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MutM, a protein that prevents G·C→T·A transversions, is formamidopyrimidine-DNA glycosylase

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ABSTRACT

We have cloned chromosomal DNA bordering an insert that inactivates *mutM*. Sequencing of this clone has revealed that the insertion element is located between the promoter and structural gene for formamidopyrimidine-DNA glycosylase (Fapy-DNA glycosylase). An overproducing clone of Fapy-DNA glycosylase complements the original *mutM* strain that had been isolated after EMS mutagenesis. Thus, we conclude that MutM is actually Fapy-DNA glycosylase. *mutM* has previously been characterized as a mutator strain that leads specifically to G·C→T·A transversions. This in vivo characterization correlates well with the mutagenic potential of one of the lesions Fapy-DNA glycosylase removes, 8-oxo-7,8-dihydro-2′-deoxyguanine (8-OxodG).

INTRODUCTION

A number of transversion-specific mutator genes have been identified in Escherichia coli (1–4). Two of these genes, *mutY* and *mutM*, lead specifically to G·C→T·A transversions (2,3). Recently MutY has been characterized as a glycosylase that removes the A from a G-A mispair (5–7). We now report that *mutM* is also a glycosylase, formamidopyrimidine-DNA glycosylase (Fapy-DNA glycosylase). It differs from MutY glycosylase in that Fapy-DNA glycosylase removes DNA damage that can lead to G·C→T·A transversions rather than removing a base from an existing mispair.

Fapy-DNA glycosylase was originally isolated by its ability to remove a ring-opened form of guanine (8). However, due to the harsh conditions necessary to generate this DNA damage (9, 10) and to the inability to detect such lesions in cellular DNA (11), it was suspected that formamidopyrimidine was not the physiological substrate. Recent studies have shown that Fapy-DNA glycosylase is active on an oxidatively damaged form of guanine, 8-oxo-7,8-dihydro-2′-deoxyguanine (8-OxodG) (12). 8-OxodG is produced under physiological conditions (13, 14) and is most likely the natural substrate for this enzyme (12). The 8-OxodG lesion is found in DNA isolated from prokaryotes and eukaryotes (15). In vitro synthesis studies have shown that dA can be misincorporated opposite an 8-OxodG lesion (16). Further, transformation of phage (17) or plasmid (18) DNA containing a site-specific 8-OxodG lesion leads primarily to G→T transversions targeted to the site of the damaged guanine. Thus, inactivation of Fapy-DNA glycosylase leads to the accumulation of oxidatively damaged guanines in cells and these lesions lead specifically to G·C→T·A transversions.

MATERIALS AND METHODS

Bacterial strains, plasmids and phage

E. coli strain CC104 is *ara*, $\Delta(gpt\text{-}lac)_5$, *rpsL* [F′*lacI378*, *lacZ461*, *proA*$^+$*B*$^+$] (19). Strain TT101 is identical to CC104 except that it has *mutM*::mini-*tet*. The recombinant phage M13mp*tet*-1 has been previously described (20), and recombination experiments were performed on a TT101 derivative that contained an F′*kan* episome. Phage clones were propagated in a JM109 host. Plasmid pKK223-3 was purchased from Pharmacia, and plasmid clones were grown in JM109.

All media and genetic manipulations, unless otherwise noted, are as described (21).

Enzymes and chemicals

Restriction enzymes were purchased from New England Biolabs. Sequenase™ sequencing kits were purchased from United States Biochemical Corp. and templates were sequenced as per manufacturer's instructions. Double-stranded DNA sequencing was performed as previously described (22). Deoxyadenosine 5′-α-(^{35}S) thiotriphosphate was purchased from New England Nuclear.

Endonuclease Assay

Crude extracts were obtained by growing JM109 containing plasmid pKK223-3 or pKK-Fapy2 in LB media containing 100μg/ml ampicillin and when necessary 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG). 15 OD_{600} units were collected by centrifugation, resuspended in 2 ml 50 mM Tris-HCl pH 7.5, 20 mM β-mercaptoethanol, 1 mM phenylmethylsulfonyl fluoride (PMSF), and frozen at −70°C. The solution was thawed and sonicated on a Fisher Sonic Dismembrator Model 300 on maximum output for two 30 sec bursts. The cell debris was removed by a 20 min, 15,000 rpm spin at 4°C. The supernatant

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was taken and glycerol was added to a concentration of 5%. The protein content of the extracts were measured with the BioRad Protein Assay Kit using bovine serum albumin (BSA) as the standard. Various amounts of extract were incubated with 100 fmol of 17-mer duplex containing an AP site in the ^{32}P-labeled strand: 5'^{32}P-dGTTTTCCC(AP)GTCACGAC-OH-3'/5'OH-G-TCGTGACCGGGAAAAC-OH-3'. The reaction was incubated at 37°C for 30 min in 20 mM Tris-HCl pH 7.6, 10 mM EDTA, 50μg/ml BSA in a 10μl volume. The reaction was stopped by the addition of 3 μl of loading dye containing 95% formamide and by heating at 100°C for 2 min. An aliquot of the reaction was loaded onto a 15% denaturing polyacrylamide gel. Autoradiographs were quantitated by measuring transmittance of substrate and product bands with a Biorad 620 Video Densitometer.

Complementation tests

Ten independent cultures containing plasmid pKK223-3 or pKK-Fapy2 were grown to saturation in LB media supplemented with ampicillin (100μg/ml) and plated on minimal lactose. After two days incubation at 37°C, Lac$^+$ revertants were counted.

RESULTS

Cloning of the *mutM* gene

The *mutM* gene was cloned in a two-step method. In the first step, we constructed strain TT101 (see Materials and Methods) by transposition of the Tn*10* derivative, mini-*tet* (23), into strain CC104 (19). Colonies with mini-*tet* insertions were screened for increased G·C→T·A transversions by a previously described papillation test (3). Mutators collected from this initial screen were then mapped by P1 transduction with the *cysE* marker, which is cotransducable with *mutM*.

The juncture between the mini-*tet* insertion element and the *mutM* gene was then cloned out from the *E. coli* chromosome by a previously described recombination procedure (20). Briefly, a recombinant M13 phage, M13mp*tet*-1, that carries a central portion of the tetracycline resistance gene from Tn*10* was forced to integrate into TT101 at the mini-*tet* insertion site. Chromosomal DNA from the resulting lysogen was digested with a variety of restriction enzymes, ligated and transformed into JM109. One of the ligations yielded the phage clone 1Xba1 (Fig. 1A). We sequenced this clone and found that the mini-*tet* transposon had inserted between the promoter and structural gene for Fapy-DNA glycosylase (Fig. 1B) (24). A stretch of 460 base pairs directly adjacent to the Tn*10* insert was identical to the *fpg* gene which, like *mutM*, maps to about 82 min on the *E. coli* chromosome. An interesting feature in the promoter region is the large inverted repeat structure preceding the gene. Boiteux *et al.* reported a 10/11 matched structure (24) whereas we find a slightly different sequence in this region that leads longer inverted repeat structure (15/16 matched). The −35 promoter region is located at the base of this potential cruciform. The inverted repeat may therefore play a role in the regulation of this gene.

Characterization of an overexpression plasmid

To clone the intact wild type gene, we used PCR primers to amplify the the *fpg* gene. The amplified fragment was cloned into the pKK223-3 plasmid, to form the overexpression plasmid, pKK-Fapy2 (Fig. 2). Expression of the Fapy-DNA glycosylase is under control of the *tac* promoter. Under inducing conditions,

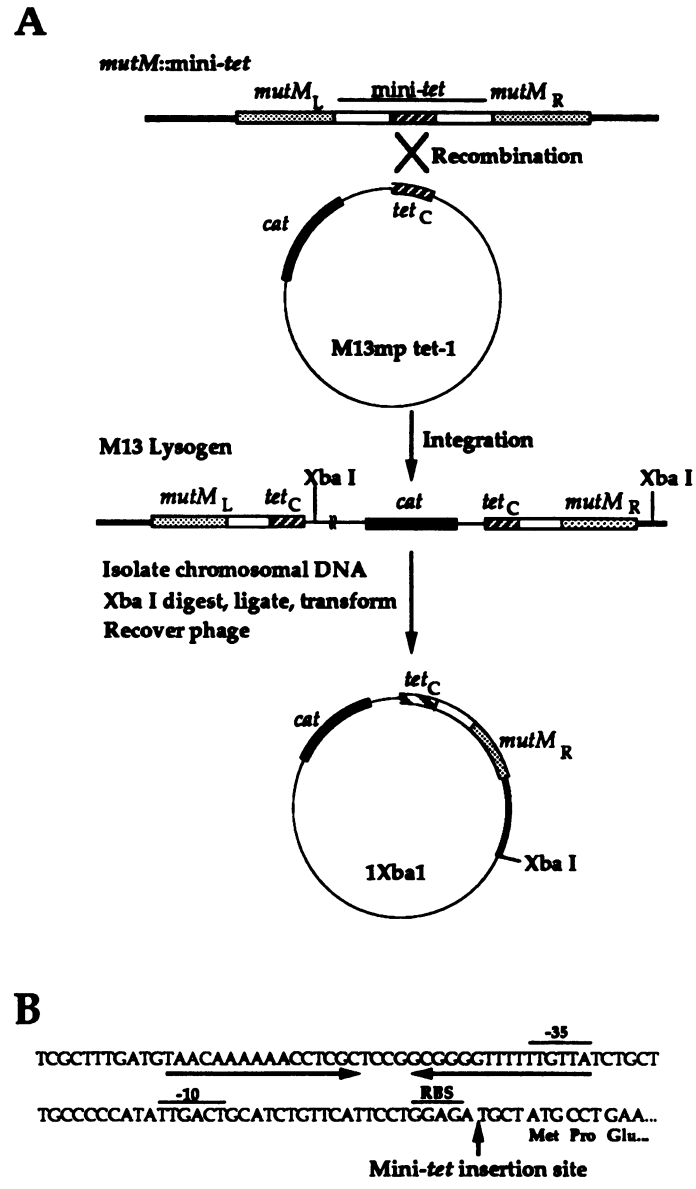

Figure 1. (A) Cloning of *mutM* sequences flanking a mini-*tet* insertion. A transposable insertion element, mini-*tet* (open and striped boxes), was used to inactivate the *mutM* gene (stippled boxes) and form *mutM*::mini-*tet*. Recombination between the central portion of the tetracycline resistance gene, tet_C (striped box), present on the chromosome and the M13mp*tet*-1 phage results in an M13 lysogen. Xba I digestion, ligation, and transformation of chromosomal DNA from the lysogen produced the clone 1Xba1, which carried approximately 3.6 kb of chromosomal DNA flanking the right side of the mini-*tet* insertion. Subscripts refer to portions of the *mutM* gene that are positioned to the left (L) or right (R) of the mini-*tet* insertion site. (B) Mini-*tet* insertion site. A detailed view of the promoter and beginning of the *fpg* gene are shown along with the exact site of insertion of the mini-*tet* element. The horizontal arrows underscore an inverted repeat that could potentially result in a cruciform structure.

the clone leads to the overproduction of Fapy-DNA glycosylase as judged by protein gel analysis (Fig. 3).

Fapy-DNA glycosylase has both glycosylase and apurinic/apyrimidinic (AP) endonuclease activity (25). We measured the AP endonuclease activity of crude lysates from a wild type strain and an overproducing strain (see Materials and Methods). We found that under inducing conditions the overproducing strain had over 400-fold more endonuclease

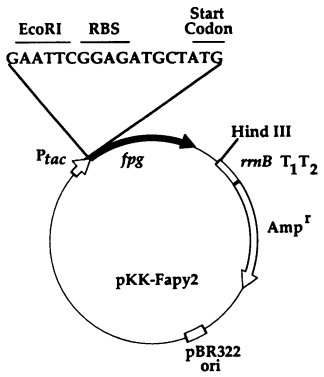

Figure 2. Construction of pKK-Fapy2. A PCR primer introduced an EcoRI site immediately upstream of the predicted ribosomal binding site (RBS) for the *fpg* gene. The EcoRI/Hind III fragment containing the entire *fpg* gene was cloned into pKK223-3 which introduces a strong inducible *tac* promoter in front of the gene and the *rrnB* transcription terminators at the end of the insert.

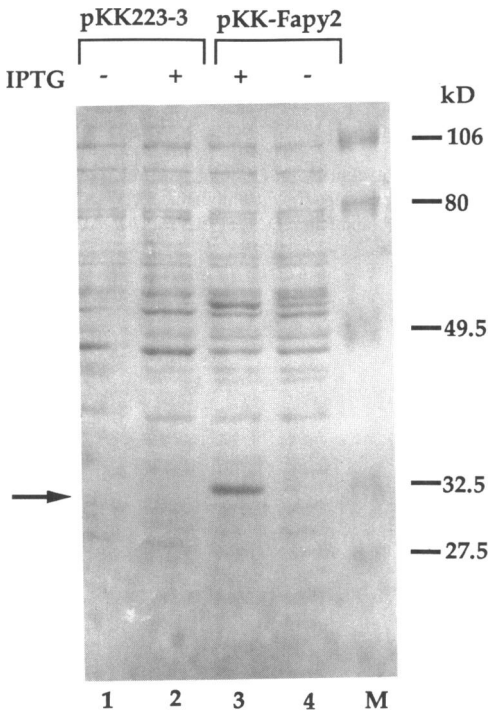

Figure 3. Overproduction of Fapy-DNA glycosylase. Crude lysates of uninduced or induced (1 mM IPTG) JM109 harboring either the wild type plasmid, pKK223-3, or the Fapy overproducing plasmid, pKK-Fapy2, were run on an 11% denaturing protein gel. Under inducing conditions, the 31 kD Fapy protein is overproduced in the culture that contains the pKK-Fapy2 plasmid (lane 3).

activity than the wild type strain (Table 1). Therefore, both the protein gel and enzyme activity analysis show that pKK-Fapy2 overexpresses Fapy-DNA glycosylase.

Complementation tests

The pKK-Fapy2 clone was tested for its ability to complement a *mutM* strain. Cultures of *mutM* strains have been shown to have a 10−15 fold higher rate of formation of Lac$^+$ revertants when tested against a *lacZ* allele that can only revert to Lac$^+$ by a specific G·C→T·A transversion (3). As shown in Table 2, the *mutM* clone, pKK-Fapy2, reduces the number of Lac$^+$ revertants to wild type levels in strain TT101. The pKK-Fapy2 clone also reduces the number of Lac$^+$ revertants in the original *mutM* strain to wild type levels. The original *mutM* strain had been obtained after EMS mutagenesis of strain CC104 (3). These complementation results show that both the original *mutM* isolate and the transposon-derived isolate are due to mutations that interfere with Fapy-DNA glycosylase expression.

Table 1. Overproduction of Fapy-DNA glycosylase

Strain	Plasmid	IPTG	nmol/mg
JM109	pKK223-3	−	0.04
		+	0.04
JM109	pKK-Fapy2	−	2.60
		+	19.0

The overproduction of Fapy-DNA glycosylase was estimated by measuring the amount of AP endonuclease activity in crude lysates from 5 separate reactions as described in Materials and Methods.

Table 2. Complementation of a *mutM* strain.

Strain	Plasmid	Lac$^+$ revertants
CC104 (wild type)	pKK223-3	2 +/− 1
	pKK-Fapy2	2 +/− 1
mutM	pKK223-3	72 +/− 12
	pKK-Fapy2	4 +/− 4

Overnight cultures of a wild type strain CC104 and the original *mutM* isolate containing the control plasmid, pKK223-3, or the *mutM* clone, pKK-Fapy2, were plated onto minimal lactose media. Lac$^+$ revertants were counted from at least 10 independent cultures. Cultures were titered and average reversion frequencies and standard deviations are expressed per 10^8 cells.

DISCUSSION

We have cloned and sequenced the juncture between the *mutM* gene and a Tn*10* derivative, mini-*tet* (Fig. 1), and found that the transposable element was inserted between the promoter and structural gene for Fapy-DNA glycosylase (*fpg*). An overproducing clone of Fapy-DNA glycosylase was constructed which put expression of the gene under the control of the *lac* promoter (Fig. 2). This clone fully complements both the transposon-generated mutant and the original *mutM* strain that had been obtained by EMS mutagenesis (Table 2). We therefore conclude that *mutM* is identical to *fpg*.

We have previously determined the specificity of the mutator character of the *mutM* strain (3). Mutations generated by *mutM* in a *lacZ* single-site reversion assay and with the more extensive *lacI* nonsense system both show that *mutM* leads specifically to G·C→T·A transversions.

How can we explain the specificity of the mutator effect in *mutM* strains in terms of the absence of the Fapy-DNA glycosylase? Fapy-DNA glycosylase has been shown to excise two types of substrates: substituted-imidazole ring-opened purines, Fapy lesions (8); and an oxidized form of guanine, 8-oxo-7,8-dihydro-2′-deoxyguanine (8-OxodG) (12). Fapy lesions generally are only formed under harsh conditions such as treatment with ionizing radiation or treatment of alkylated DNA with base for several days (9, 10, 26) and have not been detected in DNA from cells treated with alkylating agents (11). Further, Fapy lesions have been shown to block DNA replication (27). Therefore in the absence of an induced SOS system, Fapy lesions

would be potentially lethal in a strain lacking Fapy-DNA glycosylase rather than being mutagenic (28). One could argue that Fapy lesions could spontaneously depurinate, leaving an apurinic site that could lead to G·C→T·A transversions. However, the misincorporation of A opposite an apurinic site is not nearly frequent enough (59%) (29) to explain the specificity of G·C→T·A transversions in *mutM* (*fpg*) (3). Also, apurinic sites themselves block DNA replication in the absence of an induced SOS system. Therefore the data indicate that the Fapy lesion may be regarded as a cell-killing lesion but is unlikely to be a significant mutagenic lesion.

On the other hand, 8-OxodG is an important, potentially mutagenic substrate for Fapy-DNA glycosylase. Under physiological conditions active oxygen species generated by endogenous cellular oxidative processes react with guanine residues in DNA to form 8-OxodG (12−15). Treatments with chemical carcinogens and ionizing radiation can also result in 8-OxodG lesions since these treatments generate active oxygen species. In a cell free system it has been shown that polymerase I holoenzyme and Klenow fragment as well as polymerase β, α and δ, all insert both dC and dA opposite an 8-OxodG lesion (16). The rate of misincorporation varies with the enzyme tested. However, in all cases the rate of extension from a dA·8-OxodG pair was higher than extension from a dC·8-OxodG pair, suggesting that after misincorporation occurs, the mutation is not recognized by potential proof reading functions and becomes a fixed mutation. An explanation for the observed misincorporation comes from recent NMR studies which demonstrate that dA (anti)·8-OxodG (syn) forms a stable pair in the interior of a helix (30). Finally, transformation experiments using site-specifically modified 8-OxodG templates have shown that the lesion leads primarily to G→T transversions targeted to the site of the damaged guanine (17, 18). Thus, the misincorporation and structural studies on 8-OxodG directly correlates with the observed mutation spectrum obtained when the enzyme is inactivated and the lesion accumulates in DNA.

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