Mammalian assay for site-specific DNA damage processing using the human *H-ras* proto-oncogene

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ABSTRACT

The human genomic *H-ras* proto-oncogene was inserted into an Epstein–Barr virus (EBV) vector (p220.2) that replicates synchronously with the cell cycle. Unique restriction enzyme sites, 30 bp apart, were created on either side of codon 12 to enable the construction of gapped heteroduplex (GHD) DNA. Depending upon experimental protocol, the gap could be located either on the coding (non-transcribed) strand or the non-coding (transcribed) strand. GHD DNA was created using a 1.8 kb segment of *H-ras* DNA containing exon 1, into which a synthetic 30 nucleotide oligomer containing a strand- and site-specific mismatched nucleotide was annealed. The 1.8 kb segment of *H-ras* DNA containing a codon 12; middle G:T, A:C or T:C mismatch has been religated with high efficiency into the EBV vector and transfected into NIH 3T3 cells using a mild liposome-mediated protocol. Subsequent hygromycin resistant NIH 3T3 colonies have been PCR amplified and sequenced. In this study, codon 12; middle nucleotide mismatch correction rates to wild-type G:C during replication in NIH 3T3 cells were 96.4% of G:T mismatches, 87.5% of A:C mismatches and 67% of T:C mismatches.

INTRODUCTION

Over the past several years, research of mammalian DNA damage and repair mechanisms has undergone several exciting developments. An exceptional example has been the cloning and characterization of several nucleotide excision repair (NER) genes from cell lines derived from patients with xeroderma pigmentosum, Cockayne's syndrome and trichothiodystrophy (1,2). This complex repair system can recognize a broad spectrum of DNA lesions such as UV photoproducts, bulky chemical adducts, alkylating chemical adducts, crosslinks and, to a lesser extent, abasic sites and mismatches (3,4). Another recent development has been the identification of human homologues of bacterial MutS (hMSH2) and MutL (hMLH1, hPMS1, hPMS2) (5). The roles of these bacterial gene products have been well described for methyl-directed mismatch repair activity, a post-replicative DNA repair pathway critical for the maintenance of genetic stability. It now appears that the majority of hereditary

non-polyposis colorectal cancers (HNPCC) and a significant percentage of sporadic colon cancers contain a disabling mutation in at least one of the human mismatch repair homologue genes. This evidence, along with high mutation rates in microsatellite regions of HNPCC tumor cell lines, is a strong indication that loss of mismatch repair results in a mutator phenotype (6).

Biochemical mechanisms of mismatch repair in mammalian cells are not yet as well understood as prokaryotic systems. Several interesting mechanistic details have been reported, however. It is known that mammalian mismatch repair systems use one DNA repair complex to repair A:C, T:T or T:C mismatches but have at least two separate repair complexes for G:T or G:U mispairs putatively arising from endogenous 5-methylcytosine or cytosine deamination events, respectively (7,8). One pathway to correct G:T mispairs appears confined primarily to CpG sequences in mammalian cells (7,8). It is now evident that all combinations of mismatches can be repaired in mammalian cells; however, G:T mispairs are corrected with much higher efficiency than others (7,9).

Early studies of mammalian DNA repair events have been primarily limited to transfection and replication of shuttle vectors containing bacterial, viral or synthetic DNA as the target sequence. Difficulties with these experiments, such as low transfection efficiency, high cell toxicity, high spontaneous mutation rate and the necessity of additional bacterial replication for mutation analyses (10) have been circumvented by the use of selectable mammalian chromosomal sequences and direct PCR analysis (11–13). Information obtained from both types of experimental approaches have revealed a distinct difference in the repair rate of damaged bases at different sites (14–16) and, for many lesions, preferential repair on the transcribed strand of active genes (17–19).

Mutations in oncogenes and/or tumor suppressor genes consistently found in human tumors may be due, in part, to a genetic defect of a DNA repair pathway, such as NER or mismatch repair. However, to account for the high specificity and frequency of 'hot spots' of mutation, it is probable that a separate mechanism of misreplication or misrepair occurs in sensitive locations in the genome. The *ras* gene family in particular (H-, K-, N-*ras*) has an overall mutation frequency of 30% in all human tumors and >90% in human pancreatic adenocarcinomas (20). Sequence analyses of human tumors, in addition to the NIH 3T3 cell transformation assay and the nude mouse tumorigenicity test, have demonstrated that *ras* genes have activating mutations

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almost exclusively in codons 12, 13 or 61 (20,21). One hypothesis to explain such precise mutagenic targeting is that specific types of DNA damage occur more frequently at certain sites in the genome. Indeed, animal model studies have demonstrated correlations between type of chemical exposure and location and type of *ras* activating mutations in resultant tumors (22). A second hypothesis is that there is random DNA damage in the genome with decreased fidelity of replication or repair of damage at specific sites. This notion has prompted several studies using both bacterial and mammalian systems with H-*ras* codon 12 as the target sequence. Conflicting results from studies based primarily on insertion of guanine adducts at different positions within codon 12 or 13 of H-*ras* have raised several intriguing questions regarding positional effect on repair (23–25). A third hypothesis is that cells with certain activating mutations are selected for increased survival. However, arguments against natural selection playing a major role have been inferred from *in vitro* *ras* transformation assays in which several active mutational sites have been discovered that have never been found in human tumors (26–29).

The subject of this work has been the development of a unique, highly sensitive mammalian assay to study both strand- and site-specific mechanisms of mutation and repair that may contribute to cellular transformation. The system described here has several distinct advantages compared with other DNA damage and repair assays. Human H-*ras* genomic DNA with a well known oncogenic 'hot spot' as the target sequence was chosen for these studies. A precise enzymatic method was used to create gapped heteroduplex (GHD) DNA that can be purified with the gap located on either strand allowing for both strand- and site-specific studies. In addition, a mild liposome-mediated transfection methodology was used to introduce the site-specifically altered plasmid into the cell. PCR amplification was subsequently used to retrieve the DNA of interest directly from antibiotic resistant NIH 3T3 colonies. In this study, experiments were designed to investigate NIH 3T3 repair efficiency of specific base-pair mismatches located at the human H-*ras* codon 12; middle nucleotide position.

MATERIALS AND METHODS

Enzymes

T₄ DNA Ligase, calf intestinal alkaline phosphatase (CIP), *Kpn*I, *Xho*I and *Bfr*I were purchased from Boehringer Mannheim Biochemicals. T₄ polynucleotide kinase and Replitherm DNA polymerase were purchased from Epicentre Technologies. *Bam*HI and *Hpa*II were purchased from New England Biolabs. *Hind*III was purchased from US Biochemicals. Proteinase K was purchased from Sigma Chemical Company.

Cells, plasmids, oligonucleotides and other reagents

NIH 3T3 cells (mouse embryo cell line) and plasmids pbc-N1 and pT24-C3 were obtained from American Type Culture Collection (ATCC). Plasmid p220.2 was a kind gift from Dr William Sugden, University of Wisconsin. M13mp18, M13mp19, pUC18, DH5 α competent *E.coli*, Lipofectin, LipofectAMINE and Opti-MEM were purchased from Life Technologies, Inc. All synthetic oligonucleotides were purchased from Operon Technologies, Inc. Radioactively labeled nucleotides were purchased from New England Nuclear. Agarose for electrophoretic separation

and purification of DNA was purchased from FMC Bioproducts. Gene Clean II was purchased from Bio 101, Inc. Dulbecco's Modified Eagles's Medium (DMEM; 4.5 g/l glucose) and calf serum (bovine) were purchased from HyClone Laboratories, Inc. Hygromycin B was purchased from Calbiochem Biochemicals. All other reagents were purchased from Sigma Chemical Company unless otherwise noted.

Construction of Epstein-Barr virus (EBV) shuttle vectors containing human H-*ras* genomic DNA

Two separate shuttle vectors containing human H-*ras* genomic DNA were constructed by inserting either wild-type (wt) human H-*ras* genomic sequence (*Bam*HI 6.4 kb segment from pbc-N1) or human H-*ras* genomic sequence containing a codon 12; middle T:A base pair activating mutation as a positive control plasmid (*Bam*HI 6.6 kb segment from pT24-C3) into the *Bam*HI site in the polylinker region of the EBV shuttle vector p220.2, thereby creating p220.pbc and p220.T24, respectively (Fig. 1A) (29,30).

Removal of the original *Hind*III site in the polylinker region of p220.pbc was accomplished by DNA polymerase I (Klenow fragment) 5'→3' exonucleolytic digestion and subsequent blunt-end ligation by standard procedure (30). A 2 kb *Bam*HI–*Kpn*I segment of p220.pbc containing exon 1 was ligated into the polylinker region of bacteriophage M13mp19 and subjected to oligo-directed mutagenesis to create two new restriction enzyme recognition sites, *Hind*III and *Bfr*I, 30 bp apart flanking H-*ras* codon 12 in exon 1 (31,32). Three separate rounds of oligo-directed mutagenesis (31,32) were performed with the M13-*ras* plasmid to change a total of four bases: codon 6; G→T (no amino acid change to create unique *Hind*III site) and codon 15; GGC→CTT (Gly→Leu, both neutral non-polar amino acids to create unique *Bfr*I site) (Fig. 1B). Restriction digestion and dideoxy sequencing (33) were used to select M13-*ras* clones that contained only the above indicated base changes. *Bam*HI–*Kpn*I segment containing the new sites was excised from RF M13-*ras* DNA and reinserted into p220.pbc to replace the original 2 kb H-*ras* segment (Fig. 1C). This modified plasmid (p220.pbc+H/B) contains unique *Hind*III and *Bfr*I recognition sites 30 bp apart flanking codon 12; middle G, which is located 22 bp 3' of the *Hind*III site and 9 bp 5' of the *Bfr*I site.

Formation and characterization of p220.pbc+H/B containing site-specific mismatch

The 2 kb *Bam*HI–*Kpn*I H-*ras* segment from p220.pbc+H/B was first ligated into the polylinker region of pUC18, a small, high copy number plasmid, to produce *pras*BK2.0. This was done to enable a larger yield of the 1.8 kb *Xho*I–*Kpn*I H-*ras* fragment for synthesis of GHD DNA.

Restriction digestion conditions for p220.pbc+H/B and *pras*BK2.0 to release the 13.5 and 1.8 kb *Xho*I–*Kpn*I fragments, respectively, were identical: a total volume of 3.6 ml at 300–500 ng plasmid DNA per μ l and 3 U of each restriction enzyme (*Xho*I and *Kpn*I) per μ g of DNA in OPA buffer (10 mM Tris-acetate, 10 mM Mg-acetate, 50 mM K-acetate, pH 7.5) was divided into 100 μ l aliquots. Solutions were incubated at 37°C for 5 h. Separation of the 13.5 and 1.8 kb DNA fragments from each plasmid was accomplished by agarose electrophoresis using 1% Seakem Gold (FMC) in TAE buffer (30). Purification of DNA from the agarose was accomplished with Gene Clean II (Bio 101, Inc.) as described by the manufacturer. A portion of the purified 1.8 kb DNA

Figure 1. Construction of p220.pbc+H/B. (A) Complete human genomic wt (pbc-N1) or activated (pT24-C3) H-ras sequence was ligated into the *Bam*HI site of the polylinker region of p220.2 to produce p220.pbc and p220.T24 shuttle vectors containing the EBV origin of replication (EBV ori P), nuclear antigen 1 (EBNA 1), ampicillin (Amp^R) and hygromycin (Hyg^R) resistance genes. (B) A 2 kb *Bam*HI-*Kpn*I H-ras segment of p220.pbc containing exon 1 was ligated into M13mp19 polylinker region to produce M13-ras for oligo-directed mutagenesis to create two unique restriction enzyme sites: *Hind*III at codon 6 (G→T) and *Bfr*I at codon 15 (GGC→CTT). (C) The *Bam*HI-*Kpn*I segment from M13-ras containing the two new unique sites on either side of codon 12 were reinserted into p220.pbc, replacing the original *Bam*HI-*Kpn*I segment to make p220.pbc+H/B.

fragment was digested with *Hind*III and *Bfr*I and repurified through G-50 Sephadex columns to remove the original 30 bp segment containing codon 12 of H-ras. This *Hind*III-*Bfr*I cleaved DNA was mixed with undigested 1.8 kb fragment in TE (30) at a 4:1 molar ratio and a total concentration of 75 ng/μl. This mixture was divided into 200 μl aliquots and melted at 100°C for 8 min; adjusted to 0.1 M NaCl; incubated at 80°C for 2 min; cooled to 65°C over a period of 40 min and held at this temperature for an additional 30 min (34). The 1.8 kb reannealed DNA, containing a mixture of gapped and non-gapped heteroduplex DNA, was purified from smaller DNA fragments by agarose electrophoresis and Gene Clean II. This mixture was then redigested with either *Hind*III or *Bfr*I. *Hind*III cleaves all dsDNA and all DNA containing a gap on the transcribed (non-coding) strand, but not DNA containing a gap on the non-transcribed

(coding) strand. *Bfr*I, however, cleaves all dsDNA and all DNA containing a gap on the non-transcribed (coding) strand, but not DNA containing a gap on the transcribed (non-coding) strand (Fig. 2A). In this manner, strand selection was made by choice of restriction enzyme. Agarose electrophoresis and Gene Clean II were used to purify the final, strand-selected 1.8 kb GHD DNA product from all smaller DNA fragments. The purified GHD DNA was then 5' dephosphorylated, using CIP, and purified once more by Gene Clean II.

Mismatch oligonucleotides complementary to the 30 bp gapped region spanning codon 12 (middle dG replaced with dT or dA for non-transcribed strand mismatch oligomer, or middle dC replaced with dT or dA for transcribed strand mismatch oligomer) were phosphorylated and G-25 Sephadex column purified. Phosphorylated oligomers and GHD DNA were

Figure 2. (A) Formation of strand-specific GHD DNA. Step 1: an aliquot of purified 1.8 kb *Xho*I-*Kpn*I H-ras DNA segment of *praskB2.0* was cleaved with *Hind*III and *Bfr*I to produce 1.5 and 0.3 kb fragments. This was then melted and reannealed in a 4:1 molar ratio with full length 1.8 kb *Xho*I-*Kpn*I DNA. The 1.8 kb portion of the reannealed mixture, now containing gapped and dsDNA, was purified from all other DNA fragments by agarose electrophoresis. Step 2: if the purified 1.8 kb mixture of gapped and dsDNA is cleaved again with *Bfr*I, all of the reannealed DNA species will be cleaved to smaller fragments except 1.8 kb DNA that contains a gap in the transcribed strand (because the recognition sequence for *Bfr*I is missing five nucleotides in this gapped species only). Alternatively, if the purified 1.8 kb mixture of gapped and dsDNA is cleaved again with *Hind*III, all of the reannealed DNA species will be cleaved to smaller fragments except 1.8 kb DNA that contains a gap in the non-transcribed strand (because the recognition sequence for *Hind*III is missing five nucleotides in this gapped species only). (B) Selection of strand-specific GHD DNA. Lane 1 is purified 1.8 kb *Xho*I-*Kpn*I H-ras DNA segment of *praskB2.0*. Lane 2 is 1.8 kb *Xho*I-*Kpn*I DNA cleaved with *Hind*III and *Bfr*I to produce 1.5 and 0.3 kb DNA fragments. These DNA fragments are melted and reannealed at a 4:1 molar ratio with uncleaved 1.8 kb *Xho*I-*Kpn*I DNA to produce a mixture of 1.8 kb gapped and dsDNA (amongst other size fragments, Fig. 2A, step 1). Lane 3 is a purified mixture of 1.8 kb gapped and dsDNA that has been resealed with *Bfr*I (Fig. 2A, step 2) to select for 1.8 kb DNA containing a gap in the transcribed strand. Lane 4 is the same mixture of 1.8 kb gapped and dsDNA as in lane 3, but cleaved with both *Bfr*I and *Hind*III, removing all 1.8 kb DNA species. Lane 5 is the same mixture of 1.8 kb gapped and dsDNA as in lane 3, but cleaved with *Hind*III to select for 1.8 kb DNA containing a gap in the non-transcribed strand.

annealed at a 200:1 molar ratio at a concentration of 75 ng/ μ l, in a total volume of 20 μ l of T4 DNA ligase buffer at 60°C with subsequent slow cooling to room temperature. GHD DNA (1.8 kb) (now containing the mismatched oligomer) was ligated to the 13.5 kb *Xho*I-*Kpn*I portion of p220.pbc+H/B at a 3:1 molar ratio of GHD DNA to vector at 16°C overnight. Uncut and *Eco*RV digested portions of each ligation reaction were compared with circular and *Eco*RV linearized p220.pbc+H/B by agarose electrophoresis to determine efficiency of ligation (Fig. 3).

Transfection, selection and analyses of human H-ras DNA from hygromycin resistant NIH 3T3 cells

NIH 3T3 cells were grown in DMEM, 10% calf serum at 37°C, 5% CO₂. Cells were seeded at 1×10^6 per 100 mm plate in preparation for transfection experiments 16–18 h later. Plasmid DNA (100–300 ng per plate) was transfected into NIH 3T3 cells using Lipofectin or LipofectAMINE reagent as described by the manufacturer. NIH 3T3 hygromycin resistant cells were subse-

Figure 3. Final ligation of p220.pbc+H/B to contain site-specific mismatch in codon 12 of H-ras. Uncut control: p220.pbc. Uncut ligation: final ligation product containing site-specific mismatch in codon 12 of H-ras. EcoRV control: p220.pbc linearized by EcoRV cleavage. EcoRV ligation: final ligation product linearized by EcoRV cleavage. One kb standard marker is shown on the left.

quently selected by the addition of 150 U of hygromycin per ml of media, starting 24 h after transfection. After 3 weeks, hygromycin resistant colonies on each plate were methanol fixed and stained with 1% crystal violet; 20% ethanol. DNA was purified from each NIH 3T3 colony by the following method: cloning cylinders (Bellco Glass, Inc.) were adhered to the culture dish surrounding each colony using sterile silicone vacuum grease. A 100 μ l aliquot of lysis buffer (10 mM Tris-HCl pH 8.3, 50 mM KCl, 0.1 mg/ml gelatin, 0.45% NP-40, 0.45% Tween 20, 2.5 mM MgCl₂) containing 12 μ g Proteinase K (35) was added to the cloning ring and incubated for 10 min at room temperature. Each solution was then repeatedly aspirated to dislodge remaining cells from the culture plate, transferred into a 0.5 ml microcentrifuge tube and incubated for 1 h at 55°C followed by 10 min at 95°C. Samples were phenol/chloroform extracted and precipitated with ethanol. DNA pellets were resuspended in 10 μ l of sterile dH₂O. A 5 μ l aliquot from each DNA solution was then PCR amplified. PCR primers used for DNA amplification reactions were: 5' H-ras $\rightarrow$ 5'-TGA GGA GCG ATG ACG GAA

TAT-3' and 3' H-ras $\rightarrow$ 5'-CAG GCT CAC CTC TAT AGT GGG GTC-3', yielding an amplified DNA product of 129 bp containing exon 1 of human H-ras plus several base pairs of human intronic region surrounding exon 1. A 20 μ l aliquot was then restriction digested using *Hpa*II. Only PCR amplified DNA containing wt human H-ras codon 12 sequence (GGC) is cleaved by *Hpa*II, yielding an 88 and a 41 bp band from the original 129 bp sequence (Fig. 4). All PCR amplified DNA not completely cleaved by *Hpa*II was cycle-sequenced as described by the manufacturer (Perkin Elmer).

RESULTS

To study mechanisms of mutation at specific sites ('hot spots') that contribute to cellular transformation more closely, both wt (pbc-N1) and activated (pT24-C3) human genomic H-ras DNA were inserted into an autonomously replicating EBV vector, p220.2, (29,36) to create p220.pbc and p220.T24 (Fig. 1A). Two new restriction sites were created in p220.pbc by oligo-directed mutagenesis, resulting in a plasmid (p220.pbc+H/B) in which codon 12; middle bp is flanked by a new, unique *Hind*III site (22 nt 5') and a new, unique *Bfr*I site (9 nt 3') (Figs 1B and 2A). These alterations permit the facile insertion of a site-specific mismatch or adducted nucleotide at codon 12 or another location within the 30 bp region flanked by the two unique restriction enzyme sites.

Synthesis of strand-specific GHD DNA

GHD DNA was created by first cleaving an aliquot of purified 1.8 kb *Xho*I-*Kpn*I DNA segment with *Hind*III and *Bfr*I, resulting in a 1.5 kb *Xho*I-*Hind*III fragment, a 0.3 kb *Bfr*I-*Kpn*I fragment, and a 30 bp *Hind*III-*Bfr*I fragment. This mixture (minus the 30 bp fragment) was combined with uncut 1.8 kb *Xho*I-*Kpn*I fragment, melted, and allowed to reanneal. This process yields a mixture of gapped and fully double-stranded 1.8 kb DNA plus double-stranded 1.5 and 0.3 kb DNA. The mixture of 1.8 kb DNA was purified from all smaller DNA fragments (Fig. 2A, step 1), and cleaved once again with either *Hind*III or *Bfr*I (Fig. 2A, step 2). By this method, we can selectively retain only the DNA segment containing the gap either on the transcribed strand (by cutting with *Bfr*I) or the non-transcribed strand (by cutting with *Hind*III). The reason for this is that *Hind*III and *Bfr*I will cleave their recognition palindrome sequence if one nucleotide of the six base pairs in the recognition sequence is missing, but not if five nucleotides are missing. Figure 2B demonstrates that while virtually all purified 1.8 kb mixture DNA is cleaved by simultaneous digestion with *Hind*III and *Bfr*I, 10-25% of this DNA does not cleave in the presence of *Hind*III or *Bfr*I alone, indicating a reasonable yield of strand-specific GHD DNA using either restriction enzyme.

Synthesis of site-specific mismatch p220-ras plasmid

The 1.8 kb strand-specific GHD DNA fragment was purified from all other DNA fragments, and a strand-specific codon 12; middle nucleotide mismatched oligomer was annealed into the 30 bp gapped region. This was then ligated to the previously purified 13.5 kb DNA fragment of p220.pbc+H/B to produce p220.pbc+H/B with a site-specific mismatch at codon 12; middle nucleotide position. Ligation efficiency was determined by agarose electrophoresis of uncut and *Eco*RV linearized ligation products (Fig. 3). It is evident from Figure 3 that ligation of these

Figure 4. PCR amplification and *HpaII* cleavage of *H-ras* DNA. Lanes 2–9 contain PCR amplified and *HpaII* cleaved DNA from Hygro^R NIH 3T3 cells that were transfected with T:C mismatched p220.pbc+H/B (see Results). Lane 10 contains PCR amplified and *HpaII* cleaved DNA from Hygro^R NIH 3T3 cells that were transfected with p220.T24. Lane 11 contains PCR amplified and *HpaII* cleaved DNA from Hygro^R NIH 3T3 cells that were transfected with p220.pbc. Lanes 12 and 13 contain PCR amplified and *HpaII* cleaved p220.pbc DNA (0.001 ng). Lanes 14 and 15 contain PCR amplified and *HpaII* cleaved p220.T24 DNA (0.001 ng). Lanes 1 and 16 contain no DNA except primers as PCR negative controls.

DNA fragments efficiently produces relaxed circular plasmid DNA that, when digested with *EcoRV*, co-migrates with linearized control plasmid DNA.

Transfection, PCR amplification and analyses

A site-specific DNA repair assay that depends on direct PCR amplification of plasmid DNA from mammalian cells must also require a low copy number of plasmid per cell and a highly sensitive mutation detection system. In order to ascertain the sensitivity of the assay, an experiment was performed to determine the highest ratio of p220.pbc (wt *ras*) to p220.T24 (activated *ras*) to which NIH 3T3 cells could be exposed during transfection and still exhibit the transformed phenotype above background. In this experiment, even at the highest ratio of 99:1 (9.9 μ g p220.pbc:0.1 μ g p220.T24), the average number of foci per plate was increased 2-fold above background. Consistently increasing numbers of foci were seen at lower ratios of wt to activated p220-*ras* (Table 1). This appears, then, to be a highly sensitive assay since activated *ras* expression in NIH 3T3 cells is not hampered by 100-fold excess wt *ras*. Also, the results of this experiment strongly suggest a low copy number of plasmid per cell: the 2-fold increase in foci at 99:1 wt to activated *H-ras* ratio did not derive from multiple copies of wt *H-ras* expression (37), as the same concentration of wt *H-ras* alone does not produce foci above background of non-transfected NIH 3T3 cells (Table 1). In fact, liposome transfection experiments by this author have consistently resulted in an equal or lower number of foci produced by NIH 3T3 cells transfected with wt *H-ras* as compared with non-transfected NIH 3T3 cells (results not shown). Subsequent experiments to determine plasmid copy number (38,39) in hygromycin resistant NIH 3T3 colonies have consistently indicated less than three plasmid copies per cell (results not shown). This is in agreement with *HpaII* cleavage and sequencing results.

Table 1. p220.pbc (wt):p220.T24 (activated) ratios and foci

μ g DNA per plate		average # foci per plate	
(wt)	(act.)		
–	–	6	
10.0	–	5	
–	10.0	591	
(ratio)			
1:3	2.5	7.5	470
1:1	5.0	5.0	418
3:1	7.5	2.5	277
9:1	9.0	1.0	47
19:1	9.5	0.5	29
99:1	9.9	0.1	10

Different ratios of p220.pbc and p220.T24 were transfected into 1×10^6 NIH 3T3 cells per 150 mm plate. Duplicate plates were used for each ratio. Cells were incubated for 14 days, then methanol fixed and crystal violet stained. Foci were counted in each plate and the average number of foci was determined for each ratio.

PCR amplification of *H-ras* DNA and subsequent analyses were performed using hygromycin resistant NIH 3T3 colonies that had been transfected with plasmids containing mismatched, wild-type or activated *H-ras* sequences. Figure 4 illustrates the results of an experiment in which NIH 3T3 cells were transfected with p220.pbc+H/B containing a codon 12; middle T:C mismatch. Amplified DNA in sample lanes 3 and 6 clearly are not cleaved by *HpaII* and therefore do not contain wt *H-ras* DNA sequence at codon 12. However, some lanes contain partially cleaved DNA and leave some question as to the sequence. Sequencing of all PCR amplified DNA partially cleaved or not cleaved by *HpaII* was undertaken to determine nucleotide sequence at codon 12 resulting from NIH 3T3 replication/repair of the middle T:C

Figure 5. NIH 3T3 mismatch correction results of G:T, A:C and T:C. Percent (%) comparison of types of repair of each mismatch are at the top of each column in each of the three graphs.

mismatch plasmid. *HpaII* digestion and sequencing results of PCR amplified human *H-ras* DNA from 101 different hygromycin resistant NIH 3T3 colonies are included in Figure 5. G:T mismatch correction to G:C was 96.4% (27/28) and G:T→mixed G:C/A:T was 3.6% (1/28). A:C mismatch correction rate to G:C was 87.5% (35/40); A:C→A:T was 2.5% (1/40); and A:C→mixed G:C/A:T was 10% (4/40). T:C mismatch correction rate to G:C was 67% (22/33); T:C→T:A was 18% (6/33); and T:C→mixed G:C/T:A was 15% (5/33).

The highest correction rate of G:T→G:C (96.4%) agrees with previous findings by other investigators who have described more than one error correction mechanism for this mismatch, resulting in more accurate repair than in other types of mismatches (7–9). The lowest correction rate of T:C→G:C (67%) agrees with human *ras* mutation literature which cites codon 12; middle G:C→T:A transversion mutations as the most frequent (21,25,40). The majority of all mixed results appear to be a 1:1 ratio of each base pair by sequencing results. This most likely indicates a lack of mismatch repair before plasmid replication (9), although it cannot be ruled out that there is more than one type of mismatch repair of more than one plasmid within the cell.

DISCUSSION

We have devised a unique restriction endonuclease-mediated methodology that allows us to study rate and fidelity of mammalian DNA repair when a strand- and site-specific lesion is introduced at a major 'hot spot' of mutation in the human *H-ras* oncogene. We have constructed a mammalian expression vector (p220.pbc+H/B) containing the entire human *H-ras* genomic sequence for these experiments. This plasmid contains the EBV origin of replication (oriP) and EBV nuclear antigen 1 (EBNA-1), providing synchronous replication with the cell cycle and maintaining a stable plasmid copy number and low spontaneous mutation rate, unlike plasmids containing an SV40 origin of replication (29,36,41,42). In addition, transfection experiments in our laboratory have indicated less than three plasmid copies per NIH 3T3 cell. Furthermore, p220.pbc+H/B contains a hygromycin resistance gene allowing for selection and colony growth of only those mammalian cells expressing the plasmid. We chose to use the complete *H-ras*

genomic sequence (rather than bacterial, viral or synthetic sequences) to ensure the experimental results accurately represent mammalian DNA repair processes. The first exon has been altered to incorporate two unique restriction endonuclease sites to enable the preparation of gapped heteroduplex DNA (GHD) containing a 30 bp single-stranded region surrounding the codon 12 'hot spot'. Our unique restriction enzyme-mediated methodology permits selection of GHD DNA with the gap located either on the transcribed or non-transcribed strand. A complementary, single-stranded 30 bp oligomer including the desired mismatched nucleotide is then annealed into this gap. We have shown that this system provides a sensitive assay for activated *H-ras* detection (Table 1).

A mild liposomal transfection method was used to avoid high cell toxicity and background mutation, which are often associated with harsher techniques such as calcium phosphate precipitation, DEAE-dextran or electroporation. Minimizing stress to host cells should limit disruption of physiologically normal activities relevant to studies of DNA damage and repair.

After hygromycin selection, human *H-ras* exon 1 sequences were PCR amplified directly from NIH 3T3 colonies for sequence analysis, to avoid background mutation rates associated with additional bacterial replication. PCR primers were designed to have partial sequence complementarity with human intronic regions on either side of the target sequence to avoid amplification of mouse *H-ras* DNA. PCR amplified DNA was initially screened by *HpaII* cleavage, as only wt human *H-ras* contains the complete *HpaII* recognition sequence at codon 12. DNA not cleaved or partially cleaved by *HpaII* was sequenced to determine exact type and location of each mutation.

These mismatch experimental results (Fig. 5) agree with previous experiments by others. G:T mismatch results indicate a high (96.4%) correction rate to G:C, probably due to more than one error correction mechanism for this frequently occurring endogenous mismatch (10,43). It is interesting that the T:C mismatch is corrected to G:C with only 67% efficiency. Considering that human *ras* mutations at codon 12 or 13 frequently undergo G:C→T:A transversions (21,25,40), we could speculate that tumors containing this mutation have a mutator phenotype specifically involving T:C mismatches at this location.

We found mixtures of both wild-type and mutated codon 12 sequences in 3.6% of G:T mismatch samples, 10% of A:C mismatch samples and 15% of T:C mismatch samples. This could be due to a single transfected plasmid undergoing replication before mismatch repair; or to the presence of more than one plasmid in the cell, each repaired differently. As equal quantities of each base pair were consistently observed in the mixtures, we believe the former scenario to be correct. This would also agree with previous mismatch studies using the SV40 viral plaque assay (9).

We are currently expanding these experiments to elucidate DNA adduct repair events using a site-specific DNA adduct polymerase-insertion methodology (44).

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