·(GT)₁₉ heteroduplexes. As shown in Figure 5 and summarized in Table 3, SO nuclear extracts corrected the heterology within the incised strand of such heteroduplexes, with the covalently continuous strand serving as template. Repeat elements in otherwise identical (CA)₁₉·(GT)₁₉ and (CA)₂₀·(GT)₂₀ control homoduplexes were unaffected by incubation with nuclear extract. As observed with the heteroduplexes containing fewer repeat elements, extracts derived from RER⁺ H6 cells were unable to process the (CA)₁₉·(GT)₂₀ and (CA)₂₀·(GT)₁₉ heteroduplexes (Figure 5; Table 3), confirming a defect in slipped-strand mismatch repair in RER⁺ H6 cells.

H6 Cells Are Defective in Repair of Base-Base Mismatches

In *E. coli*, heteroduplexes containing small heterologies

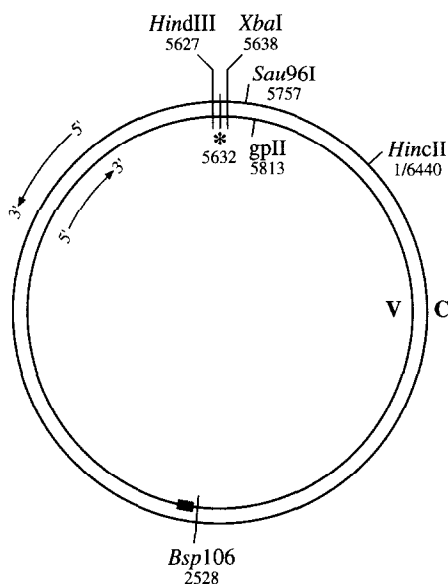


Figure 3. Structure of f1MR1 Bacteriophage and Derivatives
Base–base mismatch heteroduplexes were 6440 bp in size and contained a single mispair (asterisk) at position 5632. Heteroduplexes containing slipped-strand mispairs were constructed from f1MR1 derivatives in which synthetic oligonucleotide duplexes (Table 5) had been inserted into HindIII, XbaI-cleaved f1MR1 replicative form DNA. This method of construction places slipped-strand mismatches in approximately the same position as the base–base mispairs in the substrates alluded to above. Strand targeting of heteroduplex repair was provided by a site-specific single-strand break (Holmes et al., 1990) in the complementary (C) DNA strand at Sau96I or HincII sites or in the viral (V) strand at the cleavage site for gpII as described previously (Fang and Modrich, 1993). Restriction assay for mismatch repair (see Experimental Procedures) is based on cleavage of DNA products with Bsp106 and the appropriate endonuclease to detect correction. The stippled region corresponds to the oligonucleotide used for indirect end labeling in the analysis of mismatch-provoked excision tracts (Figure 6).

are repaired by the MutHLS-dependent, methyl-directed pathway that is also responsible for correction of base–base mismatches (Parker and Marinus, 1992). Accordingly, we have tested nuclear extracts of SO and H6 cells for repair activity on the base–base mispairs. As demon-

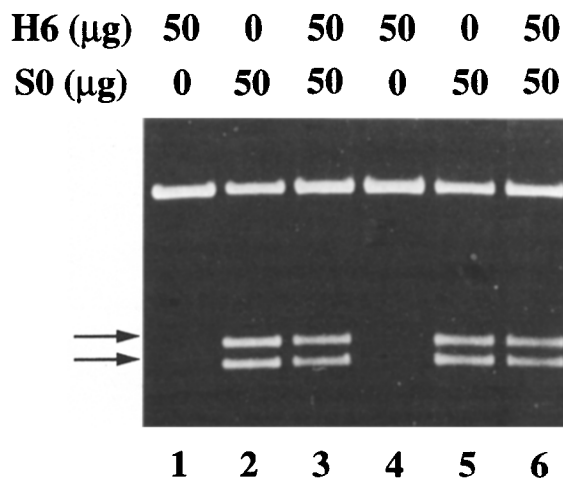


Figure 4. The H6 Repair Defect Is Not Due to a Diffusible Inhibitor
Mismatch repair reactions (see Experimental Procedures) contained H6 nuclear extract, SO nuclear extract, or both, as indicated. Lanes 1–3, repair of a circular G–T heteroduplex DNA containing a complementary strand incision at the Sau96I site (Figure 3) was scored by cleavage with Bsp106 and HindIII (Su et al., 1988); lanes 4–6, repair of a circular CA insertion heteroduplex (first row of Table 3) with a viral strand discontinuity at the gpII site. Correction of the heterology was scored by cleavage with Bsp106 and XcmI. Arrows designate repair products.

strated previously for HeLa (Holmes et al., 1990; Thomas et al., 1991; Fang and Modrich, 1993) and TK6 (Kat et al., 1993) cell lines, nuclear extracts from SO cells efficiently corrected each of the eight base–base mismatches in a strand-specific manner (Table 4). Extracts from RER⁺ H6 cells, however, were defective in repair of all of the base–base mispairs. Extract mixing experiments like those shown in Figure 4 (lanes 1–3) excluded the presence of a diffusible inhibitor as the basis of this defect. These observations suggest that, like *E. coli*, human cells largely depend on a single pathway for correction of small insertion/deletion heterologies and base–base mismatches.

The Defect in RER⁺ Cells Blocks an Early Step in Mismatch Repair

Analysis of mismatch repair in HeLa nuclear extracts has

Table 4. H6 Cells Are Deficient in Repair of Base–Base Mismatches

Mismatch	Repair of Sau96I-Nicked Heteroduplexes (fmol)		Repair of gpII-Nicked Heteroduplexes (fmol)	
	SO Cell Line	H6 Cell Line	SO Cell Line	H6 Cell Line
G–T	12	<0.3	9.8	<0.3
A–C	8.4	<0.3		
G–G	10	1.7		
A–A	8.4	0.7		
A–G	6.5	0.7		
T–T	7.9	<0.3		
C–T	8.5	<0.3		
C–C	6.3	0.3		

Mismatch repair was determined as described in Experimental Procedures using 50 μg of nuclear extract and 24 fmol of circular heteroduplex DNA containing a base–base mismatch at position 5632 and site-specific incision at either the Sau96I site in the complementary strand or the gpII site in the viral strand (see Figure 3).

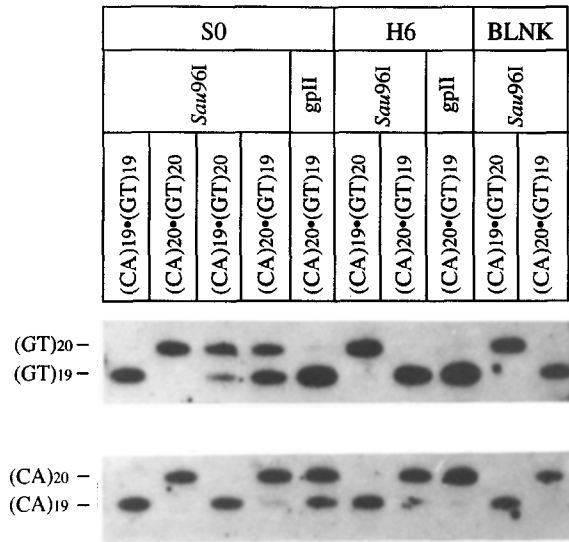


Figure 5. H6 Cells Are Defective in Repair of (CA)₁₉·(GT)₂₀ and (CA)₂₀·(GT)₁₉ Heteroduplexes

(CA)₁₉·(GT)₂₀ and (CA)₂₀·(GT)₁₉ heteroduplexes and control (CA)₁₉·(GT)₁₉ and (CA)₂₀·(GT)₂₀ homoduplexes were subjected to mismatch repair in nuclear extracts of SO or H6 as described in Experimental Procedures except that reactions (105 μl) contained 170 fmol of DNA and 0.52 mg of protein. Strand specificity was provided by incision of the complementary or viral DNA strand at Sau96I or gpII sites, respectively. Repeat sequence elements were excised from reaction products by cleavage with HindIII and XbaI (Figure 3), separated by electrophoresis through 10% sequencing gels, and DNA electrotransferred to nylon membranes as described (Fang and Modrich, 1993). Products derived from the viral strand (lower panel) were visualized by hybridization with [5'-³²P]d(GACGCTCTAGAC(TG)₁₉CAAGCTTCAGGC) and complementary strand products (upper panel) by hybridization with [5'-³²P]d(GCCTGAAGCTTG(CA)₁₉GTCTAGACGCTC). BLNK, untreated heteroduplexes.

demonstrated that intermediates in the mismatch-provoked excision reaction can be visualized by blocking repair DNA synthesis associated with the reaction (Fang and Modrich, 1993). With circular heteroduplexes (see Figure 3), excision is confined to that portion of the incised strand spanning the shorter path between the mismatch and the strand break, regardless of the polarity of the incised strand, thus implying a bidirectional excision capability (Fang and Modrich, 1993). The finding that H6 cells are defective in correction of heteroduplexes incised in either the viral or complementary DNA strand (Tables 3 and 4) therefore suggested that the repair defect blocks a step prior to excision. This possibility was confirmed by mapping of termini produced in nuclear extracts when DNA synthesis was restricted by omission of exogenous dNTPs.

The circular G-T and (CA)₂₀·(GT)₁₉ heteroduplexes used contained a HincII incision in the complementary DNA strand approximately 800 bp 5' to the mismatch (shorter path; see Figure 3), with an otherwise identical (CA)₁₉·(GT)₁₉ homoduplex serving as control. Mapping relative to the Bsp106 cleavage site placed the 5' terminus in untreated substrates at the HincII site (Figure 6). Incubation of heteroduplexes with SO nuclear extract produced two

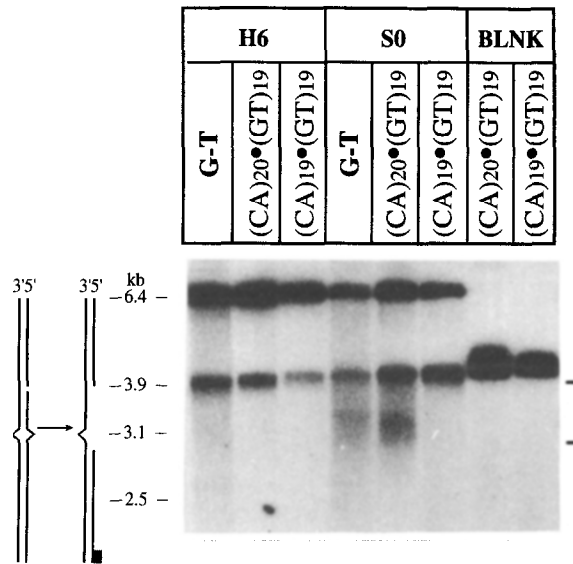


Figure 6. The H6 Repair Defect Is Manifested at or Prior to Excision Circular heteroduplexes (6.4 kb; Figure 3) contained G-T or (CA)₂₀·(GT)₁₉ mismatches and an incision in the complementary strand at the HincII site (Figure 3). A control homoduplex contained (CA)₁₉·(GT)₁₉, but was otherwise identical to the (CA)₂₀·(GT)₁₉ heteroduplex. DNAs were incubated with H6 or SO nuclear extracts in the absence of exogenous dNTPs as described in Experimental Procedures. Products were cleaved with Bsp106, electrophoresed through alkaline 1.5% agarose gels, and DNA electrotransferred to nylon membranes as described previously (Fang and Modrich, 1993). Membranes were hybridized with [5'-³²P]d(ATGGTTTCATTGGTGACGTT) to map excision tract 5' termini. The mapping method is illustrated on the left, with the stippled bar corresponding to the oligonucleotide probe. The mismatch and the strand break in the heteroduplex map 3.1 kb and 3.9 kb 5' to the Bsp106 cleavage site, respectively (Figure 3). Excision intermediates are located in the bracketed region. The 6.4 kb species is produced by ligation of the substrate (Fang and Modrich, 1993). BLNK, untreated homo- and heteroduplexes.

novel forms, a 6.4 kb species resulting from ligation of the HincII nick and a population of termini mapping 3' to the location of the mispair, results similar to those obtained previously with HeLa extracts (Fang and Modrich, 1993). Since the latter species was not observed with the control homoduplex, it has been attributed to a mismatch-provoked excision process that presumably initiates at single-strand break that directs repair. Inasmuch as this intermediate species was not observed with either the G-T or the slipped-strand heteroduplex when nuclear extracts were derived from RER⁺ H6 cells, the H6 defect must block mismatch repair at or prior to the excision stage of the reaction.

Complementation of H6 Nuclear Extracts with Partially Purified HeLa Fractions

To test the possibility that the H6 repair defect might be due to simple deficiency of a required repair component, we have examined partially purified HeLa fractions for their ability to complement RER⁺ nuclear extracts. As shown in Figure 7, repair was restored to H6 extracts by a fraction that chromatographs as a discrete species on phosphocellulose. The heteroduplex used in the column assays of Figure 7 contained a CA dinucleotide insertion

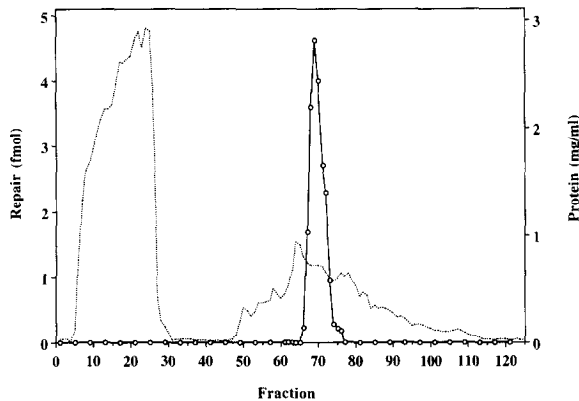


Figure 7. A HeLa Nuclear Component Restores Mismatch Repair to H6 Nuclear Extracts

HeLa cell nuclear extract was fractionated by chromatography on phosphocellulose as described previously (Kat et al., 1993), except that the column was developed with a 13 column volume gradient of KCl (0.02–0.9 M). Samples (2 μ l) of each fraction were assayed for their ability to restore mismatch repair to H6 nuclear extract (50 μ g) on a heteroduplex containing a CA insertion in the complementary strand and a site-specific nick in the viral strand at the gpII cleavage site (Figure 3; first entry of Table 3). Mismatch repair is shown by open circles, while protein concentration is indicated by the dotted line.

in the complementary strand (heteroduplex in the first entry of Table 3) and a site-specific nick in the viral strand. A coincident peak of complementing activity was observed when repair was scored using a G–T base–base heteroduplex with a complementary strand incision (data not shown). These observations therefore suggest that RER⁺ cells are deficient in a single species that is necessary for the bidirectional processing of both slipped-strand and base–base mispairs. This species could be a single activity or a complex of activities involved in repair reaction.

A Lymphoblastoid Cell Line Derived from an HNPCC-Affected Individual Is Proficient in Mismatch Repair

While most tumors developing in HNPCC patients contain mutations within microsatellite sequences, corresponding variability has not been observed in normal tissue from the same individuals (Aaltonen et al., 1993). In view of the results presented above, these tissue differences could be due to selection, or they could reflect a tumor-specific defect in mismatch correction and associated hypermutability. We have addressed these possibilities by testing the repair activity of KK cells, a lymphoblastoid line derived from an HNPCC patient whose colorectal cancer was RER⁺. The CA insertion heteroduplex illustrated in the first entry of Table 3 was repaired in nuclear extracts derived from these cells (per 50 μ g of protein: 3.2 fmol on the Sau96I-incised substrate; 3.3 fmol on the gene II protein [gpII]-cleaved heteroduplex), as was the CTG insertion shown in the fourth entry of Table 3 (2.5 fmol/50 μ g with the Sau96I-incised substrate) and a G–T heteroduplex (4.1 fmol/50 μ g for Sau96I incision). Although these values are somewhat less than those observed with extracts derived from the SO cell line (Tables 3 and 4), they demonstrate that KK lymphoblastoid cells are repair proficient. Indeed,

the specific G–T repair activity observed with these cells compares favorably with that determined previously with the TK6 lymphoblastoid cell line from a normal individual (4.2 fmol/50 μ g [Kat et al., 1993]). The mismatch repair defect in HNPCC thus appears to be tumor specific.

Discussion

As discussed above, the frequent alterations observed within simple repeats in RER⁺ tumor cells may reflect hypermutability in tumor cells or could result from selective forces. While we cannot exclude a role for selection, our results demonstrate that mutability of (CA)_n repeats is at least two orders of magnitude higher in H6 RER⁺ HNPCC cells than in RER⁻ SO colon tumor cells. The finding that H6 RER⁺ cells are also defective in mismatch repair is consistent with the known role of this process in stabilizing (CA)_n repeat sequences in *E. coli* and yeast (Levinson and Gutman, 1987a; Strand et al., 1993) and suggests a cause and effect relationship with respect to observed microsatellite instability, an idea that is further substantiated below. Since the H6 repair defect also blocks correction of base–base mismatches, RER⁺ cells may also prove to be unstable to transition and transversion mutagenesis as well. It must be emphasized that the correlations noted here are based on a small sample size, and substantiation of these points must await analysis of additional normal and transformed cell lines. However, it is noteworthy that we have observed a similar genetic instability of CA tracts using the pCAR2 vector and a comparable defect in mismatch repair in an independently derived RER⁺ colorectal tumor cell line (data not shown). Preliminary experiments suggest that the repair defect in this line may be due to deficiency of the same component(s) missing in H6 cells, as nuclear extracts from the two RER⁺ lines do not complement *in vitro*.

The accompanying paper (Leach et al., 1993) localizes the germline mutation in some HNPCC families to a chromosome 2p16 gene that encodes a homolog of bacterial MutS, the protein responsible for mismatch recognition and initiation of repair by the bacterial methyl-directed system (Modrich, 1991). Cells in unaffected somatic tissue were heterozygous for this defect, while RER⁺ tumor cells acquired a mutation in the remaining wild-type gene in at least one case (Leach et al., 1993). These observations are perfectly consistent with the nature of the mismatch repair defect described here. In contrast with the defect in tumor cells, lymphoblastoid cells derived from an affected HNPCC patient with a germline *mutS* mutation were shown to be repair proficient.

The affected chromosome 2p16 gene is also consistent with the nature of the repair block observed in H6 cells. Although the activities required for human mismatch repair are not yet available in pure form, the strand-specific reaction occurring in nuclear extracts is similar to bacterial methyl-directed repair with respect to mismatch specificity, unusual bidirectional excision mechanism, and function in mutation avoidance (Fang and Modrich, 1993; Grilley et al., 1993; Kat et al., 1993). The two systems have been postulated to be functional homologs on this basis.

Of the ten activities required for the bacterial reaction, only three (MutS, MutL, and DNA helicase II) are required for initiation of excision with the type of heteroduplexes described here (Grilley et al., 1993). Loss of function of a MutS homolog is therefore expected to lead to the type of excision defect observed in RER⁺ H6 cells. Despite the consistency of the genetic and biochemical results, it is important to note that the locus affected in the patient from whom H6 cells were derived has not been mapped, nor have mutations been identified in the genes encoding the H6 MutS homolog. HNPCC is known to be genetically heterogeneous, with loci on chromosome 2p16, 3p21, and elsewhere, but tumors from HNPCC patients are RER⁺ regardless of locus (Aaltonen et al., 1993; Lindblom et al., 1993).

H6 cells represent the second instance in which a mismatch repair defect has been demonstrated in a human cell line. The previously described MT1 mutant line (Kat et al., 1993) was derived from TK6 lymphoblastoid cells by frameshift mutagenesis and subsequently screened for resistance to the cytotoxic action of DNA-methylating agents (Goldmacher et al., 1986). MT1 cells are hypermutable in the absence of alkylating agents, displaying increased frequencies of single nucleotide insertions, transversions, and transitions within *HPRT* coding sequences (Goldmacher et al., 1986; Kat et al., 1993). As in the case of H6 cells, the MT1 defect blocks mismatch correction prior to the excision step of the reaction. Despite the similar nature of their repair defects, distinct components may be lacking in H6 and MT1 cells since preliminary experiments indicate that nuclear extracts from the two lines complement in vitro. Since MT1 cells tolerate levels of DNA alkylation that kill parental TK6 cells, it has been suggested that in addition to function in reversal of DNA metabolic errors, one or more components of the human mismatch repair system may also serve as a sensor for other kinds of genetic damage (Kat et al., 1993).

Given the known functions of mismatch repair in stabilizing bacterial genomes (Meselson, 1988; Rayssiguier et al., 1989; Modrich, 1991; Petit et al., 1991), it is not surprising that mismatch correction has significant antioncogenic function in mammalian cells. Indeed, mismatch repair defects are among the strongest mutator mutations that have been identified in bacterial systems. Furthermore, our demonstration that the human mismatch repair system stabilizes (CA)_n repeats and is capable of processing a variety of slipped-strand mispairs suggests that this pathway may also serve to stabilize tracts of trinucleotide repeats, the expansion of which has been implicated in several heritable disease states (Caskey et al., 1992; Kuhl and Caskey, 1993).

Experimental Procedures

Growth of Cell Lines and Preparation of Extracts

SO cells were grown in Leibovitz L-15 media supplemented with glutamine (4 mM final concentration), 10% fetal bovine serum (Hyclone), 100 U/ml penicillin, and 0.1 mg/ml streptomycin. Monolayers were grown at 37°C in sealed flasks. H6 cells were grown in McCoy's 5A medium (modified) with 10% fetal bovine serum (Hyclone), 100 U/ml penicillin, and 0.1 mg/ml streptomycin. Monolayer cultures were grown at 37°C in a 5% CO₂ atmosphere. The H6 and SO cell lines were

derived from the colorectal cancer cell lines HCT116 (Brattain et al., 1981) and SW480 (Leibovitz et al., 1976), respectively, by continued passage in the media described above. Lymphoblastoid KK cells, which were derived from an HNPCC patient with a germline *mutS* mutation (kindred C; Leach et al., 1993), were grown in suspension in RPMI 1640 media supplemented with 10% fetal bovine serum, glutamine (4 mM final concentration), 0.01 M HEPES-KOH (pH 7.3), 100 U/ml penicillin, and 0.1 mg/ml streptomycin.

For biochemical work, roller bottles (850 cm²) were seeded with about 10⁷ H6 or SO cells in 100 ml of the appropriate complete media, and H6 cultures were flushed with CO₂ prior to capping. Cells were maintained in a subconfluent state by splitting 1:8 by trypsinization every 3–4 days. Near confluent cultures were chilled to 4°C, and cells were collected with the aid of a rubber policeman and washed with 35 ml (per roller bottle) of 0.02 M HEPES-KOH (pH 7.5), 5 mM KCl, 0.5 mM MgCl₂, 0.2 M sucrose, 0.5 mM dithiothreitol, 10⁻³ vol of phenylmethylsulfonyl fluoride (saturated solution in isopropanol), 1 µg/ml leupeptin, 10 µg/ml soybean trypsin inhibitor, and 1 µg/ml N α -p-tosyl-L-lysine chloromethylketone. About 7 g of packed, washed cells (~2 × 10⁸ cells) were harvested from 14 roller bottles. Suspension cultures (4–5.5 l) of KK cells were harvested, and nuclear extracts from each of the three cell types were prepared as described previously (Holmes et al., 1990).

Mutagenicity Assays

The pCAR1 and pCAR2 vectors were constructed by inserting the multicloning site and β -galactosidase gene from pBluescript (nucleotides 55–975; Stratagene) into the Sall site of pCEP4, an EBV-based episomal vector (Groger et al., 1989). The resultant construct was cleaved at the multicloning site with BamHI, and the (CA)_n repeat from pSH31 (Henderson and Petes, 1992; provided by T. Petes) was inserted at this site. The resultant clones were sequenced, and two clones, containing one or two copies of the (CA)_n insert, were chosen. Plasmid DNA from these clones was transfected into H6 or SO cells using lipofectin (Life Technologies), and colonies were selected for 17–21 days with hygromycin. Of these colonies, 50–100 were pooled and DNA purified from the Hirt supernatant. This episomal DNA was digested with DpnI and used to transform DH10B cells (Life Technologies) by electroporation. Transformed cells were spread onto L-agar containing isopropyl- β -D-thiogalactopyranoside (6 µg/ml), X-Gal (20 µg/ml), and ampicillin (100 µg/ml), and the number of blue colonies (resulting from α complementation [Ullman et al., 1967]) and total colonies were counted.

Microsatellite Analysis

DNA was purified from H6 and SO clones and used as template for amplification with oligonucleotides specific for microsatellite markers (Weissenbach et al., 1992). Conditions for polymerase chain reaction amplification and denaturing polyacrylamide gel electrophoresis have been described previously (Peltomäki et al., 1993). DNA from colorectal cancers and normal colonic mucosa was purified from the paraffin-embedded tissues of the patients from whom H6 and SO cells had originally been derived. The RER status of the primary cancers from these patients was determined by the methods previously described (Peltomäki et al., 1993) using DNA purified according to Goelz et al. (1985).

Heteroduplex DNAs and Mismatch Repair Reactions

Heteroduplexes representing the eight base–base mispairs were prepared using a previously described set of f1MR bacteriophage constructed for this purpose (Su et al., 1988). Each member of this set contains a mismatch at position 5632 (Figure 3) and a site-specific single-strand break either in the complementary DNA strand at the Sau96I or HincII site or within the viral strand at the site cleaved by the phage fd gpII (Fang and Modrich, 1993; Kat et al., 1993). The base–base mismatches within these molecules reside in overlapping sites for two restriction endonucleases, with strand-specific repair scored as conversion of the substrate, which is resistant to cleavage by either activity, to a form sensitive to one endonuclease or the other (Su et al., 1988).

We have also prepared derivatives of phage f1MR1 (Su et al., 1988) that allow construction of heteroduplexes containing slipped-strand mispairs. This was accomplished by insertion of synthetic oligonucleo-

Table 5. Construction of f1MR Derivatives Containing Repetitive Sequences

Phage	Oligonucleotide Insert	Restriction Marker
f1MR23	5'-AGCTGCAGCCAGCCTGTGTGGC . . . V 3'- . . . CGTCGGTCGGACACACCCGGATC C	BglI
f1MR24	5'-AGCTGCAGCCAGCCTGTGTGTGGC . . . V 3'- . . . CGTCGGTCGGACACACACCCGGATC C	XcmI
f1MR25	5'-AGCTGGTACCAGCCTGTGGGCT . . . V 3'- . . . CCATGGTCGGACGACCCGAGATC C	BglI
f1MR26	5'-AGCTGGTACCAGCCTGTGTGGGCT . . . V 3'- . . . CCATGGTCGGACGACACCCGAGATC C	XcmI
f1MR27	5'-AGCTGCAGCCAGCCTGTGGC . . . V 3'- . . . CGTCGGTCGGACACCCGGATC C	PfIMI
f1MR28	5'-AGCTTG (CA) ₁₉ GT . . . V 3'- . . . AC (GT) ₁₉ CAGATC C	—
f1MR29	5'-AGCTTG (CA) ₂₀ GT . . . V 3'- . . . AC (GT) ₂₀ CAGATC C	—

Oligonucleotide duplexes shown were inserted into f1MR1 replicative form (Su et al., 1988) that had been cleaved with HindIII and XbaI (see Figure 3). In each case the nature of the resulting construct was confirmed by sequence analysis of the region of interest. V and C indicate phage viral and complementary strands, respectively. In those cases noted, oligonucleotide insertion generates unique XcmI (CCA(N)_nTGG), BglI (GCC(N)_nGGC), or PfIMI (CCA(N)_nTGG) restriction sites that can be used to monitor correction of heteroduplexes produced by annealing viral and complementary strands from an appropriate phage pair. Repeat sequence elements are shown in bold.

tide duplexes (Table 5) between the HindIII and XbaI sites of f1MR1 (Figure 3), with the resulting molecules being of two types. One class was based on insertion of recognition sites for restriction endonucleases with interrupted recognition sequences (e.g., XcmI, CCA(N)_nTGG), with repeat elements placed within the interior of the sequence (Table 5). Since such molecules permit construction of heteroduplexes in which slipped-strand mispairs reside within overlapping restriction sites (Table 3), repair can be scored by endonuclease cleavage assay. During the course of this work, we found that BglI, when used in the buffer recommended by the manufacturer, displayed a high level of background cleavage on heteroduplexes containing the recognition site for the enzyme. This problem was eliminated when hydrolysis by this enzyme was performed at 37°C in 0.05 M Tris-HCl (pH 7.0), 0.06 M NaCl, 2 mM MgCl₂, 0.1 mg/ml bovine serum albumin.

Two phage containing (CA)₁₉ or (CA)₂₀ repeat elements (Table 5) were also prepared. Correction of heteroduplexes constructed with these two DNAs was scored by cleavage of product molecules on each side of the repetitive element, resolution of the two strands of interest on DNA sequencing gels, transfer to nylon membranes, and blotting with appropriate oligonucleotide probes (Figure 5). Repair was quantitated by analysis of blots with a phosphorimager.

Unless indicated otherwise, mismatch repair assays were performed as described previously (Holmes et al., 1990; Fang and Modrich, 1993) in 10 µl reactions containing 24 fmol of heteroduplex and 50 µg of nuclear extract protein at 37°C for 15 min, conditions under which product formation is largely limited by rate of repair. Repair results are averages of several determinations, which in most cases used independent extract preparations. Assay variability was typically less than ± 20%.

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