Inefficient in vivo repair of mismatches at an oncogenic hotspot correlated with lack of binding by mismatch repair proteins and with phase of the cell cycle

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Repair rates of mismatched nucleotides located at an activating hotspot of mutation, H-ras codon 12, have been analyzed in vivo in mammalian cells. Repair rates at codon 12 are significantly improved in cells synchronized to the G1 stage of the mammalian cell cycle as compared with non-synchronous cells, demonstrating that mismatch repair mechanisms are active in G1. Repair rates in non-synchronous cells for the same mismatches at a nearby non-hotspot of mutation, H-ras codon 10, are also significantly improved over repair rates at codon 12 in non-synchronous cells, demonstrating that DNA mismatch repair rates can differ depending on the sequence context. These results suggest that inefficiencies in mismatch repair are responsible, at least in part, for the well documented hotspot of mutation at codon 12. Further experiments involving gel-shift analysis demonstrate a mismatch-specific binding factor for which the degree of binding correlates with in vivo repair rates for each mismatch tested at the codon 12 location. This binding factor appears to be the hMutSα heterodimer as identified by monoclonal antibody assay and inhibition of binding by ATP. Furthermore, a lack of binding is observed only for G:A mismatches at the codon 12 location. This lack of binding correlates with the low rate of repair observed in vivo for G:A mismatches at codon 12 versus the improved repair rates for G:A mismatches at codon 10. This may have biological relevance in that G:C→T:A transversions are a common mutation at this location in naturally occurring human tumors. These results suggest that there is lowered efficiency in the kinetics of mismatch repair at codon 12. Mismatches at this location are therefore more likely to be replicated before repair, thus resulting in a mutation.

Introduction

It has been well documented that the frequency and spectra of tumor-associated mutations are not random. Indeed, recent reviews have suggested that a major portion of human gene mutations are found at mutagenic hotspots, similar to the mutational spectra seen in bacteria (1,2). For example, direct sequence analysis has demonstrated that there are activating mutations in the ras oncogene family in nearly 30% of human tumors, and that these mutations occur almost exclusively in codons 12, 13 and 61 (3–5). Clearly, a better understanding of the mechanisms responsible for such precise mutagenic targeting is important for our understanding of the processes responsible for cell transformation to the neoplastic phenotype.

Three general hypotheses have been put forward to explain tumor-associated hotspots of mutation. First, hotspots could be due to the increased susceptibility of specific DNA sequences to damage. Numerous animal model studies have demonstrated correlations between type of chemical exposure and location and type of ras activating mutations in resultant tumors (3,6). Also, recent studies have shown preferential binding by carcinogens and formation of benzo[a]pyrene adducts at mutational hotspots in the p53 tumor suppressor gene (7,8). A second hypothesis is that activating mutations are selected for due to the increased survival rates that they confer. However, several additional activating mutations have been found in ras genes in vitro that have never been found in vivo in human tumors or animal model studies (3–5). This would suggest that the selective advantage conferred by an activating mutation is not a sufficient explanation for the existence of tumor-associated hotspots of mutation. The third hypothesis to explain mutagenic hotspots is that there is random DNA damage with decreased fidelity of repair, depending on the sequence context. For example, repair of pyrimidine dimers and benzo[a]pyrene adducts has been shown to occur more slowly at mutational hotspots in the p53 tumor suppressor gene (9,10). Results from studies examining repair of inserted guanine adducts at different positions in codon 12 or 13 of H-ras have suggested that repair rates can vary at different locations (11–13). These hypotheses attributing different causative events to the process of cell transformation are not mutually exclusive, and, as observed in the p53 studies, a combination of events may contribute to the significantly increased frequency of mutation observed at specific locations.

The effect of the third hypothesis, decreased fidelity of DNA repair at a mutagenic hotspot, has been the focus of these experiments. Several studies have now demonstrated defects in DNA repair mechanisms resulting in a mutator phenotype in inherited predispositions to cancer (for reviews see 14,15). For example, the molecular basis of hereditary non-polyposis colon cancer has been shown to involve genetic instability associated with defects in the genes involved in long-patch mismatch repair (15–18). We are interested in determining if inefficient repair of mismatches can also play a role in mutations at a tumor-associated hotspot in individuals who do not carry mutations in mismatch repair genes.

A number of partially redundant systems have been demonstrated to repair mismatched DNA in eukaryotic cells (15,19,20). In long-patch mismatch repair, hMSH2 and hMSH6 (GTBP) form a heterodimer, hMutSα, which has been shown to interact with mismatched DNA (21–23); however, it has not yet been determined whether this interaction is an initial recognition step or whether repair is facilitated directly by this complex. In addition to long-patch mismatch repair, there is also evidence for short-patch DNA mismatch repair systems in mammalian cells. A thymine DNA glycosylase specific for G:T mismatches has been identified in HeLa cell extracts which removes thymine in G:T mispairs to form G:C (20,24).
hMYH, the mammalian homolog of Escherichia coli mut Y, has also been identified, which removes adenine from A:G mismatches and A:OH-G (20,25). Additional factors contributing to correct repair rates are transcriptional activity of the gene and location of the damage on the transcribed versus non-transcribed DNA strand (26).

There have been relatively few studies in relation to effect of the stage of the eukaryotic cell cycle on correct mismatch repair rates. Mismatch repair activity has been demonstrated in mammalian G2-synchronized cells (27), and in extracts from both Drosophila embryos and adults (28). Other studies have found long-patch mismatch repair proteins expressed at all stages of the cell cycle (29–31). Also, nucleotide excision repair has been shown to be active during the G1 and G2 stages of the mammalian cell cycle (32).

We have focused on examining the mechanism(s) responsible for mutagenic targeting to the codon 12 hotspot in H-ras. Previously, we demonstrated significant differences in repair rates for different mismatches at the middle nucleotide position of codon 12 within replicating NIH 3T3 cells (33). All clones in which the mismatch at codon 12 had not been correctly repaired to wild-type (G:C) contained a mixture of wild-type and mutated base pairs (G:C and T:A or A:T depending on the mismatch used). The mixtures most probably resulted from a single originally transfected cell in which DNA replication occurred before repair. The significant differences we observed between the different mismatches in efficiency of correct repair before replication indicated that the kinetics of cellular repair may vary at this sensitive location depending on the specific mismatch.

We report here results from analyses of mismatch repair at a nearby non-hotspot of mutation, codon 10, and also the repair rates at codon 12 in G1-synchronized cells. We demonstrate almost complete correct repair at codon 12 in G1-synchronized cells. We also demonstrate significantly increased repair rates in non-synchronized cells for the same mismatches when located at codon 10 as compared with codon 12. The amount of repair observed in vivo correlates with gel-shift experiments in which the degree of binding by mismatch-specific factors varies depending on the location and type of mismatch. Based on these data, we propose that the increased incidence of mutation observed at the codon 12 hotspot in H-ras is due in part to a lack of mismatch repair before replication.

**Materials and methods**

**Enzymes and reagents**

All site-specific mismatch synthetic oligonucleotides were purchased from Operon Technologies (Alameda, CA). Amplitherm DNA Polymerase was purchased from Epicentre Technologies (Madison, WI). Nael and Nal restriction endonucleases and T4 polynucleotide kinase were purchased from Promega (Madison, WI). Shrimp alkaline phosphatase (SAP), exonuclease I (Exo I) and thermosequenase were purchased from Amersham (Piscataway, NJ). Radioactively labeled nucleotides were purchased from NEN Life Science (Boston, MA). DH5α competent E.coli cells, Dubecco’s modified Eagle’s medium (DMEM; 4.5 g/l glucose), LipofectAMINE and Opti-MEM were purchased from Gibco BRL (Gaithersburg, MD). NIH 3T3 cells were obtained from the American Type Culture Collection (ATCC) (Rockville, MD). Clonfectin was purchased from Clontech (Palo Alto, CA). Bovine calf serum was purchased from Sigma (St Louis, MO) unless otherwise noted.

**Heteroduplex preparation**

Mismatch plasmid construction was performed essentially as described previously (33,34). Briefly, gapped heteroduplex DNA was constructed by restriction digest of a plasmid containing 2 kb of the H-ras sequence, followed by annealing to single-stranded M13 DNA containing either the coding or non-coding strand of H-ras. A unique 1.8 kb fragment containing a 30 bp gap at the codon 10 and codon 12 region was then isolated by restriction digest and gel electrophoresis, and annealed with a 200 molar excess of oligonucleotides containing a strand- and site-specific mismatch position either of codon 12 or codon 10. The mismatch-containing fragment was then ligated to a vector containing the remaining H-ras genomic sequence, Epstein–Barr virus origin of replication, antibiotic resistance and bacterial replication sequences (35). This procedure produces a plasmid that contains the majority of introduced nicks (four of six) on the same strand as the incorrect base. Under the conditions used, ligation is <100% efficient and occurs with equal probability on both strands, thus biasing repair to that strand containing the incorrect base (34).

**Cell lines, G1 synchronization, fluorescence-activated cell sorter (FACS) analyses and transfection**

DH5α competent E.coli cells were transformed with 50 ng of mismatch-containing plasmid as described by the manufacturer (Gibco BRL). Non-synchronous NIH 3T3 mismatch repair experiments were performed as described previously (33,34). For G1 synchronization experiments, NIH 3T3 cells were seeded at 4.9×10^4 cells per 100 mm plate in media containing 0.25% calf serum and incubated for 3 days at 37°C in 5% CO2. On day 4, 1 h prior to the start of transfection, cells were re-fed with fresh medium containing 10% calf serum. Mismatch-containing plasmid DNA (125 ng per plate) was transfected into G1-synchronized cells using Clonfectin reagent as described by the manufacturer (Clontech) for a 4 h period of time. Hygromycin-resistant cells were subsequently selected by the addition of 124 U hygromycin per ml media starting 48 h post-transfection for 3–4 weeks. FACS analyses of the NIH 3T3 cell cycle synchronization parameters were performed at the Flow Cytometry Laboratory, University of Washington (Seattle, WA) using 4,6-diamidino-2-phenylindole reagent for DNA quantification.

**Mismatch repair analysis**

DNA was purified from each hygromycin-resistant colony and amplified by PCR as described previously (33,34), yielding an amplified plasmid DNA product of 128 bp containing exon 1 of human H-ras plus several human intronic nucleotides, thus precluding amplification of NIH 3T3 genomic DNA. An aliquot of each amplified product was digested with NaeI restriction enzyme for codon 12 analysis or NalI for codon 10 analysis. Only PCR-amplified DNA containing wild-type human H-ras at the relevant codon is cleaved by these restriction enzymes. All PCR-amplified DNA not completely cleaved was treated with the enzymes SAP and Exo I and cycle-sequenced to determine the exact mutation, as described by the manufacturer (Amersham).

**Gel-shift assays**

For these experiments, 32mer oligonucleotides were derived from the H-ras sequence surrounding codons 10 and 12. These have the sequence of GGTGTTGGCXCCCGGYYGCTTGTTGGCAAGAGTGCGC or GGTGGGCCTCCMCGGTTGGCCAAAGATGGCGC, in which X represents the middle base of H-ras codon 10 and Y represents the middle base of H-ras codon 12. Oligos with a single specific mismatch were 5’ end-labeled using [32P]ATP and T4 polynucleotide kinase as described by the manufacturer (Promega), and purified using a sephadex G-25 spin column. The labeled mismatch-containing single-stranded oligos were then annealed to the complementary wild-type oligo at a 1:3 molar ratio in a final volume of 100 μl in 10 mM Tris–HCl (pH 8.0), 10 mM MgCl2. The solution was heated to 95°C for 5 min and then allowed to cool to room temperature over 2 h. The specific activity of each purified double-stranded oligo was determined by liquid scintillation counting. Nuclear protein–DNA binding reactions were carried out at 4°C in a total volume of 10 μl using 35 fmol of radioactively labeled oligo, 0.9 pmol of a double-stranded wild-type competitor (an ~1.25 molar ratio) and 10 μg of nuclear extract. Nuclear extracts were purchased from Promega or were prepared essentially as described by Dignam (36) with final resuspension in a buffer consisting of 20 mM HEPES (pH 7.9), 20% glycerol, 0.1 M KCl, 0.2 mM EDTA, 0.2 mM PMSF, 0.5 mM DTT. Reactions were incubated for 30 min at 4°C in binding buffer containing 20% glycerol, 5 mM MgCl2, 2.5 mM EDTA, 2.5 mM DTT, 250 mM NaCl, 50 mM Tris–HCl (pH 7.5) and 2.5 fmol of normal poly(dI–dC)–poly(dI–dC). Following incubation, 10% non-denaturing loading buffer [250 mM Tris–HCl (pH 7.5), 0.2% bromophenol blue, 40% glycerol] was added and the samples were loaded onto a 6% polyacrylamide non-denaturing gel (39:1 acrylamide:bisacrylamide). Electrophoresis was carried out at 4°C at 100 V (10 V/cm) in TBE. Gels were then dried and exposed to Biomax film (Kodak). In experiments to assess efficiency of annealing to different radiolabeled substrates, equal amounts of radioactive activity were used. For antibody-binding assays, 1 μg of monoclonal anti-mMsh6 antibody (Santa Cruz Biotechnology, Santa Cruz, CA) was allowed to incubate with nuclear extracts for 20 min prior to addition of DNA. For ATP inhibition...
assays, 1 mM ATP was added to the binding buffer prior to addition of nuclear extract and DNA.

Results

Comparison of repair rates at codon 12 in non-synchronized and G1-synchronized cells

Previously we determined in vivo rates of repair for specific mismatches at the middle nucleotide position of H-ras codon 12 in non-synchronized NIH 3T3 cells (33) (summary of results shown in Figure 1 for comparison). The G:T, T:C, A:C and G:A mismatches were selected as those deemed most likely to result from DNA damage or replication errors at this site within mammalian cells. To determine whether our previously observed differences in repair rates are the result of replication before repair, and to determine if mismatch repair is active in the G1 stage of the cell cycle, we transfected heteroduplex DNA into G1-synchronized cells. FACS analysis was used to determine the percentage of cells in G1 at the time of transfection and for the next 24 h (Table I). As shown, >96% of the cells were in G1 throughout the 5 h transfection process and for 7 h post-transfection. Analysis of in vivo mismatch repair by individual cells was performed by selecting for the growth of hygromycin-resistant colonies, subsequent PCR amplification of a 128 bp segment from the plasmid containing H-ras codon 12 and analysis by restriction digest and DNA sequencing to determine the sequence at codon 12 (33,34).

A number of controls were performed to assess the quality and purity of our mismatched plasmid preparations and repair results. Each mismatch plasmid was prepared at least twice to verify reproducibility of results. Each mismatch preparation was also transformed into E.coli (Tables II and III), as we have demonstrated previously that E.coli is not transformed by gapped plasmid (minus the mismatch oligo) and that codon 12 of H-ras is not a hotspot of mutation in bacteria. Further controls in which mismatch-containing plasmid was transformed into mismatch-deficient bacteria (NR9161, mut L-) yielded only unrepaired mixtures in the resultant colonies (34). Also, control transfections of plasmid containing either the wild-type or mutated sequence were included with each experiment to ensure that analysis of the sequence at codon 12 yielded the expected results after PCR amplification.

As seen in Table IV, we observed almost complete correct repair resulting from transfection of mismatches into the G1-synchronized cells, compared with rates as low as 35% correct repair for mismatches at codon 12 in non-synchronized cells (see Figure 1 for comparison). This indicates that DNA mismatch repair mechanisms are active and accurate during the G1 stage of the mammalian cell cycle. Interestingly, G1 synchronization was the only experimental situation in which we observed a single incident of unequivocal incorrect repair (G:T to A:T), rather than lack of repair before replication (mixture of G:C and A:T or T:A) in NIH 3T3 cells. It is important to note that the G1-synchronized cells have significant amounts of time to repair the mismatched DNA before entering S phase, while in non-synchronized cells, the amount of time before replication is quite variable. Our results indicate that given enough time before replication, the cells have a significantly increased capability to correctly repair all mismatches tested at the codon 12 location. Comparison of the above results with E.coli mismatch repair during log phase growth (Table II) indicates that E.coli has a correct repair rate that is similarly high or higher than the G1-synchronized NIH 3T3 cells (with the exception of G:A mismatches which are repaired most accurately in G1-synchronized cells). These results further indicate that H-ras codon 12 is not a hotspot of mutation for E.coli (34). It is interesting to note that E.coli has a significant rate of incorrect repair leading to mutation, while the majority of mutations that result in NIH 3T3 cells occur due to replication before repair, with only one example of incorrect repair.

Table I. Percentage of NIH 3T3 cells in G1 stage of the cell cycle with and without serum starvation

<table>
<thead>
<tr>
<th>Day and time harvested</th>
<th>Percentage of cells in G1a</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Serum starvedb</td>
</tr>
<tr>
<td>Day 2</td>
<td>85.2</td>
</tr>
<tr>
<td>Day 3</td>
<td>94.3</td>
</tr>
<tr>
<td>Day 4:</td>
<td></td>
</tr>
<tr>
<td>0 h</td>
<td>96.8</td>
</tr>
<tr>
<td>2 h</td>
<td>96.9</td>
</tr>
<tr>
<td>4 h</td>
<td>96.7</td>
</tr>
<tr>
<td>6 h</td>
<td>97.8</td>
</tr>
<tr>
<td>8 h</td>
<td>98.0</td>
</tr>
<tr>
<td>10 h</td>
<td>97.9</td>
</tr>
<tr>
<td>12 h</td>
<td>97.5</td>
</tr>
<tr>
<td>14 h</td>
<td>78.8</td>
</tr>
<tr>
<td>16 h</td>
<td>52.3</td>
</tr>
<tr>
<td>18 h</td>
<td>8.4</td>
</tr>
<tr>
<td>20 h</td>
<td>9.6</td>
</tr>
<tr>
<td>22 h</td>
<td>22.7</td>
</tr>
<tr>
<td>24 h</td>
<td>38.8</td>
</tr>
</tbody>
</table>

nd = not determined.
aPercentages of cells in G1 were determined by FACS. Plates for each time point were collected in duplicate.
bCells were serum starved in 0.25% calf serum/DMEM beginning on day 1. On day 4 at 0 h, this media was replaced with 10% calf serum/DMEM.
cControl cells were grown in 10% calf serum/DMEM. This media was replaced with fresh 10% calf serum/DMEM on day 4 at 0 h.

Inefficient mismatch repair in vivo

Fig. 1. Overall comparison of mismatch repair rates of H-ras codon 10 and codon 12 in non-synchronized and G1-synchronized cells. Repair rates for codon 12 in non-synchronized cells were reported by Arcangeli and Williams (34) and are shown here for comparison (correct repair: G:A→35%, A:C→58%, T:C→80%, G:T→100%). Repair rates include all mismatches either repaired correctly to G:C or incorrectly to T:A or A:T (in the case of G:T in G synchronized cells, the 100% repair rate reflects 92% correct repair and 8% incorrect repair), but not mismatches which are replicated before repair and thus are found as mixtures in the resulting colony.
Table II. Repair of mismatches at codon 12 of H-ras in E.coli

<table>
<thead>
<tr>
<th>Mismatch</th>
<th>Correctly repaired (total assayed)</th>
<th>Incorrectly repaired (total assayed)</th>
<th>Unrepaired mismatch (total assayed)</th>
</tr>
</thead>
<tbody>
<tr>
<td>G:A</td>
<td>89% (65/73)</td>
<td>3% (2/73)</td>
<td>8% (6/73)</td>
</tr>
<tr>
<td>A:C</td>
<td>97% (38/39)</td>
<td>0% (0/39)</td>
<td>3% (1/39)</td>
</tr>
<tr>
<td>T:C</td>
<td>100% (35/35)</td>
<td>0% (0/35)</td>
<td>0% (0/35)</td>
</tr>
<tr>
<td>G:T</td>
<td>95% (42/44)</td>
<td>5% (2/44)</td>
<td>0% (0/44)</td>
</tr>
</tbody>
</table>

* A portion of each mismatch preparation used in NIH 3T3 studies was used to transform mismatch proficient E.coli as a control (see Results).

Table III. Repair of mismatches at codon 10 of H-ras in E.coli

<table>
<thead>
<tr>
<th>Mismatch</th>
<th>Correctly repaired (total assayed)</th>
<th>Incorrectly repaired (total assayed)</th>
<th>Unrepaired mismatch (total assayed)</th>
</tr>
</thead>
<tbody>
<tr>
<td>G:A</td>
<td>94% (31/33)</td>
<td>0% (0/33)</td>
<td>6% (2/33)</td>
</tr>
<tr>
<td>A:C</td>
<td>100% (41/41)</td>
<td>0% (0/41)</td>
<td>0% (0/41)</td>
</tr>
<tr>
<td>T:C</td>
<td>98% (59/60)</td>
<td>0% (0/60)</td>
<td>2% (1/60)</td>
</tr>
<tr>
<td>G:T</td>
<td>100% (51/51)</td>
<td>0% (0/51)</td>
<td>0% (0/51)</td>
</tr>
</tbody>
</table>

* A portion of each mismatch preparation used in NIH 3T3 studies was used to transform mismatch proficient E.coli as a control (see Results).

Table IV. Repair of mismatches at codon 12 of H-ras in G1-synchronized NIH 3T3 cells

<table>
<thead>
<tr>
<th>Mismatch</th>
<th>Correctly repaired (total assayed)</th>
<th>Incorrectly repaired (total assayed)</th>
<th>Unrepaired mismatch (total assayed)</th>
</tr>
</thead>
<tbody>
<tr>
<td>G:A</td>
<td>100% (27/27)</td>
<td>0% (0/27)</td>
<td>0% (0/27)</td>
</tr>
<tr>
<td>A:C</td>
<td>100% (18/18)</td>
<td>0% (0/18)</td>
<td>0% (0/18)</td>
</tr>
<tr>
<td>T:C</td>
<td>100% (21/21)</td>
<td>0% (0/21)</td>
<td>0% (0/21)</td>
</tr>
<tr>
<td>G:T</td>
<td>92% (12/13)</td>
<td>8% (1/13)</td>
<td>0% (0/13)</td>
</tr>
</tbody>
</table>

* >96% of cells were in G1, as determined by FACS analysis.

Table V. Repair of mismatches at codon 10 of H-ras in non-synchronized NIH 3T3 cells

<table>
<thead>
<tr>
<th>Mismatch</th>
<th>Correctly repaired (total assayed)</th>
<th>Incorrectly repaired (total assayed)</th>
<th>Unrepaired mismatch (total assayed)</th>
</tr>
</thead>
<tbody>
<tr>
<td>G:A</td>
<td>69% (25/36)</td>
<td>0% (0/36)</td>
<td>31% (11/36)</td>
</tr>
<tr>
<td>A:C</td>
<td>94% (47/50)</td>
<td>0% (0/50)</td>
<td>6% (3/50)</td>
</tr>
<tr>
<td>T:C</td>
<td>96% (47/49)</td>
<td>0% (0/49)</td>
<td>4% (2/49)</td>
</tr>
<tr>
<td>G:T</td>
<td>96% (49/51)</td>
<td>0% (0/51)</td>
<td>4% (2/51)</td>
</tr>
</tbody>
</table>

* Codon 10 is not a hotspot of mutation in H-ras.

Comparison of repair rates at codons 10 and 12 in non-synchronized cells

Repair rates were also determined in vivo in non-synchronous cells for each of the specific mismatches when located at the middle nucleotide of codon 10 of H-ras (Table V; see Figure 1 for comparison with codon 12 mismatch repair results). Codon 10 has the same primary sequence as codon 12 (GGC) with the middle nucleotide of each separated by only 6 bp. However, codon 10 does not appear to be a hotspot of mutation, as there have not been mutations at this sequence found in any in vivo activation assay or in naturally occurring human tumors (3–5). As seen in Figure 1, in non-synchronized cells there is a significant improvement in correct mismatch repair rates for codon 10 as compared with codon 12 for G:A, A:C and T:C mismatches, while the repair rate for G:T remains at a high level. These results demonstrate clearly that repair of mismatched nucleotides is more efficient at codon 10 than at codon 12 in non-synchronous cells, indicating that codon 12 is a hotspot of mutation, at least in part, due to inefficiencies in accomplishing mismatch repair. Furthermore, as the only difference between the mismatches at codons 10 and 12 was the sequence context surrounding the GGC codon, these results indicate that sequence context effects mismatch repair efficiency at these locations in H-ras.

Nuclear protein binding to mismatches at the codon 12 hotspot and at codon 10

Gel-shift nuclear protein–DNA binding assays were used to assess potential differences in mismatch-specific binding to the different mismatches at the codon 12 hotspot and at codon 10. 32mer double-stranded oligonucleotides were constructed
Inefficient mismatch repair in vivo

Fig. 2. Relative amounts of nuclear protein binding to specific mismatches at codon 10 or codon 12 of H-ras. Gel-shift assays were performed as described in Materials and methods using equal amounts of radioactivity for each mismatch. Arrows indicate mismatch-specific bands. (A) HeLa nuclear extract binding to mismatches at codon 10 or codon 12. (B) NIH 3T3 nuclear extract binding to codon 12 mismatches. (C) Comparison of the relative amounts of HeLa mismatch-specific nuclear extract binding at codon 12 and codon 10. (D) Comparison of the relative amounts of NIH 3T3 mismatch-specific nuclear extract binding at codon 12. Autoradiographs were scanned and analyzed using NIH Image software. Relative amounts of each mismatch were compared with the amount bound to the G:T containing oligonucleotide which was arbitrarily set at 100%. Averages were determined from three separate experiments.

Based on the human H-ras genetic sequence with a specific mismatch located at the middle nucleotide of either codon 12 or 10. To ensure that the proximate location of codon 10 or 12 to the 3′ or 5′ end of the oligo was not affecting binding, an additional set of oligos was used in which the codon 12 position was identical, in relation to the 3′ and 5′ end, to the position of codon 10 in the first set of oligos, with similar results (Materials and methods). All experiments were performed with nuclear extracts from both NIH 3T3 cells and from the human cell line HeLa, which is known to be mismatch proficient (Figure 2A and B) to rule out unknown mismatch repair deficiencies in the NIH 3T3 cell line. Two mismatch-specific bands were observed when using HeLa nuclear extracts, and one band was observed when using NIH 3T3 nuclear extracts. Densitometry measurements were performed to determine the relative amounts of mismatch-specific protein binding after incubation with NIH 3T3 and HeLa nuclear extracts (Figure 2C and D). As shown in Figure 2B and D, the relative amounts of mismatch-specific binding in NIH 3T3 extracts correlates closely to our observed in vivo mismatch repair rates for the H-ras codon 12 site. Additionally, as shown in Figure 2A and C, there is virtually no mismatch-specific binding to the G:A mismatch located at codon 12, while at codon 10 there are observable levels of binding of the slower migrating band to this same mismatch. This demonstrates a sequence context effect in binding by the mismatch-specific factor, and is especially intriguing in light of the low levels of repair of G:A mismatches at the codon 12 position and correspondingly higher levels of repair at codon 10.

To determine the identity of the binding factor, we included monoclonal antibodies specific for hMSH6 (GTBP), a component of the hMutSα heterodimer. As seen in Figure 3A, incubation of anti-hMSH6 antibody with HeLa nuclear extract before the addition of mismatch DNA results in a marked decrease in binding by especially the slower migrating band to this same mismatch. This demonstrates a sequence context effect in binding by the mismatch-specific factor, and is especially intriguing in light of the low levels of repair of G:A mismatches at the codon 12 position and correspondingly higher levels of repair at codon 10.
specific mismatches at both codons 10 and 12 with either NIH 3T3 or HeLa nuclear extracts (results not shown). Competition assays using an excess of non-radioactively labeled mismatch oligo demonstrated inhibition of mismatch-specific binding for each mismatch when using either NIH 3T3 or HeLa nuclear extracts, indicating that the same binding factor was responsible for the mismatch-specific binding in each cell line (results not shown). Taken together, the above results indicate that the mismatch-specific binding factor we observe in NIH 3T3 and the slower migrating band we observe with HeLa nuclear extract is the hMutSα complex.

Discussion

Although the existence of hotspots of mutation at oncogenic locations has been well documented, the mechanisms contributing to the increased mutation frequency at these hotspots within human tumors are still in question. Our data demonstrate that for the biologically relevant codon 12 location of H-ras, there is inefficient repair of specific mismatched basepairs within NIH 3T3 cells, resulting in replication before repair. We have found that repair rates vary depending on the precise location, exact composition and stage of the cell cycle in which the mismatch is introduced.

Our results for mismatch repair at the middle nucleotide hotspot of codon 12 in G1-synchronized cells demonstrate that repair of mismatched DNA is active in the G1 stage of the mammalian cell cycle. Our observance of repair activity is in accordance with previous studies demonstrating expression of mismatch repair proteins in G1 (29,30). Furthermore, we have observed correct repair of virtually all codon 12 mismatches transfected into G1-synchronized cells, with the single exception of one incorrectly repaired G:T mismatch (Table IV). In contrast, we previously found high rates of unpaired mismatches at codon 12 in non-synchronous cells (Figure 1) (34). Although it is theoretically possible that the increased correct mismatch repair observed in G1-synchronized cells is due to increased repair activity during the G1 stage of the cell cycle, this seems unlikely for what is generally considered a post-replicative repair process. Also, recent reports have indicated that mismatch repair protein expression is fairly consistent throughout the eukaryotic cell cycle (30) and may even be slightly decreased in G1 (29). Instead, we believe the increased overall rate of mismatch repair observed in G1-synchronized cells is due to the length of time available for the correction process prior to replication. FACS analysis has shown that >96% of the synchronized cells are still in G1 as long as 7 h after completion of transfection. Therefore, the increased correct mismatch repair during G1 probably indicates a function of increased time for correct repair before replication rather than an increase in repair activity. Thus, our results indicate that repair of specific mismatches at codon 12 in non-synchronous cells is inefficient, rather than inaccurate.

Our results for mismatch repair at the middle nucleotide position of codon 10 in non-synchronous cells demonstrate that G:A, A:C and T:C mismatches at this location are repaired significantly more efficiently than when located at the codon 12 middle nucleotide hotspot of mutation in non-synchronous cells. As the only difference when comparing repair rates for each specific mismatch at these two locations is the sequence context surrounding the codon containing the mismatch, these results indicate a sequence context effect on the cell’s ability to repair mispaired DNA. These results are similar to studies in which differential nucleotide excision repair at hotspots of mutation has been observed (9,10,40). Indeed, in studies examining repair of O6-methylguanine adducts opposite thymine, a consensus sequence for lack of repair was derived that has significant similarity to the sequence surrounding codon 12 of H-ras (40). It is also intriguing that previous primer extension studies have demonstrated both DNA polymerase β and α to have a strong pause site at the wild-type codon 12 location (41), suggesting steric hindrance, a difficulty in the gap-filling stage of different DNA repair processes, or a general difficulty in enzymatic reactions at this site.

In agreement with previous studies, we also observed significant differences in the repair rates for specific mismatches when located at the same codon 10 location, indicating that different mismatches are repaired at different efficiencies, even when at the same location (42–47). As seen in Table V, only 69% of G:A mismatches were correctly repaired at codon 10, while A:C, T:C and G:T were all repaired at a high rate (94–96%) at this location. We previously found G:A mismatches to be repaired at the even lower rate of 35% at codon 12 in non-synchronous cells. Other investigators have also found G:A to be particularly difficult to repair in both in vitro and in vivo mismatch repair studies (43,45–48). It appears that there are slower kinetics of recognition and/or repair specifically for G:A mismatches in our system. Lack of repair of G:A mismatches at codon 12 in H-ras may be biologically relevant, as the resulting T:A transversion is a common mutation found at this location in naturally occurring human tumors (3–5).

We next performed gel-shift analysis using nuclear extracts to determine if binding by specific DNA mismatch repair proteins could be a rate-limiting step. As seen when comparing Figures 1 and 2D, the relative amount of mismatch-specific binding to each mismatch at codon 12 closely correlates with specific mismatch repair abilities within non-synchronized cells. We believe the observed binding is due to the long-patch mismatch repair hMutSα complex, as: (i) this has been shown to be the major mismatch binding activity in HeLa
nuclear extracts (15); (ii) pre-incubation of nuclear extract with antibody to hMSH6 destroyed this binding (23); and (iii) addition of ATP, which has been shown to disrupt binding of the hMutSα complex (22,37–39), resulted in a complete lack of binding by the mismatch-specific factors (Figure 3). Western blotting has also demonstrated both hMSH2 and hMSH6 as components of the mismatch-specific binding (results not shown). Although other mismatch-specific binding factors such as DNA topoisomerase I and deoxyoxime 3′′ endonuclease have been reported (49,50), these do not bind to DNA with the same sequence specificity and do not exhibit the same sensitivity to ATP.

Furthermore, we observed mismatch-specific binding to oligos that contain G:A at the codon 10 position but not for oligos with G:A at the codon 12 position. This demonstrates that the sequence context surrounding a mismatch can effect hMutSα binding. Local sequence context effects on hMutSα binding have also been demonstrated recently for one and two base pair loop structures (51). This lack of binding to oligos containing G:A mismatches at codon 12 correlates with the very low efficiency of repair observed in vivo for G:A mismatches at codon 12 in non-synchronous cells. Recent reports have demonstrated that hMutSα recognizes and binds to mismatched DNA when in an ADP-bound form and that subsequent ATP binding results in dissociation of the heterodimer from the mismatched DNA (37–39). This had led to the suggestion that repair is modulated by ATP binding rather than mismatch recognition. However, our studies would suggest that at sensitive locations such as the H-ras codon 12 hotspot of mutation, there may be deficiencies in recognition of specific mismatches that result in low levels of repair.

The lower rates of repair for a variety of different mismatches (T:C, A:C, G:A) at codon 12 in non-synchronous cells, combined with the differential binding by hMutSα, implicates an inefficiency in the general long-patch mismatch repair system. We have also observed almost 100% correct repair for all mismatches tested in G1-synchronized cells. Therefore, we believe that there are slower kinetics of recognition and/or repair at codon 12 as compared with codon 10, rather than a completely non-functioning long-patch mismatch repair system at this location. Variable kinetics of repair that result in altered in vivo repair rates due to plasmid replication before repair have been observed previously in studies using DNA adducts (52). Although mutations at codon 12 are activating and thus likely to be selected for in tumors, selection for increased proliferative ability alone is not sufficient to explain the incidence of mutation at codon 12, as there are activating mutations at other locations that have never been found in vivo. Our results demonstrate that codon 12 is a hotspot of mutation, at least in part, due to a rate limiting step in the mismatch repair process. This inefficient repair results in replication before repair, thus fixing the mutation in the genome.

The importance of understanding mechanisms of site-specific mutation is demonstrated by the prevalence of hotspots of mutation in naturally occurring tumors. In this study, we have demonstrated differences in cellular mismatch repair rates for mismatches at a hotspot and at a non-hotspot location and for different cell cycle parameters. We have also observed differences in binding by hMutSα in vitro that correlate with mismatch repair efficiency in vivo. It is interesting to note that these experiments were performed using two mismatch proficient cell lines, and thus may have ramifications for initiation of tumorigenesis in individuals who are not hereditarily predisposed to cancer. Further work is clearly necessary to define the precise enzymatic mechanisms responsible for the lack of repair at oncogenic hotspots.

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References


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