AN ABSTRACT OF THE THESIS OF

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John B. Hays

Mismatch repair is one of the mechanisms by which cells ensure genomic stability. Deficiencies in mismatch repair (MMR) increase mutation rates and cancer risks. In the well-characterized methyl-directed *Escherichia coli* system, MMR is initiated by MutS, Mut L, and MutH proteins. The single MutS protein and the single MutL protein in prokaryotes are diverged into several MSH (MutS homolog) proteins and MLH (MutL homolog) proteins in eukaryotes. Several germline mutations in human mismatch repair genes, mainly *hMSH2* and *hMLH1*, have been associated with hereditary non-polyposis colorectal cancer (HNPCC). MMR-deficient cells show a higher resistance to some anti-tumor reagents. Early detection of mismatch repair defects might be useful in anti-tumor drug selection.

In this study, I wanted to develop an assay for screening MMR-proficient cells. First, I constructed a gapped plasmid, employing the tandem-nick method (Wang & Hays, 2001) and generated G/A base-base mismatched substrates by annealing a synthetic oligomer into the gapped molecules. The plasmid with the incorrect

adenine on the template strand encodes a truncated non-functional protein, and the repair of this incorrect adenine to the correct cytosine would produce an active enzyme. A strand-specific and site-specific nick site was generated by a DNA single-strand nicking enzyme, N.*Bpu*10I endonuclease. This repair-reporter plasmid was transfected to a number of different cells, including lymphoblastoid (TK6 and MT1) cells, mouse fibroblast (mc2 and mc5) cells, and tumor (HCT116) cells. Luciferase activities in cell lysates were assayed to determine the efficiency of correction mismatched G/A to G/C, which encodes an active protein. To normalize transfection efficiencies as well as lysate preparation variations, plasmid pCH110, which encodes full-length *E. coli* β-galactosidase, was used as second reporter gene in co-transfection experiments. The apparent repair efficiencies proved to be independent of the mismatch-repair genotype in lymphoblastoid cells and were slighter higher in mismatch-repair-proficient mc5 mouse cells than in mc2 mismatch-repair-deficient cells but were low in general. The results indicate that the G/A base-base mismatch is very likely repaired via another activity. A likely possibility is the hMYH DNA glycosylase, which can cleave adenine from a G/A mismatch as well as from A/8-oxo-guanine. I was able to quantify the following repair efficiencies for a G/A mismatch, in supercoiled DNA: 50% in lymphoblast cells, 5-14% in mouse fibroblast cells, and about 11% in tumor cells. However, I also found that a G/A mispair may not be a good substrate for screening MMR proficiency *in vivo*.

AN ASSAY FOR SCREENING CELLS FOR MISMATCH REPAIR PROFICIENCY *IN VIVO*

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CHAPTER 1. INTRODUCTION

1.1 INTRODUCTION

Macromolecules in living cells, such as DNA, RNA and proteins, are constantly challenged by endogenous and exogenous damaging agents, such as UV light, radiation, reactive oxygen species leaking out of the electron transport chain in mitochondria or generated by radiolysis of water, and dietary or environmental mutagens. To be able to survive and grow normally, it is essential for cells to accurately replicate DNA and to repair any damage in DNA. The main repair pathways for correcting damaged DNA are mismatch repair (MMR), base excision repair (BER), nucleotide excision repair (NER), and DNA double strand break repair. DNA double strand breaks (DSB) could result from ionizing radiation or a replicative DNA polymerase encountering a single strand break. The two pathways for repair of DNA DSB are homologous recombination and non-homologous end-joining (reviewed by Jackson, 2002). During homologous recombination, the damaged chromosome invades and forms a synapsis with regions of high sequence homology on the undamaged

DNA molecule. DNA polymerase then replicates past the damage, using the non-damaged DNA strand as a template. Non-homologous end-joining involves the ligation of two DNA DSB, but does not require extensive sequence homology. BER acts on single bases damaged through oxidation, such as 8-oxo-guanine, or chemical modification during normal cellular process, including deamination of cytosine to uracil or 5-methylcytosine to thymine. The damaged base is removed by a specific DNA glycosylase leaving an apurinic or apyrimidinic (AP) site. This must be further processed by an AP endonuclease, which incises the phosphodiester backbone. The deoxyribose phosphate residue is removed by the action of a DNA deoxyribosephosphodiesterase. The resulting gap is finally filled by a DNA polymerase and sealed by a DNA ligase (reviewed by Nilsen and E.Krokan, 2001). The process of nucleotide excision repair requires the concerted effort of a large suite of proteins. Bulky DNA adducts such as UV-induced pyrimidine dimers or other helix-distorting lesions are repaired. After initial recognition, the lesion-containing strand is nicked on both sides of the lesion at sites approximately 25-29 bp apart. Excision of the lesion-containing fragment is followed by re-synthesis via a DNA polymerase and a DNA ligase (reviewed by de Laat *et al*, 1999).

A very important role of MMR is post-replication error correction. MMR proteins recognize base-base mismatches (non-Watson-Crick base pairs) that occur as a result of DNA polymerase replication error. They also repair insertion-deletion loopouts (IDL) that result from DNA template slippage

during replication (Dohet *et al*, 1985). MMR also serves as a species barrier, by antagonizing sequence-divergent homologous recombination. Genetic recombination provides a means for the transfer or exchange of genetic information between homologous regions of DNA, as well as the repair of DNA. Thus, if the sequence differences between two homologous regions are too high, the recombination cannot occur. A study showed that intergeneric recombination occurs efficiently between two MMR-defective bacteria strains, which are about 20% divergent in DNA sequence (Rayssiguier *et al*, 1989). The evidence that overexpression of MMR proteins (Msh2 and Mlh1) induces apoptosis and the reduction of apoptosis in response to N-methyl-N′-nitro-N-nitrosoguanidine treatment seen in MMR-defective cells suggests that MMR might also play a role in signaling to apoptosis (programmed cell death), when excess DNA damage occurs in cells (Zang *et al*, 1999; Li 1999).

1.2 MISMATCH REPAIR IN PROKARYOTIC AND EUKARYOTIC CELLS

1.2.1 MMR in Prokaryotes

MMR has been intensively studied in *Escherichia coli* (*E. coli*). The initiation of MMR requires MutS, MutL, and MutH proteins, and repair is bi-directional (Lu *et al*, 1983; Su *et al*, 1988; Lahue *et al*, 1989; Au *et al*, 1992; Grilley *et al*, 1993). MutS proteins form a homodimer that recognizes base-base mismatches or small insertion/deletion loopouts (IDL). MutL

homodimer binds to the MutS/DNA complex and activates MutH, an endonuclease. It is critical for the MMR system to distinguish between the template strand and the nascent DNA strand during error correction. The facts that *E. coli* methylates adenines in GATC sequences, and that newly replicated strands are not immediately methylated, provides a mechanism for this distinction. Activated MutH recognizes GATC sequences and nicks the non-methylated (nascent) strand just 5′ of the GATC sequence. GATC nicking can occur 5′ or 3′ to the mismatch. With the help of helicase II, one of the four exonuclease (ExoI or RecJ for 5′ to 3′ excision, Exo VII and ExoX for 3′ to 5′ excision) excises nucleotides from GATC to a point approximately 150 bp past the mismatch. Finally, DNA polymerase III holoenzyme re-synthesizes DNA, and the gap is sealed by DNA ligase (Grilley *et al*, 1990).

1.2.2 MMR in Eukaryotes

Mismatch repair has been highly conserved through evolution, but in eukaryotes the protein components have increased in number and become more specialized (reviewed by Jiricny, 1998; Buermeyer et al, 1999). A single *mutS* gene and a single *mutL* gene in prokaryotes have diverged into several homologs in eukaryotes. Seven MSH (MutS homolog) proteins have been identified so far. MSH2 can form at least two heterodimers, MutSα (MSH2-MSH6) and MutSβ (MSH2-MSH3) in eukaryotic nuclei. Both *in vitro* binding assays and mutational studies indicate that MutSα recognizes and

initiates repair for base-base mismatches and one nucleotide IDLs, while MutSβ is responsible for small IDLs composed of 2-4 bases. Four MutL homolog (MLH) proteins have been identified: MLH1, MLH2 (yeast only), MLH3, and PMS1 or PMS2 (PMS1 is the *S. cerevisiae* homologue to human PMS2). MLH1 forms heterodimers with other MLH proteins, such as MutLα (MLH1-PMS2). MutLα can bind to an MSH-DNA complex. The mechanism of discrimination between the parental and daughter strands remains unknown, but EXOI exonuclease and DNA polymerase δ and ε are involved in the excision and re-synthesis steps.

1.3 DEFECTIVE MISMATCH REPAIR AND CANCER

Both humans and mice deficient in MMR exhibit an elevated spontaneous mutation rate and cancer risk. The spontaneous mutation rate exhibits a 100- to 1000-fold increase in the MMR-deficient mutants (Cox, 1976). A common phenotype for MMR-deficient cells is microsatellite instability (MSI), characterized by accelerated alterations in the length of simple repetitive microsatellite sequences that occur throughout the genome. MSI likely results from misalignment of the template strand and nascent strands in regions of mono-, di-, or tri-nucleotide repeats after transient disassociation. Another phenotype common to MMR-deficient cells is higher resistance to methylation agents such as N-methyl-N′-nitro-N-nitrosoguanidine (MNNG) and 6-thioguanine than wild type cells (Goldmacher *et al*, 1986; Armstrong and

Galoway, 1997). One hypothesis to explain this phenotype is that MMR in a wild-type cell recognizes the lesion and tries to repair it but the repair becomes stuck in futile cycles, leading to a double strand break, a strong signal for apoptosis. A cell deficient in MMR fails even to attempt repair, is not subject to futile cycling and tolerates the lesions.

A link between cancer development and nonfunctional MMR has been implicated. Inactivated MMR genes have been identified in hereditary non-polyposis colorectal cancer (HNPCC), a common colon cancer. The majority of MMR mutations found in HNPCC are in either *MSH2* or *MLH1*. However, *PMS2*, *PMS1* and *MSH6* mutations were also observed (Kane *et al*, 1997; Fishel, 2001). The germlines in those patients are normally heterozygous in the MMR genes. Additional mutations in the functional allele occur in somatic cells. These mutations abolish mismatch repair function in those cells or tissues, and increase mutation rates. Because carcinogenesis is presumed to be a multi-step phenomenon, a high mutation rate may be a necessary precursor to the acquisition of sufficient mutations for cancer.

1.4 ANALYSIS OF MMR ACTIVITY

A cell with a defective MMR system often exhibits miscrosatellite instability (MSI) and resistance to methylation agents. Therefore, analyses of these two phenotypes are used to characterize MMR status. MSI is a hallmark for HNPCC tumor cells. MSI analysis requires PCR amplification of gene

segments with tandem-repeat sequences (1-4 nucleotides in length) and electrophoretic analysis of PCR products. A panel of five microsatellite repeats is recommended as an initial screen for MSI (Boland *et al*, 1998). However, this method is restricted to the analysis of the specific microsatellite sequence which may not be representative of overall mutation rate, and the sensitivity is limited by the need for the frameshift to occure early in colonial expansion. Another phenotypic analysis for MMR is measurement of resistance to methylation agent treatment, described in the previous section. For example, MT1 cells have a higher resistance than their nearly isogenic TK6 cells because the MT1 cells have a non-functional Msh6 protein (Goldmacher *et al*, 1986; Drummond *et al*, 1995).

Direct measurements of MMR status have been made *in vitro*, using nuclear or whole cell extracts, utilizing base-base mismatches or loopouts as substrates. The error correction end-products can be assayed as restored restriction endonuclease sites or reverted mutations in reporter genes. Holmes *et al* (1990) measured mismatched-base correction in HeLa and *Drosophilia melanogaster* cell extracts. They used phage DNA to prepare circular heteroduplex substrates with a site-specific single strand break and found that different mispairs were repaired with different efficiencies (G/T > G/G ≅ A/C > C/C). The preparation of mismatched substrates involves annealing circular phage ssDNA to linearized phage dsDNA. The breaks in the dsDNA provide the preexisting excision-initiation nicks. Holmes *et al* showed that a nick was

necessary for mismatch repair *in vitro*. Thomas *et al* (1991) used a variety of M13mp2 DNA (phage DNA) substrates containing single-base mismatches and a small loopout, incubated them with human HeLa cell extracts, and subsequently tranfected the treated phage DNA into bacteria cells. By analyzing the plaques, they measured extensive repair and confirmed that the presence of a nick is required for efficient repair in HeLa cell extracts.

Brown and Jiricny (1988, 1989) transfected monkey kidney cells with covalent closed circular viral SV40 DNA containing a single base-base mispair in the intron of the gene coding for large T antigen. Transfection of mismatch-containing SV40 DNA into host cells produced plaques. Error corrections were assayed by restriction digestion of DNA isolated from the plaques. Mismatches inactivate endonuclease digestion; however, the correction of the mismatch would restore different endonuclease restriction sites, depending upon which strand is repaired. The restored endonuclease site provides a basis for screening. They found that repair efficiencies for G/T, A/C, C/T, and A/G to G/C were 96%, 78%, 76%, and 39% respectively. Williams and colleagues (1997, 1999) constructed vectors with four different mismatched bases on codons 10 or 12 of the H-ras oncogene, with nicks, to test repair in the mouse cells. The mismatched substrates were transfected into mouse NIH 3T3 cells; DNA was purified from each cell colony, amplified by PCR, and analyzed by digestion. The repair efficiencies at codon 12, a hot spot for mutation, were 35% for G/A, 60% for A/C, 80 % for T/C, and 100% for G/T. The repair rates

were higher at codon 10, which is not a hot spot for mutation.

In this study, I attempted to develop an assay to determine MMR function *in vivo*. This would be useful for screening human populations and also might facilitate anti-tumor drug selection, due to the fact that MMR-deficient cells are more resistant to some alkyation reagents. I employed a new method for substrate preparation (Wang & Hays, 2001), which makes it easier to prepare large quantities of a purified covalently closed circular form of DNA containing a mismatch. Specific single-strand-nicking enzymes were then employed to generate defined nicks after supercoiled mismatched substrate was purified. For the purpose of the study, a G/A mismatch was constructed. To quantify mismatch repair, the G/A mismatch was created in the template strand of the luciferase gene. If the mismatch were not corrected or corrected to T/A, then the plasmid would encode a truncated protein. Only if the mismatch were corrected to G/C would the plasmid then encode a functional luciferase. By measuring luciferase activity from transfected cells, I was able to quantitatively measure the degree of error correction. The repair efficiencies for the G/A substrate were relatively high, but did not seem to directly reflect the known MMR genotype in lymphoblast cell lines. There was a slight increase in the repair efficiency for the G/A mismatched substrate in MMR-proficient mc5 mouse fibroblast cells versus the efficiency in the MMR-deficient cells mc2, but the difference was small. The results indicate that G/A may not be a good substrate to measure MMR proficiency *in vivo*.

CHAPTER 2. MATERIALS AND METHODS

2.1 PLASMID CONSTRUCTION

Plasmid pCMVLUC was constructed by M.Hedayati (1997) starting from pRL-CMV (Promega). Gapped plasmids were generated using the tandem-nicking method (Wang & Hays, 2001). This required that all eight original N.*Bst*NBI endonuclease sites located at basepairs 565, 926, 1042, 1683, 1915, 2792, 3974, and 4478 in the pCMVLUC vector be removed by means of QuickChange™ site-directed mutagenesis Kit (Stratagene). Numbering in this plasmid begins at the sequence AGATCTTCAA--- at the CMV enhancer promoter. The DNA-nicking enzyme N.*Bst*NBI recognizes the DNA sequence GAGTC and nicks the fourth nucleotide in the 3' direction of the end of the same strand. Briefly, Turbo Pfu DNA polymerase (Stratagene) was used to extend two complementary primers with the same desired mutation and to replicate the whole plasmid during PCR cycles. After PCR, template plasmids with methylated GATC sites were cleaved by *DpnI* endonuclease (Stratagene) digestion; therefore, only the PCR products containing the desired mutation remained as nicked plasmids. Digestion products were then transformed into either XL10-Gold (Stratagene) ultracompetent cells or XL1-Blue competent cells. Mutants were screened by *HinfI* endonuclease digestion, which recognizes the sequence GANTC similarly to N.*Bst*NBI endonuclease, except

for flexibility of the center nucleotide. Mutant fragments with desired mutations were subcloned back to the original vector to avoid spontaneous mutations generated during Pfu polymerase replication of the whole plasmid.

The sequences of N.*Bst*NBI endonuclease sites were changed from GAGTC to GGGTC, GAGTT, and GAGTT, on the sense strand at positions 915, 2792 and 3974, respectively. On the coding strand (with reference to the luciferase gene) the sequences were changed to TAGTC, CAGTC, CAGTC, GATTC, and CAGTC at positions 565, 926, 1042, 1683, and 4483, respectively. The mutations in the luciferase coding region were confirmed by both digestion and direct sequencing. Luciferase activities from each intermediate were checked by *in vitro* transcription and translation lysate (Promega). The plasmid lacking all N.*Bst*NBI endonuclease sites was designated pCMVLUCns (see Table 1).

Two tandem N.*Bst*NBI sites 30 nucleotides apart were re-introduced back into the template strand of the luciferase coding region to generate plasmid pCMVLUCbb1. The sequence at codons 193 and 194 was changed from GCA TCG to GGA CTC (5′-3′). The sequence at codons 203 and 204 was changed from GGT CTG to GGA CTC (5'-3'). The modification altered the encoded amino acid at codon 193 from glycine to alanine. Digestion by the N.*Bst*NBI enzyme (New England Biolabs) results in two nicks at position 1698 and 1728 of plasmid pCMVLUCbb1 (see Table 1).

Another unique nicking site recognized by N. *Bpu*10I endonuclease

(Fermentas) was introduced either upstream (3′ end, refer to template strand of luciferase gene) or downstream (5′end) of the two N.*Bst*NBI endonuclease sites. N.*Bpu*10I endonuclease recognizes the sequence CCTNAGC and nicks only on the complementary strand. The upstream N.*Bpu*10I endonuclease site located in the T7 promoter region required changing the sequence at position 1063-1069 from CCC GGG C to CCT GAG C. Introduction of the downstream N. *Bpu*10I endonuclease site required altering codon 346 from CAT to CCT without changing the encoded amino acid. The plasmids with upstream N.*Bpu*10I endonuclease site and downstream N.*Bpu*10I endonuclease site of the potential base/base mismatch were designated as pCMVLUCbb1bpu and pCMVLUCbb1bpd, respectively (see Table 1). N.*Bpu*10I endonuclease digestion generates a nick site on the template strand, which mimics the initiation of the excision in mismatch repair. Figure 1 illustrates plasmids pCMVLUC and pCMVLUCbb1bpu. The plasmid pCMVLUCbb1bpu encodes two new N.*Bst*NBI endonuclease restriction sites in the luciferase coding region for generating a mismatch and a defined nicking site for initiating excision in MMR.

2.2 MISMATCH SUBSTRATE PREPARATION

Mismatched DNA substrates were prepared as described by Wang & Hays (2001). This involved taking advantage of the DNA nicking enzyme N.*Bst*NBI. In general, plasmid pCMVLUCbb1bpu was propagated in *Escherichia coli*

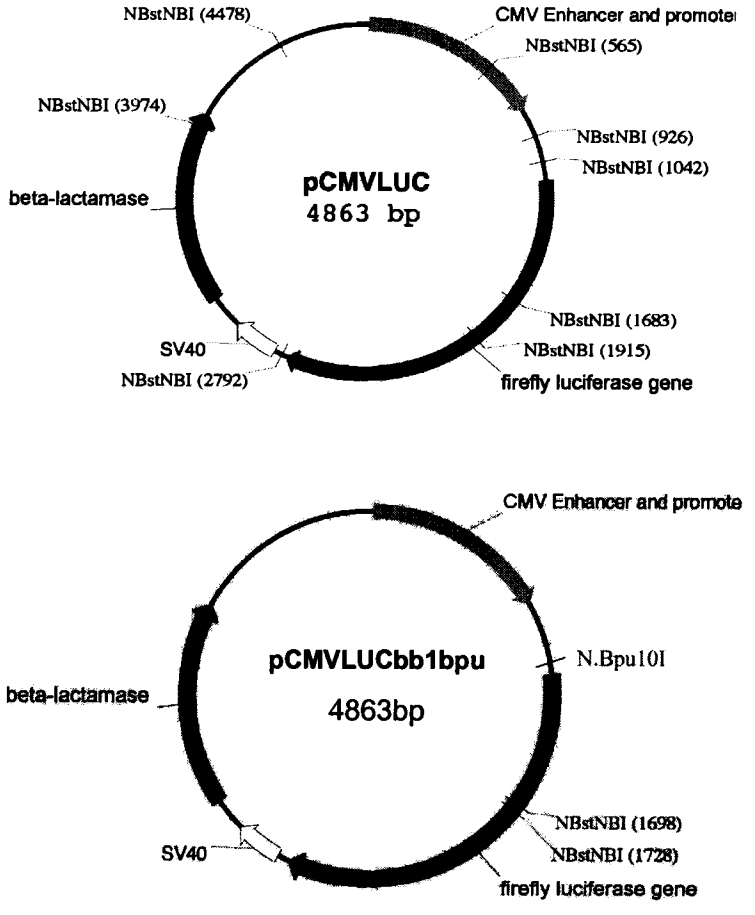

Figure 1. Construction of plasmid pCMVLUCbb1bpu. All eight N.*Bst*NBI endonuclease sites on the plasmid pCMVLUC were removed, and two new N.*Bst*NBI endonuclease sites were re-introduced in the plasmid pCMVLUCbb1bpu. This plasmid also includes a unique site for N.*Bpu*10I endonuclease, which is introduced at bp 1063 on the luciferase template strand, near the 3′ end of the gene.

Table 1. Plasmids and derived DNA substrates

Name	Nick sites generated by N.BstNBI endonuclease	Nicking by N.Bpu10I endonuclease	Base pair at code 193	Protein activity
pCMVLUC[a]	8	No site	G/C	*luc*$^+$
PCMVLUCns	No site	No site	G/C	*luc*$^+$
pCMVLUCbb1	sites (1698 and 1728)	No site	G/C	*luc*$^+$
pCMVLUCbb1bpd	sites (1698 and1728)	2252 (5′ end of mismach)	G/C	*luc*$^+$
pMVLUCbb1bpu (G/C)[a]	sites (1698 and 1728)	1063 (3′ end of mismach)	G/C	*luc*$^+$
pMVLUCbb1bpu (G/A)[a]	sites (1698 and 1728)	1063 (3′ end of mismach)	G/A	*luc*$^-$
pMVLUCbb1bpu (T/A)[a]	sites (1698 and 1728)	1063 (3′ end of mismach)	T/A	*luc*$^-$

[a] Plasmids used in transfection studies

(*E. coli*) strain XL1-Blue in 1 liter of Luria-Bertani Medium (1 liter of LB Medium: 10 grams of tryptone, 10 grams of sodium chloride, and 5 grams of yeast extract) at 37°C with vigorous shaking for over night. Cells were lysed by alkaline lysis and crude DNA was purified through cesium chloride (CsCl)/ethidium bromide isopycnic sedimentation at 20°C and 65,000 rpm for 16 hours (Beckman VTi 80 rotor, L8-M Ultracentrifuge, Beckman). Molecules with the same density could be purified together; therefore, supercoiled plasmids and nicked plasmids can be separated. After purification, 0.4 mg of plasmids pCMVLUCbb1bpu was completely nicked by 300 units of N.*Bst*NBI endonuclease at 55°C for 2 hours, as determined by gel electrophoresis of an aliquot. Another 300 units of N.*Bst*NBI endonuclease were added and incubation continued at 55°C for another 4 hours. Gapped molecules were generated by adding 50-fold excess of synthetic oligomers complementary to the 30 nucleotides in the template strand sequence between the two N.*Bst*NBI endonuclease sites of the digested plasmids. The mixture was heated to 85°C for 5 minutes and slowly cooled to room temperature. The resulting gapped plasmids were separated from 30-nucleotide oligomers and 30-nucleotide duplex by four passages through a Centricon100 filter (Millipore) at 4°C, reducing the sample to 10% of its original volume. The gapped molecules were further purified by BND (benzoylated and napthoylated DEAE) cellulose chromatography. BND cellulose was prepared as a 50% suspension in TE Buffer (10 mM Tris-HCl, pH 8.0; 1 mM ethylenediamine-tetraacetic acid

[EDTA]) containing 0.3 M NaCl. Gapped plasmids were incubated 30 minutes at room temperature with 20 mL of BND-cellulose resin in 1 M NaCl, with mixing on a Barnstead/Thermolyne Labquake (model 400110) rotator. The mixture was loaded on a 30-mL (2.5 X 7.5 cm) column, and the column was washed with 100 mL TE Buffer containing 1.0 M NaCl at a flow rate of 1 mL/min. Intact plasmids or singly nicked plasmids with those 30 nucleotides remaining on the template strand should be washed away at this step. Gapped molecules were eluted by CFS Buffer (2% caffeine 50% formamide, 1.0 M NaCl, 10 mM Tris-HCl, pH 8.0, 1 mM EDTA). The eluted gapped plasmids were subject to overnight dialysis against three changes of 1 liter TE Buffer. Four hundred pmoles of synthetic oligomer, 5'-AGA TCC AGA GGA GTT CAT GAT GAG TCA AAT-3', were incubated with 10 units of T4 polynucleotide kinase (Fementas) in 1X forwarding buffer (50 mM Tris-HCl, pH 7.6, 10 mM $MgCl_2$, 5 mM dithiothreitol [DTT], 1 mM spermidine and 1 mM EDTA) in the presence of 0.9 mM adenosine 5′ triphosphate (ATP) at 37°C for 2 hours. T4 Polynucleotide kinase catalyzes the transfer of the γ-phosphate from ATP to 5′-OH group of DNA; therefore, the kinased oligomers can be ligated to gapped molecules. Kinased lucbt oligomer was annealed in 10-fold excess to 200 μg of gapped molecules by incubation at 80°C for 5 minutes in 1X Ligation Buffer (50 mM Tris-HCl, pH 7.6, 10 mM $MgCl_2$,, 1 mM ATP, 1 mM DTT, 5% (w/v) polyethylene glycol-8000) and slowly cooled. After the mixture was cooled to room temperature, 50 units of T4 DNA ligase (Invitrogen) and fresh

ATP (1 mM to final concentration) were added to the mixture. Ligation was carried out at 16°C water bath overnight. T4 DNA ligase catalyzes the formation of a phophodiester bond between 5′-phosphate of oligomer and 3′-hydrozyl termini of gapped molecules; as a result, covalent closed circle plasmids can be formed after ligation. The ligation of synthetic oligomers to gapped molecules generated a G/A mismatch in the first nucleotide of codon 193, which would encode a truncated luciferase if the mismatched base pair is not repaired. The ligation products were purified by CsCl-ethidium bromide isopycnic sedimentation, using the same conditions described above, to separate supercoiled plasmids from nicked or unligated plasmids. Supercoiled heteroduplex pCMVLUCbb1bpu (G/A) was dialyzed against three changes of one liter TE Buffer at 4°C overnight and then used in the following transfection experiments.

2.3 IN VITRO TRANSCRIPTION AND TRANSLATION

T_NT® T7 Quick Coupled Transcription/Translation System (Promega) uses rabbit reticulocyte lysate combined with RNA polymerase, nucleotides and salt to transcribe and translate the gene cloned downstream from the T7 promoter. Plasmid pCMVLUC and its derivatives all contain a T7 promoter; therefore, the protein encoded in those plasmids can be expressed in this *in vitro* Transcription/Translation (IVTT) System. To test the luciferase protein encoded in each intermediate plasmid, 250 ng of plasmid and 1μl of

methonione (1 mM) were added to 10 µl of proprietary IVTT lysates and adjusted to a final volume of 15 µl. The mixture was incubated at 30°C for 60 minutes. A 2-µl aliquot of IVTT lysate was added to 50 µl of luciferase reagent (Promega). The highest luminometer (LKB Wallac 1250 luminometer) reading was recorded.

2.4 CELL CULTURE

TK6 Lymphoblast cell line was a gift from Niels de Wind (University of Leiden, Netherland) and was designated as TK6 (D). Additional TK6 and MT1 lymphoblast cell lines were generously given by William Thilly (Massachusetts Institute of Technology) and designated as TK (T) and MT1 (T). MT1 cell line is MMR-deficient due to the mutation on both *hMSH6* gene and is nearly isogenic to the TK6 cell line, which is MMR-proficient (Goldmacher *et al*, 1989; Drummond *et al*, 1995). The lymphoblast cells, TK6 and MT1, were cultured in medium composed of 90% RMPI 1640 (proprietary medium, GIBCO) with 10% Fetal Bovine Serum (FBS) supplement and 2% of streptomycin/penicillin (each 200 µg/ml.) Cells were cultured in a 75cm^2 polystyrene flask (Corning) and the medium was changed every 2 to 3 days to maintain the cell concentration between 2 x 10^5 and 1 x 10^6 cells/ml.

Mouse fibroblast cells mc2 (MLH1 deficient cells) and mc5 (wild type), generously provided by Dr. Andrew Buermeyer (Oregon State University), and were cultured in 90% DME Medium with 10% Calf Bovine Serum, 1% non-

essential amino acid (cellgro) and 0.1 % of gentamycin. Every 2 to 3 days, old medium was aspirated out and fresh culture medium was added into the flasks.

HCT116 was a generous gift from Brad Preston (University of Washington). The HCT116 human colon cancer cell line is known to have homozygous inactivating mutations in the DNA MMR repair gene *hMLH1* on Chromosome 3, and is defective in MMR (Hemminki *et al.*, 1994). Cells were cultured in medium containing 90% of Dulbecco's modified Eagles Medium [DME Medium] (GIBCO) with 10% FBS supplement and 2% streptomycin/penicillin (each 200 μg/ml). Every 2 to 3 days, old medium was aspirated out and fresh culture medium was added into the flasks to ensure normal growth. All cells were maintained in a humidified atmosphere with 5% CO_2 and 95% air at 37°C.

2.5 TRANSFECTION AND PREPARATION OF LYSATE

Lymphoblast cell lines, TK6 and MT1, were transfected by using DEAE-Dextran according to a protocol supplied by Dr. Lawrence Grossman (The Johns Hopkins University). Cells were counted by hemocytometer and two million cells were used in each determination. Cells were washed and resuspended in TBS Buffer (100 mM Tris-HCl, 150 mM NaCl), pH 7.3 with a final concentration of one million cells/ per 100 μl TBS Buffer. For each determination, 5 μl of TE Buffer containing 300 ng of luciferase-encoding plasmids and 500 ng of β-galactosidase-encoding plasmid, if co-transfected,

were added into DEAE-Dextran (0.5 mg/ml) in TBS Buffer to obtain a final volume of 50 µl. Afterward, the mixture was added into 200 µl of cell suspension and incubated for 15 minutes. At the end of a 15-minute incubation period, one ml of RPMI1640 complete medium was added to each determination, and the suspension was centrifuged at 2000 rpm for 10 minutes. Cell pellets were resuspended in 1 ml of fresh complete medium and transferred to 12 x 75 mm polystyrene culture tubes and incubated at 37°C, 5% CO_2 for 24 hours, unless otherwise specified.

Cell lysates were prepared by washing cells twice with 1 ml of TBS and centrifuging at 4000 rpm for 10 minutes in a microfuge. The pellets were further compacted by centrifugation at 14,000 rpm for 1 minute. Cell pellets were lysed by adding 120 µl of Cell Culture Lysis Buffer (25mM Tris-phosphate, pH 7.8, 2 mM DTT, 2 mM 1,2-diaminocyclohexane-N,N,N′,N′-tetraacetic acid, 10% glycerol, 1% Triton X-100) (Promega), vortexing for 10 seconds to help lyse the cells, and centrifuging at 4°C for 30 minutes in a MICROSPIN 24S microfuge (DU PONT) . The supernatant was transferred to a new Eppendorf tube and stored at -70°C to await luciferase and β-galactosidase activity determinations.

Mouse fibroblast cell lines (mc2 and mc5) were transfected using Lipofectamine2000 (Invitrogen). One day prior to transfection, $3x10^5$ cells were plated in 6-well cell culture plates. For each transfection, 3 µl of Lipofectamine was diluted in 50 µl of DME Medium and then mixed with

another 50 µl of DME Medium containing 300 ng of luciferase-encoding plasmids. The mixture was incubated for 30 minutes at room temperature and then added to each well. Cells were continuously incubated at 37°C for 24 hours without changing medium. After 24 hours, cells were washed twice with TBS Buffer, harvested by scraping out the plate, and lysed by adding 120 µl of Cell Culture Lysis Buffer, followed by a 10-seconds of vortexing. Cell lysates were collected after centrifugation at 4°C for 30 minutes and stored at –70°C to await luciferase assays.

HCT116 cells were transfected using DEAE-Dextran. One day prior to transfection, $3x10^5$ cells were plated in 6-well cell culture plates. Cells were washed twice with TBS Buffer and incubated with 200µl of TBS Buffer in each well until transfection. For each determination, 5 µl of TE Buffer containing 300 ng of luciferase-encoding plasmid were added into DEAE-Dextran/TBS Buffer mixture (0.5 mg/ml) to obtain a final volume of 50 µl. The mixture was added into each well and incubated for 15 minutes. At the end of a 15-minute incubation period, DEAE-Dextran mixture was aspirated out from each well, and 1 ml of complete DME Medium was added to each well. Cells were incubated at 37°C for 24 hours. After 24 hours, cells were washed twice with TBS Buffer, harvested by scraping out the plate, and lysed by adding 120 µl of Cell Culture Lysis Buffer followed by a 10-seconds of vortexing. Cell lysates were collected after centrifugation at 4°C for 30 minutes and stored at –70°C to await luciferase assays.

2.6 LUCIFERASE ASSAY

The proprietary luciferase reagent (Promega) was thawed at room temperature for one hour before each assay. Cell lysates were thawed on the bench at room temperature for 30 minutes before assaying. The highest reading from the luminometer (LKB Wallace 1250 luminometer) was recorded when a 20-μl aliquot of cell lysate from each transfection was added to 50 μl of luciferase reagent. The specific luciferase activity of each transfection was normalized by protein concentration, which was determined by Detergent Compatible Lowry Assay (BioRad). The relative luciferase activity was obtained by dividing the specific luciferase activity of each sample by the activity from a supercoiled homoduplex *luc*$^+$ plasmid. When cells were co-transfected with lacZ-encoding plasmid pCH110, luciferase enzyme activities were first divided by β-galactosidase activities. The luciferase/β-galacotosidase ratios for each determination were further compared to ratios for homoduplex *luc*$^+$ plasmid pCMVLUCbb1bpu (G/C).

2.7 β-GALACTOSIDASE ASSAY

The plasmid pCH110 (GenBank accession number U13845), which encodes the entire *E. coli lacZ* gene and uses SV40 early promoter to express β-galactosidase (β-Gal) in the mammalian cells, was used as a second reporter gene to normalize transfection efficiency. Cells were co-transfected with luciferase-encoding plasmids and plasmid pCH110 and were harvested as

described above. Cell extracts were aliquoted to carry out the luciferase assay and the β-Gal assay separately. The compound 4-methylumbrelliferyl β-D-galactopyranoside, 4-MUG, (Sigma), which can be hydrolyzed by β-Gal and produces a fluorescent 4-methylumbelliferone (4-MU) moiety, was used to measure enzyme activities. After transfection, 20 µl of cell lysate was incubated at 45°C for one hour, then mixed with 20 µl of 2X assay buffer (200 mM sodium phosphate buffer, pH 7.3, 2 mM $MgCl_2$, 100mM β-mercaptoethanol) and 0.85 mM 4-MUG. Every 30 minutes, 5-µl aliquots were taken out and added to 200 µl of cold Stop Solution (0.2M sodium carbonate) in a 96-well microtiter plate. Excitation at 365 nm resulted in fluorescent emission at 455 nm measured, in a SpectraMax Gemini. The fluorescent intensity of each time point is the average of duplicate measurements. The data were converted to a plot of fluorescent intensity versus time for each sample. The rate of β-galactosidase activity was determined as the slope (fluorescent intensity (FI) /minute) of the plot. Specific β-galactosidase activity was calculated as FI/per minutes/µg protein. Assays of cell culture lysis buffer incubated with 4-MUG for 4 hours produced less than 10 fluorescent units.

CHAPTER 3. RESULTS

3.1 PLASMID CONSTRUCTION AND SUBSTRATE PREPARATION

Mismatched substrate preparation from phage DNA is tedious, time-consuming, and results in low yields. I constructed a gapped molecule employing the tandem-nicking method (Wang & Hays, 2001); mismatched substrates are easier to prepare in a large, pure, supercoiled form. The nick can be created after the supercoiled plasmid has been purified. From plasmid pCMVLUC, I generated plasmid pCMVLUCbb1bpu, which has only two tandem N.*Bst*NBI endonuclease sites on the template strand of the luciferase-coding region. These N.*Bst*NBI sites are used for generating a gapped molecule and a unique N.*Bpu*10I nick site on the 3′ end of the luciferase template strand. This allowed for the generation of nicks that mimic the strand-discrimination signal for *in vitro* mismatch repair in the eukaryotic extracts. Figure 1 (page 13) shows the original pCMVLUC plasmid and the modified pCMVLUCbb1bpu plasmid.

Because modifications in the luciferase-coding region might alter the protein activity, it is important to monitor protein activities encoded by each of the intermediate plasmids. Plasmid pCMVLUC and its derivatives all contain a T7 promoter region upstream of the luciferase gene. Activities of proteins encoded by the intermediate vectors were examined by synthesizing them in

TNT T7 Quick Coupled Transcription and Translation system (Promega) after each modification. Figure 2 shows luciferase activities encoded by pCMVLUC and its derivatives expressed in *in vitro* transcription and translation (IVTT) lysate. Each manipulation decreased luciferase activity, but the final constructs, pCMVLUCbb1bpu and pCMVLUVbb1bpd, still retained approximately 50% of the activity encoded by plasmid pCMVLUC. The heteroduplex pCMVLUCbb1bpu (G/A) and homoduplex pCMVLUCbb1bpu (T/A) plasmids encode truncated luciferase proteins and yield less than 0.01% of activity relative to protein expressed from plasmid pCMVLUCbb1bpu (G/C). The parentheses indicate the first base pair in codon 193 in the luciferase gene [(sense-nucleotide)/(template-nucleotide)]. G/C codes for a functional luciferase protein.

3.2 OPTIMIZATION OF TRANSFECTION CONDITION

Plasmid transfection is the first step toward analysis of gene expression in living cells. Maximizing the transfection efficiency and expression of plasmid encoded proteins in cells is critical to success. Both DNA input and incubation time after transfection were varied to determine luciferase expression in lymphoblast cells. Lymphoblast cells were difficult to transfect but were chosen over other cultured cells, because primary lymphocyte can be easily isolated from human blood; therefore, this method can be applied to clinical screening.

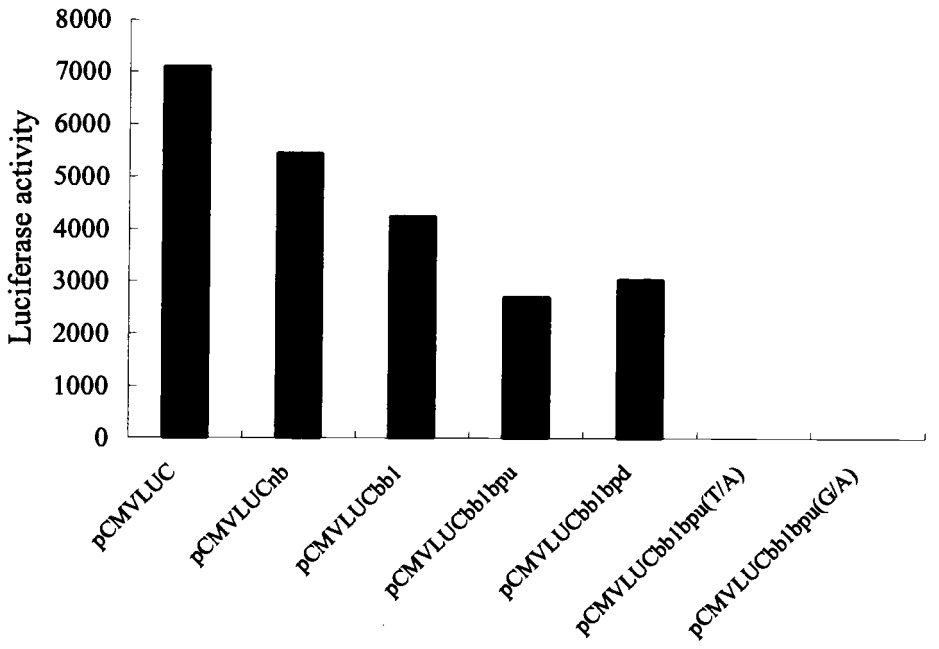

Figure 2. Activities of luciferase proteins synthesized *in vitro*. Luciferase proteins encoded in plasmid pCMVLUC and its derivatives were synthesized by coupled *in vitro* transcription/translation (IVTT) as described under Material and Methods. Each modification decreased luciferase activity, but the plasmid pCMVLUCbb1bpu, used for transfection studies still encoded a protein with about 50% of the activity of the original protein encoded by plasmid pCMVLUC. Proteins encoded by pCMVLUCbb1bpu (G/A) or by pCMVLUCbb1bpu (T/A) showed less than 0.01% of the activity of protein encoded by pCMVLUCbb1bpu. Parentheses indicate the first base pair in codon 193 in the luciferase gene [(sense-nucleotide)/(template-nucleotide)], G/C for coding active protein.

3.2.1 Determination of DNA levels for transfection

To determine the optimal DNA levels to be used for transfection of TK6 cells, differing amounts of plamsid pCMVLUC were tested for luciferase expression in lymphoblast cells. Figure 3 illustrates the resulting specific luciferase activities when different amounts of pCMVLUC plasmid were transfected by DEAE-Dextran into TK6 cells. DEAE-Dextran transfection method was chosen because it is used for transient expression of cloned genes, and in lymphoblast cells it achieves higher transfection efficiency than lipofection mediated transfection. Since both 300 and 500 ng of input DNA yielded similar luciferase activities, 300 ng of DNA was used as the standard amount in subsequent experiments, to conserve limited mismatched substrate DNA.

Uptake and expression of exogenous DNA and lysate preparation might be different from experiment to experiment; therefore, a second reporter gene was co-transfected into cells to normalize these variations. Plasmid pCH110 was chosen as a second reporter and co-transfected with luciferase-encoding plasmids pCMVLUCbb1bpu in lymphoblast cells. To determine optimal concentration of input plasmids, 300 ng of luciferase-encoding plasmid was co-transfected with various amounts of plasmid pCH110, which encodes the full-length *E. coli* β-galactosidase gene. Figure 4 shows specific luciferase/β-galactosidase activity ratio for co-transfection of MT1 and TK6 cells. In both cell lines, 500 ng of plasmid pCH110 co-transfected with 300 ng of plasmid

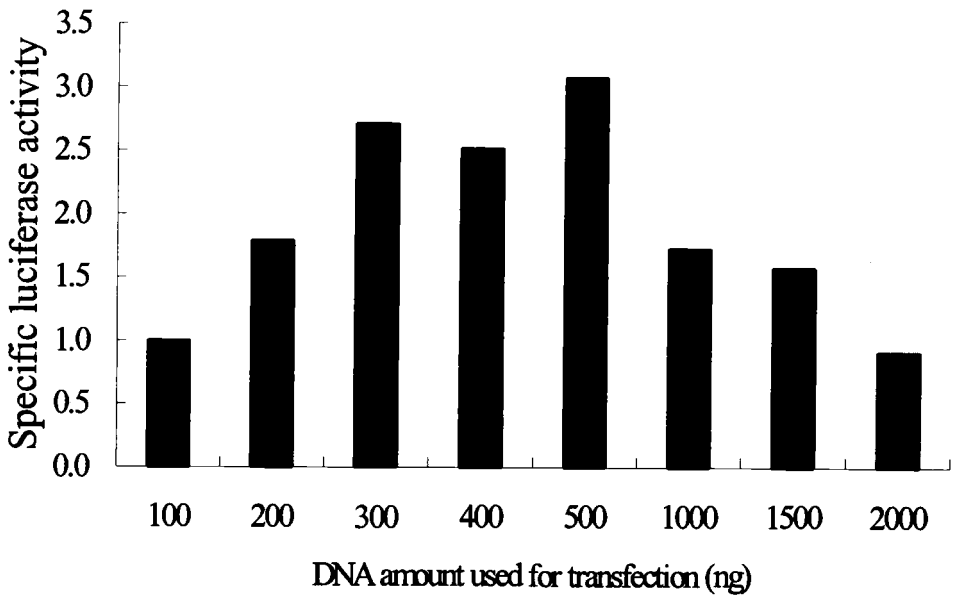

Figure 3. Determination of optimal levels of DNA for transfection of TK6 cells. Various DNA doses, 100 to 2000 ng of supercoiled plasmids pCMVLUC, were tested by DEAE-Dextran transfection to determine the dose for maxminum *in vivo* expression. A 40-hour incubation period was allowed for luciferase reporter gene expression. Each datum is the average of two measurements at the same dosage. Luciferase activities were normalized by protein concentration, both were determined and described under Materials and Methods.

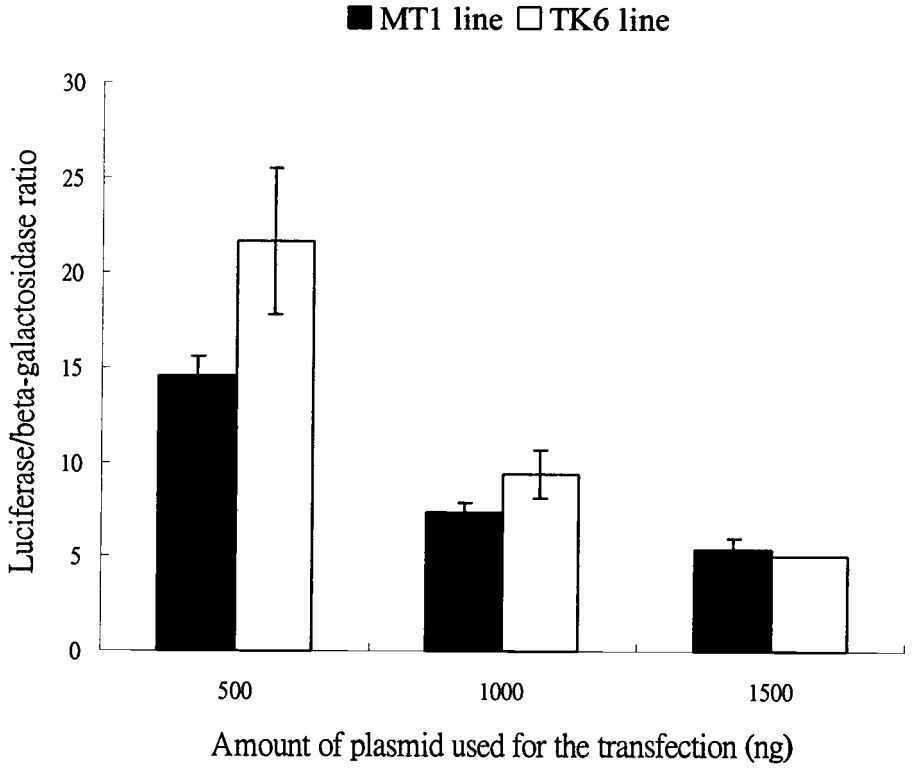

Figure 4. Determination of optimal DNA levels for two-reporter co-transfection of lymphoblast cell lines. Amounts of plasmid pCH110, which encodes the full-length *E.coli* β-galactosidase, were varied, alone with a fixed 300 ng of luciferase-encoding plasmid pCMVLUCbb1bpu, to determine the optimum level of pCH110 plasmids used for expression of both reporter protein in lymphoblast cells. After 24-hour incubation, cells were harvested, and lysates were prepared and assayed for luciferase and β-galactosidase activities as described under Materials and Methods. Each bar corresponds to the ratio of luciferase to β-galactosidase activities from duplicate transfections.

pCMVLUCbb1bpu yielded the highest luciferase activity. Therefore, I used 500 ng of plasmid pCH110 for subsequent co-transfections.

3.2.2 Determination of optimal post-transfection incubation time

Incubation time subsequent to transfection is another factor that can also affect the expression of luciferase. To determine when to harvest cells to obtain the highest luciferase activity, TK6 cells were transfected with luciferase-encoding plasmid pCMVLUC and incubated at 37°C for various periods of time. Figure 5 shows typical luciferase expression at different incubation times after transfection. Individual bars indicate averages for triplicate determinations for various incubation periods. Luciferase activity could be detected 16 hours after transfection, and remained stable or slightly higher until 24 hours post transfection. From 24 to 40 hours, there was an obvious decline of enzyme activity; the specific activity at 40 hours was only 20-30% of the activity at 16 hours. Even though 16- and 24-hour incubation periods produced similar luciferase activities, we chose 24 hours as our standard incubation time.

3.2.3 Optimization for β-galactosidase (β-Gal) Assay

To normalize for transfection efficiency, plasmid pCH110 was co-transfected with luciferase-encoding plasmids in lymphoblast cells. However, I observed a high endogenous level of β-galactosidase in the lysate, even when transfected with non-β-galactosidase-encoding plasmids. Thus, it was

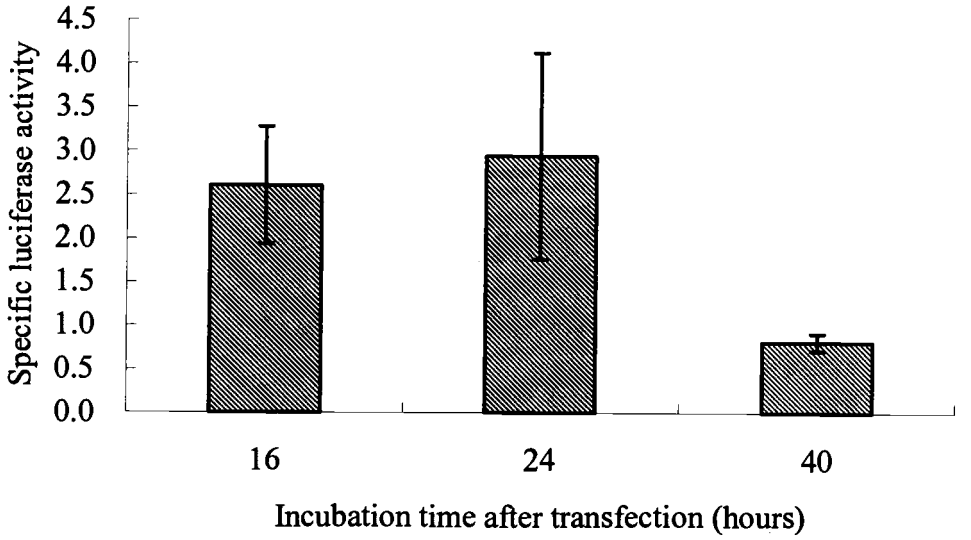

Figure 5. Determination of optimal assay time after transfection. TK6 cells were transfected with 300 ng of pCMVLUC and incubated at 37°C for different periods of time before harvest. Preparation of lysates and measurement of luciferase activities and protein concentrations were described under Materials and Methods. Luciferase activities were normalized by protein concentration. Each bar represents the average of three independent transfections.

necessary to suppress endogenous β-Gal activity if we wanted to use transgenic *E.coli* β-Gal to normalize transfection efficiency. Figure 6 shows both endogenous β-gal and transgenic β-Gal activities measured at 37°C and 45°C. When cell lysates were first incubated at 45°C for one hour, and then subsequently assayed at various times at the same temperature, the endogenous mammalian β-Gal activity was reduced to background level. Transgenic *E.coli* β-Gal activity showed slightly more activity at 45°C than at 37°C. Thus, β-galactosidase activity measured at 45°C was used to normalize transgenic luciferase activity for transfection efficiency.

3.3 RESULTS FROM TRANSFECTION OF LYMPHOBLAST CELLS

The strategy I used to screen MMR-proficient and MMR-deficient cells involved transiently transfecting cells with the repair-reporter plasmids, luciferase-encoding plasmids in this study (either functional luc^+ pCMVLUCbb1bpu (G/C) or luc^- pCMVLUCbb1bpu (G/A)). The mispaired adenine in codon 193 of plasmid pCMVLUCbb1bpu (G/A) located on the luciferase template strand would produce a truncated protein if it were not corrected. Because the plasmid pCMVLUCbb1bpu does not have a mammalian replication origin, the correction of G/A to G/C should proceed through DNA repair only and not through DNA replication. Therefore, restored luciferase activities should measure the repair efficiencies.

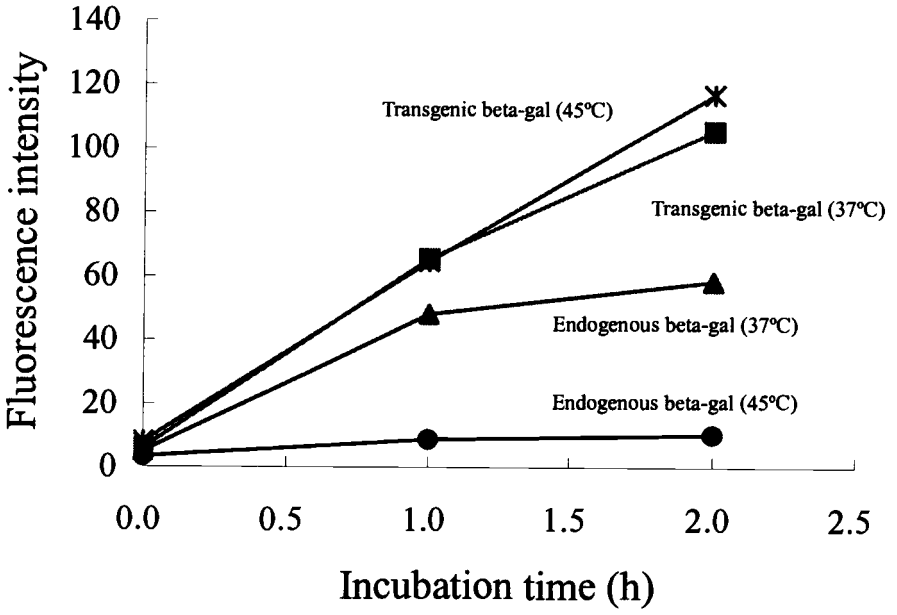

Figure 6. β-galactosidase time courses at different temperatures. TK6 cells were transfected with plasmid pCH110 (■ and ×) or plasmid pCMVLUCbb1bpu (▲ and ●). A 24-hour period was allowed for the foreign gene expression. The compound 4-methylumbelliferylgalactoside (4-MUG) was used to assay β-galactosidase (β-Gal) activity in the cell lysates as described under Materials and Methods. Both endogenous mammalian and transgenic *E.coli* β-Gal can hydrolyze 4-MUG at 37°C. Lysates were previously treated at indicated temperature for one hour and subsequently incubated at the same temperature and assayed at the indicated time. Hydrolysis of the substrate 4-MUG yielded less than 10 fluorescent units during 4 hours incubation with Cell Culture Lysis Buffer alone.

3.3.1 TK6 CELLS

3.3.1.1 One-reporter system

To test whether the mismatched substrate can be repaired in lymphoblast cells, the MMR-proficient TK6 (D) cells were transfected with either homoduplex or heteroduplex luciferase-encoding plasmids using DEAE-Dextran. Figure 7 shows results of a typical transfection of TK6 cells with homoduplex pCMVLUCbb1bpu (G/C), heteroduplex pCMVLUCbb1bpu (G/A) or homoduplex pCMVLUCbb1bpu (T/A) plasmids, either supercoiled or nicked. Supercoiled homoduplex *luc*$^+$ plasmid yielded the highest luciferase activity, while the nicked functional *luc*$^+$ showed a reduction in luciferase expression, to only 10 % of the activity of the supercoiled substrate. The defined nick was created to mimic the site where initiation of excision by the mismatch repair pathway occurs; presumably, the nicked heteroduplex would be repaired better than supercoiled mismatched substrate. However, the nicked heteroduplex (G/A) substrate showed only 12% of the activity displayed by the supercoiled *luc*$^+$ plasmid. Surprisingly, the supercoiled (G/A) plasmid yielded approximately 35% as much activity as supercoiled homoduplex *luc*$^+$ substrate. Two independent transfections of supercoiled (G/A) yielded 40% and 60% of the activity of the supercoiled homoduplex (G/C), see Table 2. Heteroduplex (G/A) might be repaired in either direction, to G/C or T/A, when there is no defined nick to initiate strand-specific excision. If so, full-length functional luciferase protein or truncated protein would be expressed. The luciferase

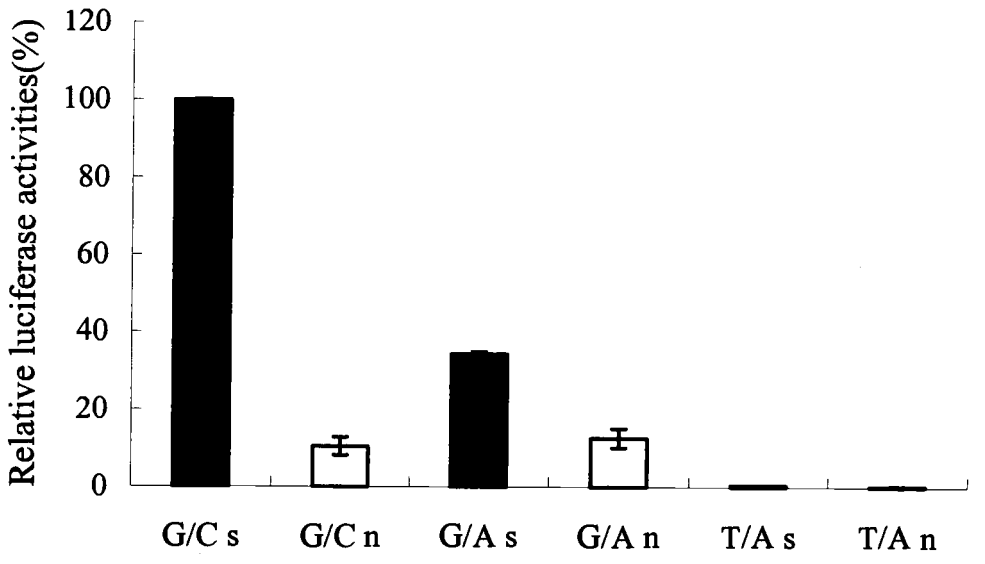

Figure 7. Luciferase activity in MMR-proficient cells. TK6 cells were transfected with plasmid pCMVLUCbb1bpu (G/C), pCMVLUCbb1bpu (G/A), or pCMVLUCbb1bpu (T/A), either supercoiled (s) or nicked (n). Each bar represents the average of duplicate transfections. After 24 hours, cells were harvested, and lysate were prepared and assayed for luciferase activities and protein concentration as described under Materials and Methods. Luciferase activities were normalized for protein concentrations. The truncated luciferase substrates T/A s and T/A n showed less than 0.5% of the activities of supercoiled *luc*$^+$ substrates. Heteroduplex supercoiled plasmid (G/A s) yielded nearly 40% of the activity of functional luciferase DNA (G/C s). Nicked substrates showed reduced activities.

Table 2. Transfection results for TK6 (D) cells

Plasmid used in the transfection	Relative luciferase activity		
	Experiment I	Experiment II	Experiment III
Supercoiled pCMVLUCbb1bpu (G/C)	100% ± 22%	100% ± 8.7%	100% ± 0.03%
Nicked pCMVLUCbb1bpu (G/C)	20% ± 2.0%	24.9% ± 2.0 %	10.5% ± 2.4%
Supercoiled pCMVLUCbb1bpu (G/A)	39% ± 1.8%	59.7% ± 3.3%	34.3% ± 0.6%
Nicked pCMVLUCbb1bpu (G/A)	N/A	16.3% ± 1.7%	12.6% ± 2.5%

1. Relative luciferase activity was calculated as described under Materials and Methods.
2. Experiment III was also shown as Figure 7.

activities yielded by supercoiled or nicked pCMVLUCbb1bpu (T/A) plasmids were less than 0.5% of the activity yielded by supercoiled *luc*$^+$ DNA.

3.3.1.2. Two-Reporter system

To compare luciferase activities from independent transfections, I used a different reporter gene as a control to monitor the transfection efficiency. Presumably, the ratio of activities from co-transfection of two reporters should be similar if fixed amounts of two plasmids were used each time. This provides the basis for using a second reporter to normalize transfection efficiency. Plasmid pCH110 was selected as the second reporter, because the plasmid encodes a full-length *E. coil* β-galactosidase whose activity is easy to measure. Plasmid pCH110 was co-transfected with either supercoiled homoduplex pCMVLUCbb1bpu (G/C) or supercoiled heteroduplex pCMVLUCbb1bpu (G/A) to TK6 (D) cells. Aliquots of lysates were assayed for luciferase and β-galactosidase activity separately. Luciferase/β-galactosidase activity ratios for each determination were compared to ratios for control determinations using homoduplex *luc*$^+$ plasmid pCMVLUCbb1bpu. Figure 8 shows two independent experiments; each bar represents the mean of parallel transfections in each experiment. The average activities resulting from transfection of the mismatched substrate were 55% of the luciferase activity from transfection of the *luc*$^+$ substrate, suggesting that the mismatch substrate was corrected in TK6 (D) cells.

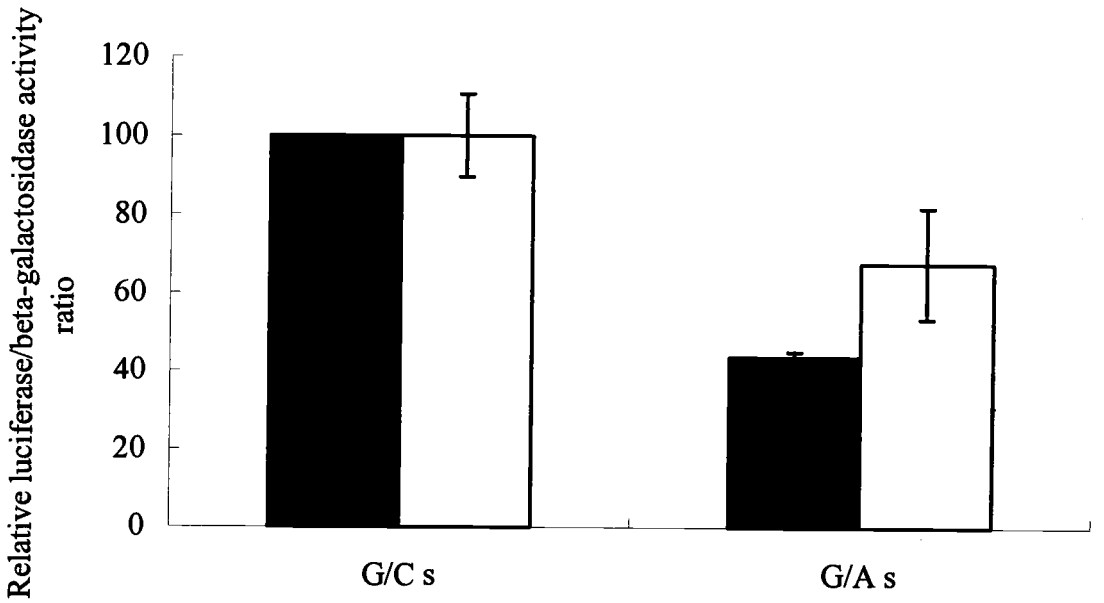

Figure 8. Luciferase/β-galactosidase activity ratio from two-reporter transfection of MMR-proficient cells. TK6 (D) cells were transfected with supercoiled homoduplex pCMVLUCbb1bpu (G/C) or supercoiled heteroduplex pCMVLUCbb1bpu (G/A). The filled and empty bars correspond to two independent transfections. Each bar represents the average of two to three parallel transfections. After 24 hours incubation, cells were harvested, and the lysates were prepared and assayed for luciferase and β-galactosidase activites as described under Material and Methods. Ratios of luciferase to β-galactosidase are expressed relative to the ratios for transfected with *luc*$^+$ substrate, pCMVLUCbb1bpu (G/C). The luciferase activities were first normalized with β-Gal activities and then converted to the percentage of normalized luciferase activities in G/C substrates. Heteroduplex (G/A) DNA yielded activities about 55% of those for *luc*$^+$ (G/C) DNA.

3.3.2 MT1 cell lines

Mismatch-repair-deficient cells have a higher tolerance to killing effects by DNA-alkylating reagents. MT1 cells were isolated from TK6 cells after low dosage MNNG alkylation reagent treatment (Goldmacher *et al* 1989). The cell extract from MT1 cells is MMR-deficient, but purified hMutSα can restore its mismatch repair function (Drummond *et al*, 1995). To examine whether our mismatched substrate could be repaired in MMR-deficient cells, they were co-transfected with luciferase-encoding plasmids and β-galactosidase-encoding plasmid. Supercoiled pCMVLUCbb1bpu (G/C) or pCMVLUCbb1bpu (G/A) plasmids were transfected into MT1 cells with plasmid pCH110, and luciferase/β-galactosidase activity ratios were compared. Figure 9 shows results from three independent transfections of MT1 cells. Contrary to our expectation, supercoiled heteroduplex (G/A) substrates appeared to be efficiently repaired, resulting in high luciferase activity. The average apparent repair efficiency was almost 50%, very close to those of TK6 (D) cells.

3.3.3 TK6 (T) cell line

Even though TK6 cells from various sources are expected to be identical with respect to functionally active MMR, there might be some variation during preparation in different laboratories. My goal is to establish an assay that can be used to screen MMR function *in vivo*. Therefore, it is better to compare repair efficiencies of two cell lines, positive MMR-proficient and negative MMR-

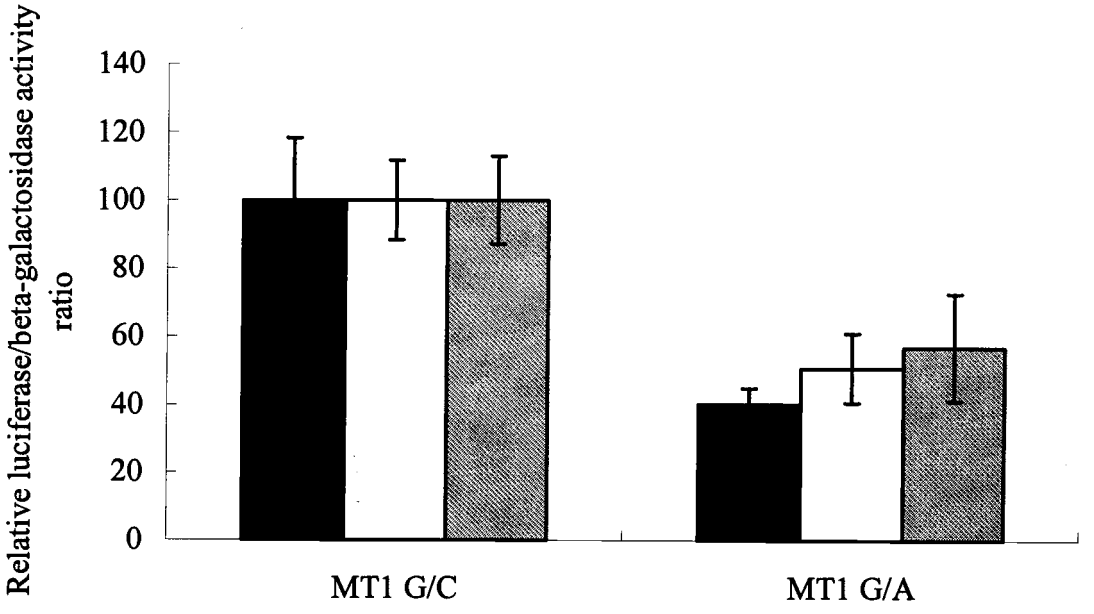

Figure 9. Relative luciferase/β-galactosidase activity ratio from two-reporter transfection of MMR-deficient lymphoblast cells. MT1 cell lines were transfected with supercoiled plasmids pCMVLUCbb1bpu (G/C) and pCMVLUCbb1bpu (G/A). Filled, empty, and hatched bars correspond to three independent transfections. Each bar represents the average of two to three parallel transfections of the same culture. After 24 hours incubation, cells were harvested, and lysates were prepared and assyed for luciferase and β-galactosidase activities as described under Materials and Methods. Relative activity ratios were determined and calculated as described in Fig. 8 legend. Luciferase activities for heteroduplex substrates (G/A) were about 50% of those for homoduplex *luc*$^+$ substrates (G/C) in this MT1 cell line.

deficient cells, from the same laboratory. TK6 (T) cells (obtained from Thilly's laboratory at the same time as MT1 cells) were co-transfected with both reporter genes, and the luciferase/β-galactosidase activity ratios were calculated as described above. Figure 10 shows results of two independent experiments. The luciferase/β-galactosidase activities ratio from cells transfected with supercoiled heteroduplex (G/A) showed only 20% of the activity of those cells transfected with supercoiled homoduplex (G/C). The repair efficiencies of G/A mismatched substrates in lymphoblast cells seemed to be independent of the MMR genotype of cells, suggesting that G/A might be repaired through a pathway independent of MMR.

3.4 TRANSFECTION RESULTS IN MOUSE FIBROBLAST CELLS

Mouse fibroblast cell line mc2 is derived from *MLH1* knockout mice (Prolla *et al*, 1998); mc5 is isogenic to mc2 but with functional MMR activity. Figure 11 shows results for transfection of both mouse fibroblast cell lines with homoduplex *luc*$^+$ plasmid pCMVLUCbb1bpu (G/C), or heteroduplex *luc*$^-$ plasmid pCMVLUCbb1bpu (G/A), either in supercoiled or nicked form. The transfection efficiencies in mc2 and mc5 cells were similar. The same reduction in luciferase activity, expressed by a nicked plasmid, relative to that by supercoiled plasmid, was seen in both cell lines, for both homoduplex or heteroduplex substrates. The nicked *luc*$^+$ plasmid yielded only about 20% as much luciferase activity as the supercoiled *luc*$^+$ DNA. In the MMR-deficient

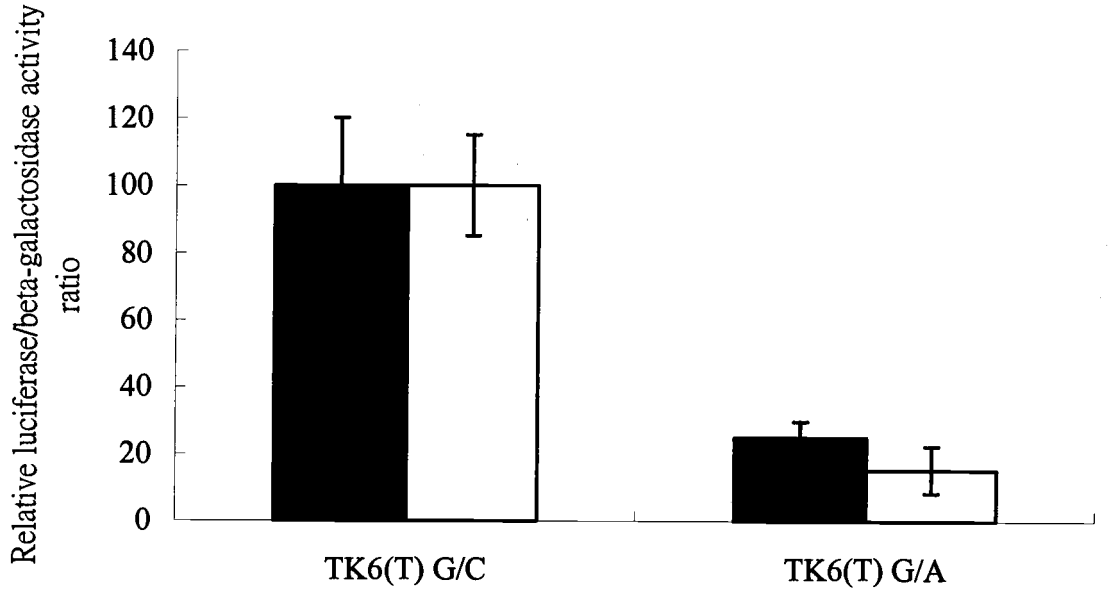

Figure 10. Relative luciferase/β-galactosidase activity ratio from two-reporter transfection of a second MMR-proficient cell line. TK6 (T) cells were transfected with supercoiled *luc*$^+$ (G/C) and *luc*$^-$ (G/A) substrates. Filled and empty bars represent two different independent transfections. Each bar corresponds to the average for three (filled bars) or four (empty bars) parallel transfections of the same culture. After 24 hours incubation, cells were harvested, lysates were prepared and determined for luciferase and β-galactosidase activities as described under Materials and Methods. Relative luciferase to β-galactosidase activity ratios were calculated as described in Fig. 8 legend. Luciferase activities for heteroduplex substrates (G/A) were about 20% of those for homoduplex *luc*$^+$ substrates (G/C) in this TK6 (T) cell line.

mouse cell, mc2, the supercoiled heteroduplex plasmid pCMVLUCbb1bpu (G/A) generated only 5% of the luciferase activity of the supercoiled *luc*$^+$ plasmid pCMVLUCbb1bpu (G/C). Luciferase activity yielded by the supercoiled mismatched substrate increased up to 11% in the MMR-proficient mc5 cells. In another experiment with supercoiled pCMVLUCbb1npu (G/A), the transfection of mc2 cells yielded about 5% activity, and transfection of mc5 cells yielded about 17% activity relative to supercoiled *luc*$^+$ plasmid (see Table 3). The difference between apparent efficiencies of correction of G/A mismatched substrates in MMR-proficient and MMR-deficient cells is approximately 2-3 fold. However, the overall repair efficiencies in mouse fibroblast cells were low.

3.5 RESULTS FROM TRANSFECTION OF TUMOR CELLS

HCT-116 cells, which originated from tumors, are MMR-deficient, due to mutations in both alleles of the MLH1 gene (Hemminki *et al.*, 1994). DEAE-Dextran was chosen to facilitate transfection of transiently expressed, non-replicating plasmids to cells. To determine whether the mismatched substrate pCMVLUCbb1bpu (G/A) could be repaired in MMR-deficient cells, HCT116 cells were transfected with plasmids pCMVLUCbb1bpu (G/C) and pCMVLUCbb1bpu (G/A), either as supercoiled or nicked DNA. Figure 12 shows that supercoiled *luc*$^+$ plasmid pCMVLUCbb1blu (G/C) has the highest activity among all substrates used. The nicked *luc*$^+$ substrate

pCMVLUCbb1bpu (G/C) showed only about 35% of the activity of the supercoiled *luc*$^+$ plasmid. The supercoiled heteroduplex pCMVLUCbb1bpu (G/A) mismatch substrate was poorly repaired, and showed only about 10 % of the luciferase activity of the supercoiled *luc*$^+$ plasmid. The nicked heteroduplex pCMVLUCbb1bpu (G/A) showed a slightly lower activity than the supercoiled pCMVLUCbb1bpu (G/A), but the difference is not statistical significant ($p = 0.41$ in T test).

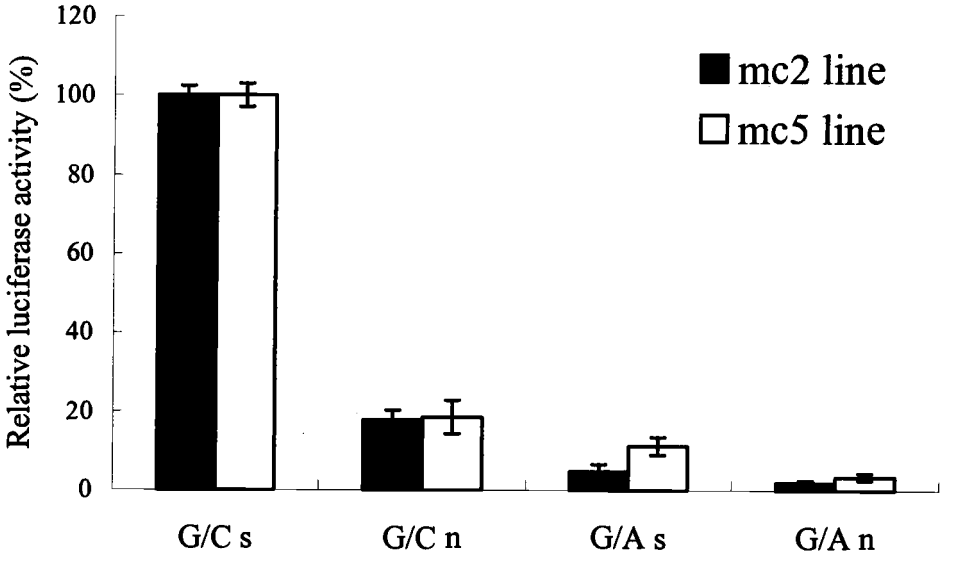

Figure 11. Relative luciferase activity in mouse fibroblast cell lines. MMR-proficient mc5 cells and MMR-deficient mc2 cells were transfected with plasmid pCMVLUCbb1bpu (G/C) or pCMVLUCbb1bpu (G/A), either supercoiled (s) or nicked (n). After 24 hours incubation, cells were harvested, and lysates were prepared and assayed for luciferase activities and determination of protein concentrations as described under Material and Methods. Transfection efficiencies in mc2 and mc5 are similar. Activities are expressed relative to those cells transfected with the supercoiled luc^+ plasmid (G/C s). Luciferase activity of supercoiled heteroduplex yielded about 5% of the activity of the supercoiled (G/C) *luc*$^+$ DNA in MMR-deficient cells and yielded about 11% of the activity of the supercoiled homoduplex (G/A) in MMR-proficient cells. Nicked substrates showed reduced activities.

Table 3. Transfection results for mouse fibroblast cells

Plasmid used in the transfection	Relative luciferase activity			
	Experiment I		Experiment II	
	Mc2	mc5	mc2	Mc5
Supercoiled pCMVLUCbb1bpu (G/C)	100% ± 29%	100% ± 8.5%	100% ± 2.4%	100% ± 2.9%
Nicked pCMVLUCbb1bpu (G/C)	N/A	N/A	18% ± 2.4%	18% ± 4.2%
Supercoiled pCMVLUCbb1bpu (G/A)	5% ± 0.6%	17% ± 5.6%	4.8% ± 1.8%	11% ± 2.2%
Nicked pCMVLUCbb1bpu (G/A)	N/A	N/A	2% ± 0.6%	3.5% ± 0.9%
Supercoiled pCMVLUCbb1 (T/A)	0.01%	0.02%	N/A	N/A

1. Experiment II was also shown in Figure 11.
2. Relative luciferase activity was calculated as described under Material and Methods.
3. mc2 is MMR-deficient cell line and mc5 is MMR-proficient cell line.

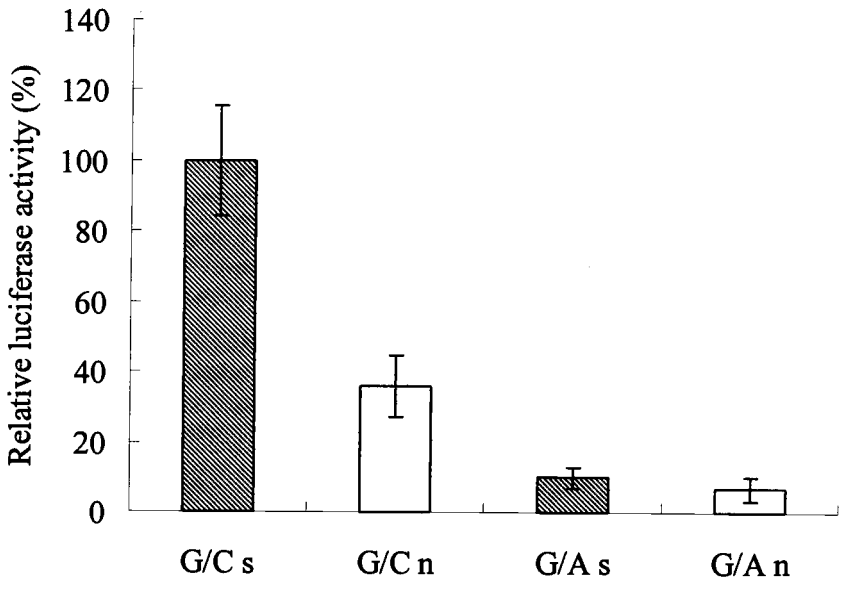

Figure 12. Luciferase activity in MMR-deficient cells. HCT116 cells were transfected with plasmid pCMVLUCbb1bpu (G/C) or pCMVLUCbb1bpu (G/A), either supercoiled (s; hatched bars) or nicked (n; empty bars) plasmids. Each bar represents the average of three parallel transfections. Cells were harvested and lysed after 24 hours incubation, and luciferase activities and protein concentrations were determined as described under Material and Methods. Activities are expressed relative to those cells transfected with the supercoiled luc^+ plasmid (G/C s). Luciferase activities of supercoiled heteroduplex (G/A) showed only 10% of the activities of supercoiled homoduplex (G/C). Nicked substrates showed a great reduction in luciferase activity, especially for the *luc*$^+$ plasmid.

CHAPTER 4. DISCUSSION AND CONCLUSION

I constructed a vector in which a template-strand adenine encodes a termination (anticodon 193: 3′ ACT), but the sense strand encodes the amino acid glycine (codon 193: 5′ GGA). The vector does not replicate inside mammalian cells (Promega technical bulletin 237); therefore, protein function can only be restored by DNA repair. Failure to correct G/A mismatch or correction to T/A would result in truncated luciferase. However, mismatch repair (MMR) to G/C would result in functional luciferase, providing the basis for an *in vivo* MMR activity. When I transfected with luc^+ (G/C) positive control plasmid, I observed that luciferase expression declined after 24 hours after transfection of lymphoblast cells. One explanation is that plasmids were slowly degraded inside the cell because they were not integrated into the chromosome and remaining luciferase activity decayed [the decay rate of the firefly luciferase protein is estimated to be 3-4 hours (Leclerc *et al*, 2000)]. Thus, once plasmids began to degrade, luciferase activities would decline. Incubation for 16-24 hours yielded similar luciferase expression; however, to give cells more time to accomplish the repair, I chose 24 hours as the standard incubation time in this study (see Figure 5, page 31).

The *E. coli* β-galactosidase (β-Gal) gene is one of the commonly used reporter genes for monitoring promoter activity in transfected cells (Howcroft,

et al, 1997; Rakhmanova and MacDonald, 1997; Gee, *et al*, 1999). The plasmid pCH110, which encodes the entire bacterial β-galactosidase gene, was selected as a second reporter gene to normalize luciferase expression in transfected cells. Initial studies of the TK6 cell lysates from cells transfected with vectors encoding green fluorescent protein or luciferase protein demonstrated a substantial basal level of β-Gal activity. Since lysis buffer itself generated less than 10 fluorescent units after 4 hours of incubation with 4-MUG, the β-galactosidase activities of lysates transfected from empty vectors should be equal to levels of mammalian endogenous β-Gal activity. Young, *et al* (1993) found that unlike mammalian β-Gal, bacterial β-Gal is stable at 50°C. However, in my study bacterial β-Gal was inactivated at 50°C. I tested for lower temperatures at which the mammalian but not the bacterial β-Gal was inactivated. When incubated at 45°C, both commercially purified *E. coli* β-Gal and lysates from cells transfected with the β-galactosidase-encoding plasmid pCH110 exhibited the same activity levels as at 37°C; the cell lysate containing only endogenous mammalian β-Gal lost most activity at 45°C. The cell lysate transfected with plasmid pCH110 contained both transgenic and endogenous β-Gal activity. When the endogenous β-Gal was selectively suppressed by heat treatment 45°C, the sum of activities should have been lower. However, as Figure 6 (page 32) shows, the cell lysates containing transgenic β-Gal have a slightly higher activity at 45°C. One possible explanation for the slightly higher activity at 45°C might be that bacterial β-Gal is more active at the higher

temperature.

MMR has been intensively studied in bacteria, where hemi-methylated DNA, just after replication, provides a mechanism for strand discrimination. However, the mechanism for strand discrimination in eukaryotes is still unknown. *In vitro* studies used circular DNA with a defined nick, to mimic the end of a newly synthesized DNA strand, in order to provoke the initiation of the excision step in MMR (Holmes *et al*, 1990). Beginning with plasmid pCMVLUC, I designed plasmid pCMVLUCbb1bpu, which has an enzyme nicking site that is position 635 bp of the plasmid. When plasmid pCMVLUCbb1bpu (G/A) is digested with N.*Bpu*10I endonuclease, the nick would be generated on 3′ end of mismatched adenine on the template strand. Since nick-dependent mismatch repair occurs efficiently *in vitro*, I expected that the nicked heteroduplexes would provoke higher levels mismatch repair than supercoiled substrates. However, the nicked heteroduplex substrates yielded reduced enzyme activities relative to supercoiled heteroduplexes both in lymphoblast cells and mouse fibroblast cells. Furthermore, the nick-related reduction was also seen in the positive-control of *luc*$^+$ substrates; the nicked *luc*$^+$ substrates yielded only 20-35% of the activity of their supercoiled counterparts. Supposedly, the nicked *luc*$^+$ substrates would not exhibit significantly reduced protein expression if DNA ligase seals the nick allowing RNA polymerase to transcribe the gene in the same manner as the supercoiled plasmid. A possible reason for the nicking-related reduction in protein expression would be that

RNA polymerase can not bypass an unligated template strand nick inside the transcribed region (from the CMV early promoter to the SV40 polyA signal). It is also possible that nicked substrates are more subject to DNA degradation inside the living cell than outside the cell.

In this study, the supercoiled plasmids were repaired more efficiently *in vivo*. Brown and Jiricny (1989) also observed extensive repair of supercoiled mismatched substrates. However, i*n vitro* studies using mammalian nuclear extracts (Holmes *et al*, 1990; Thomas *et al*, 1991) showed that the excision of continuously closed heteroduplex DNA is insignificant compared to excision of a substrate with a defined nicking site. Therefore, it is possible that adventitious nicks were generated during transfection, and these adventitious nicks were used to initiate the mismatch-repair excision. Presumably, these adventitious nicks were randomly generated, so repair of the heteroduplex DNA should be close to 50% in favor of either strand. As a result, the restored luciferase activity should be no greater than 50% of the homoduplex DNA; if repair occurred on the sense strand, the produced gene would encode a truncated non-functional protein. This might explain why in TK6 cells, the average repair efficiency was very close to 50%, see Figure 7 (page 35) and 8 (page 38).

There is also the possibility that the repair I observed in this study may not be nick-dependent or even MMR-dependent. My data showed that the repair efficiency for supercoiled G/A substrates, TK6 (D), was about 55%; that is, normalized luciferase activities were 55% of those cells transfected with luc^+

(G/C) substrates. If no nicks were generated during transfection, then perhaps a nick-independent repair pathway was responsible for repair of mismatched substrate. Furthermore, the mismatch-repair defective cell line MT1 also showed similar repair efficiency for the supercoiled G/A substrate to those of TK6 (D). Contrary to expectation, TK6 (T), which should be genetically identical to TK6 (D) showed only 20% apparent repair efficiency for the same mismatched substrate. Since MT1 and TK6 are reported to be MMR-deficient and MMR-proficient respectively, it is clear that G/A substrates can be repaired through a MMR independent pathway. Su *et al* (1988) demonstrated that six out of eight possible base-base mispairs could be repaired in a methyl-directed manner in *E. coli* extracts, except C/C and G/A mismatches. MMR machinery corrects the mispaired base only on the non-methylated strand. C/C was poorly repaired in bacterial extracts; however, G/A could be repaired in either a MutHLS-dependent, methyl-dependent pathway or in a pathway independent of MutHLS and of methylation of the heteroduplex substrate. A study using *Drosophilia* embryo and adult extracts showed repair of T/G and G/G mispaired substrates to be nick dependent, but repair of A/A, C/A, G/A, C/T, and T/T to be nick independent, suggesting a role for another activity (Bhui-Kaur et al, 1998). *E. coli* MutY is a DNA glycosylase which can cleave adenine from mispaired G/A or 8-oxo-G/A and ultimately yield the correct G/C base pair (Au *et al*, 1988; Lu *et al*, 1990). The MutY homolog (MYH), identified in nuclear extracts of calf thymus and human HeLa cells is also a DNA glycosylase that specially

removes mispaired adenine from A/C, A/G, and A/8-oxo-guanine base mismatches (Yeh *et al*, 1991; McDoldrick *et al*, 1995; Gu and Lu, 2001).

The oxidized base 8-oxo-guanine is a common endogenous product of reactive oxygen species (Cadet *et al*, 1997). Reactive oxygen species and free radicals generated in the mitochondria during ATP synthesis, as byproducts, could escape from the electron transport chain and damage DNA in various ways (Wei *et al*, 1998). During DNA replication, 8-oxoG can pair with either cytosine or adenine with in high efficiency; therefore, 8-oxoG is highly mutagenic. The MutY pathway in *E. coli* removes misincorporated adenines opposite 8-oxoG and prevents the G → T mutation (Michaels *et at* 1992). Mobility-shift assays show that the specific binding of purified hMYH on 8-oxoG/A is 7-fold stronger than to G/A; binding affinities for A/A, C/8-oxoG, and C/G substrates are very low (McGoldrick *et al*, 1995). Even though hMYH has lower affinity for G/A than 8-oxo-G/A, adenine opposite normal guanine still can be efficiently cleaved. It therefore seems possible that the G/A mismatched substrates I used in this experiment were repaired by both mismatch repair and base excision initiated by hMYH.

In another set of experiments, two mouse fibroblast cell lines, MMR-deficient mc2 cells and MMR-proficient mc5 cells, were used to test repair efficiency of G/A mismatch. Mouse fibroblast cells are easier to transfect than lymphoblast cells. Because fibroblast cells form an adhesive monolayer, I was able to use a milder transfection reagent (lipofectamine) than DEAE-Dextran,

which is cytotoxic, and still achieve high transfection efficiency. Due to the different transfection methods used, results for mouse fibroblast cells and human lymphoblast cells may not be directly comparable. The specific luciferase activities obtained by transfection of mouse fibroblast cells were 100~200 fold higher than those obtained with transfected lymphoblast cells. However, the higher transfection efficiencies did not result in higher repair efficiencies in the mouse fibroblast cells. Instead, I saw much lower apparent repair efficiencies for the G/A substrates in the mouse fibroblast cell lines mc2 and mc5 than efficiencies in the lymphoblast cells. In mc2 MMR-deficient cells, the average repair efficiency was about 5%, and only increased to an average of 14% the mc5 MMR-proficient cells. The G/A mismatch substrate might be poorly repaired in mouse cells. A previous *in vivo* study of site- and strand-directed repair of mismatches in the human oncogene, H-ras, at codon 12 (a hot spot for mutation), measured the repair rate of the specific base-base mismatches in *E. coli* and NIH 3T3 (mouse fibroblast MMR-proficient cells) (Arcangeli *et al*, 1997). They found that unlike G/T mismatches which showed 100 % repair rates in both *E. coli* and NIH 3T3 cells, only 35 % of G/A substrates were corrected to G/C at codon 12 in NIH 3T3 cells, and 87% were correctly repaired in *E. coli*. However, if the cells were synchronized in G1 stage, the repair rate of all 4 mismatched substrates approached 100 % at the same site (Matton *et al*, 1999). *In vitro* studies measured MMR protein binding affinities in NIH 3T3 nuclear extracts for DNA substrates with base-base

mismatch in H-ras codon 12. G/A mismatched substrate showed the lowest binding affinity among 4 substrates (G/T, T/C, A/C, and G/T) in the mobility-shift assay. To sum up their studies, the repair efficiency of G/A mismatched substrate depends on the type of cells, the stage of the cell cycle, the location of the base-base mismatches in the gene, and the repair is sometimes inefficient.

In conclusion, the repair efficiencies for G/A mismatched heteroduplex DNA in lymphoblast cells appeared high but might have involved a pathway other than mismatch repair. The most likely pathway would be BER initiated by hMYH DNA glycosylase. This suggests that the G/A mismatched substrate may be not an appropriate substrate for mismatch-repair measurements.

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