MutY, an adenine glycosylase active on G-A mispairs, has homology to endonuclease III

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ABSTRACT

The *mutY* gene of *Escherichia coli*, which codes for an adenine glycosylase that excises the adenine of a G-A mispair, has been cloned and sequenced. The *mutY* gene codes for a protein of 350 amino acids ($M_r = 39,123$) and the clone genetically complements the *mutY* strain. The protein shows significant sequence homology to *E. coli* endonuclease III, an enzyme that has previously been shown to have glycosylase activity on damaged base pairs. Sequence analysis suggests that, like endonuclease III, MutY is an iron-sulfur protein with a $[4Fe-4S]^{2+}$ cluster.

INTRODUCTION

The G-A mispair can assume three relatively stable G-A mispair conformations in duplex DNA [*G(anti)-A(anti)*, *G(anti)-A(syn)* and *G(syn)-A(anti)*] (1–5). Therefore, it is not surprising that *E. coli* has several pathways to exclude it from incorporation into replicated DNA. The pathways include the well-characterized DNA polymerase III editing function and methyl-directed mismatch repair system (6–8), as well as the less-defined pathways encoded by the products of the *mutT* (9–11) and *mutY* genes (12–14). The *mutT* and *mutY* systems are both *dam*-independent and appear to have complementary functions. Evidence suggests that MutT may interact with the DNA replication complex to block the incorporation of G opposite a template A, and thus prevent G-A mispairs that would lead specifically to A·T → C·G transversions (10,11). MutY, on the other hand, codes for a glycosylase that removes the A from a G-A mispair in duplex DNA (14). Presumably, MutY removes the A because MutT does not prevent the misincorporation of A opposite a template G. In this way, MutY prevents G-A mispairs that lead specifically to G·C → T·A transversions (13,14).

In this paper we report the cloning and sequencing of the glycosylase encoded by the *mutY* gene. Several mutagenesis tests show that the clone complements a *mutY* strain. Further, the deduced amino acid sequence correlates with the first seven known N-terminal amino acids (K. Au and P. Modrich, personal communication). We find significant homology between the MutY protein and *E. coli* endonuclease III but no other glycosylases.

MATERIALS AND METHODS

Bacterial strains, plasmids and phage

E. coli YN1 *ara*, $\Delta(lacproB)_{XIII}$, *rpsL*, *mutY::mini-tet*. [*F'**lacI378*, *lacZ461*, *proB*]. The recombinant phage M13mptet-1 has been previously described (15), and recombination experiments were performed on a YN1 derivative that contained an *F'**kan* episome. Phage clones were propagated in a JM109 host. Plasmid pBR329 was purchased from IBI, and plasmid clones were grown in JM109.

All media and genetic manipulations, unless otherwise noted, are as described (16).

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 (17). Deoxyadenosine 5'- α -(³⁵S) thiotriphosphate was purchased from New England Nuclear.

Complementation tests

Ten independent cultures were grown to saturation in LB media supplemented with the appropriate antibiotic (chloramphenicol 30 μ g/ml; ampicillin; 100 μ g/ml) and plated on LB + rifampicin (300 μ g/ml) + chloramphenicol (30 μ g/ml) and minimal lactose + ampicillin (100 μ g/ml).

RESULTS

Cloning of the *mutY* gene

The *mutY* gene was cloned in a two-step method. In the first step, we constructed strain YN1 (see Materials and Methods) by transposition of the *Tn10* derivative, *mini-tet* (18), into strain CC104 (19). Cells were screened by a previously described papillation test (12), and mutators collected from this initial screen were then mapped by P1 transduction with the *metC* marker, which is cotransducible with *mutY*. *mutY* sequences flanking the *mini-tet* insertion were then cloned out by a procedure that employed a recombinant M13 phage (15). The M13 phage, M13mptet-1, carries a chloramphenicol acetyltransferase gene (*cat*) as well as a central portion of the tetracycline resistance

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Figure 1. Cloning of *mutY* sequences flanking a mini-*tet* insertion. A transposable insertion element, mini-*tet*, was used to inactivate the *mutY* gene and form *mutY::mini-tet*. Recombination between the central portion of the tetracycline resistance gene, *tet_C*, present on the chromosome and the M13mp ϕ ter-1 phage results in an M13 lysogen. Pst I digestion, ligation, and transformation of chromosomal DNA from the lysogen produced the clone 8Ps2, which carried approximately 2.2 kb of flanking chromosomal DNA, including the N-terminal region of *mutY*. Subscripts refer to portions of the *mutY* gene that are positioned to the left (L) or right (R) of the mini-*tet* insertion site.

gene. Because M13mp ϕ ter-1 also contains amber mutations in phage genes *I* and *II*, which are required for phage replication, infection of a strain that lacks an amber suppressor leads to the loss of the replicon containing the *cat* gene unless homologous recombination occurs between the *tet* sequences present on the phage and chromosome (Fig. 1). A chromosomal DNA preparation of the M13 lysogen was digested with a variety of restriction enzymes, ligated and transformed into JM109. Several of the ligations yielded clones. One of the clones, 8Ps2, was sequenced and an open reading frame matched the partial N-terminal amino acid sequence of MutY, M-Q-A-X-Q-F-X₃-V-L (K. Au and P. Modrich, personal communication).

To clone the intact wild type gene, a primer extended from 8Ps2 was hybridized with a subset of clones from an ordered *E. coli* library (20). One of the clones, λ 476, gave a strong positive signal. The λ 476 clone has been physically mapped to roughly the same location that *mutY* maps genetically (64 min) (12). A 6.8 kb *EcoRV* fragment of the λ 476 clone containing the entire *mutY* coding sequence and flanking DNA was subcloned into pBR329 to form p476EV.

Table 1. Complementation of a *mutY* strain. Overnight cultures containing the control plasmid, pBR329, or the *mutY* clone, p476EV, were plated onto LB+rifampicin+ chloroamphenicol and minimal lactose + ampicillin. Rif^R revertants and Lac⁺ revertants were counted and averaged from 10 independent cultures. Cultures were titrated and reversion frequencies are expressed per 10⁸ cells.

Strain	Plasmid	Rif ^R revertants	Lac ⁺ revertants
CC104 (wild type)	pBR329	6	3
	p476EV	3	1
<i>mutY</i>	pBR329	145	62
	p476EV	2	3

Figure 2. Sequencing strategy and partial restriction map of the *mutY* gene. Arrows indicate the direction and extent of each sequence determination. The blackened box represents the *mutY* structural gene.

Complementation tests

The p476EV clone was tested for its ability to complement a *mutY* strain. Cultures of *mutY* strains have been shown to have a 15–40 fold higher rate of formation of rifampicin-resistant (Rif^R) colonies than a wild type strain (12). *mutY* also induces a higher rate of Lac⁺ revertants when tested against a *lacZ* allele that can only revert to Lac⁺ by a specific G·C → T·A transversion (12). As shown in Table 1, the *mutY* clone, p476EV, reduces the number of Rif^R revertants and Lac⁺ revertants to wild type levels.

Nucleotide sequence of *mutY*

The sequence of a portion of the complete *mutY* clone, p476EV, was determined from overlapping DNA fragments subcloned into M13 vectors. A detailed restriction map and the sequencing strategy are presented in Fig. 2. The DNA sequence of 1231 bp including the *mutY* gene is shown in Fig. 3. An open reading frame starts at nucleotide +1 with an ATG codon and ends at nucleotide 1050 with a TAG stop codon. The ORF of the *mutY* gene would be predicted to code for a protein of 39.1 kDa. The DNA sequence obtained from the recombinant phage clone, 8Ps2, matched identically with the corresponding region in the full-length plasmid clone, p476EV (nucleotides -16–243). This confirmed that the p476EV clone contained *mutY*, since the sequence of 8Ps2 had already been shown to correspond to the N-terminal amino acid sequence of MutY.

A potential ribosome binding site precedes the *mutY* gene at nucleotides -7–-3 (21). However no sequences similar to a consensus *E. coli* promoter were found upstream of the transcription start site (22).

Protein sequence alignment

A computer search of the EMBL protein database using the TFasta program (23) revealed that only endonuclease III has extensive homology with MutY. The endonuclease III protein is encoded by the *nth* gene and maps to 36 min on the *E. coli*

-147
CT TGTGA AAGTG TTCTG AAAAC GGGCA TTATC -116
C A A G T T A G T T G C C G G A T G C A A G C A T G A T A A G G C C T G T G C G G A A A G T -66
T C C G G T T T A C A C C C T G C C G T C G C T G T G C C A A T C T T G C C C C C A A C A C A -16
G T G A A T T C G G T G A C C A T G C A A G C G T C G C A A T T T C A G C C A G G T T 30
M Q A S Q F S A Q U 10
C T G G A C T G G T A C A A T A C G G C G A A A A C T C T G C C C T G G C A A 75
L D M Y D K V G R K T L P M Q 25
A T T G A C A A G A C G C C C T A C A A G T A T G G C T C A G A A G T G A T T T G 125
I D K T P V K U W L S E U M H L 40
C A A C A A A C T C A G G T T G C G A C C G T T A T C C C C T A T T T G A A C G C T T 165
Q Q T Q U A T U I P V F E R F 55
A T G G C G C G C T T C C G A C G T G A C C G A T C T C G C C A A T G C G C C G C T C 210
M A A R F P T U T D L A M A P L 70
G A C G A A G T T C T C A C T T G T G A C C G G G C T T G G C T A T T A C G C C G C 255
D E U L H L M T G L G Y V Y A R 85
G C G C G C A A T C T G C A T A A A G C G C A A C A A G T G C G A C C T T A C A C 300
A R M L H K A A Q Q U A T L H 100
G G C G G T A A A T T C C G G A A C C T T T G A G G A A G T T G C A G A C T G C C G 345
G G K F P E T F E E U A A L P 115
G G C G T C G G G C G T T C C A C C G C A G G C G C G A T T C T C T G C T T C T C T G 390
G U G A S T A G A I L S L S L 130
G G T A A G C A C T T T C C G A T T C T C G A C G G T A A C G T C A A C G C G T G C T G 435
G K H F P I L D G M U K R U L 145
G C G C G C T G C T A T G C T A A G C G G C T G G C C T G G A A A A G A G G T C 480
A R C Y A U S G M P G K K E U 160
G A G A A T A A A T T A T G G A G T T G A G C A G G A G A C G C C C G C G T T 525
E M K L M S L S E Q U T P A U 175
G G C G T G A A C G G T T T A A T C A G G C G A T G A T G A T T T G G T G C G A T G 570
G U E R F M Q A M M D L G A N 190
A T T T G T A C G C G C T C G A A C C G A A T G T T C G C T C T G T C C G C T A C A A 615
I C T R S K P K C S L C P L Q 205
A A C G G A T G T A T T G C C G C C A A C A A T A G C T G G C G C T T A T C C G 660
M G C I A A A M M S W A L Y P 220
G G C A A A A C C G A A C A G A C G C T G C C G A G C G C A C C G G C T A C T T T 705
G K K P K Q T L P E A T G Y F 235
T T G C T A T T A C A C C A A G A T G A A G T A T T G C T G C G C A G C G T C C G 750
L L L Q H E D E U L L A Q R P 250
C C G A G C G G A T T G T G G C G G T T A T A C T G T T T C C G C A G T T T G C C 795
P S G L M G G L Y C F P Q F A 265
G A C G A A A A G T T T G C G C A G T G G C T G C G C A A C G G C A G A T T G C T 840
D E E S L R Q M L A Q R Q I A 280
G C C G A T A A C C T G A C C A A C T G A C C G C G T T C G G C A T A C C T T C A G C 885
A D M L T Q L T A F A H T F S 295
C A T T T C A C T T A G A T A T T G T C C T A T G T G C T T C C C G T G T C A 930
H F H L D I U P M M L P U S S 310
T T C A C C G G C T G C A T G A T G A A G G C A A T G C G C T G G T A T A A C T T A 975
F T G C M D E G M A L M Y M L 325
G C G C A A C C G C C G T A G T T G G C C T A G C G C T C C C G T C G A G C G T T T G 1020
A Q P P S U G L A A P U E A L 340
T T A C A G C A G T T A C G C A C T G G C G C C G G T T A G C G T G A G T C A A A G 1068
L Q Q L R T G A P U A M B
A C G A C G A T T T A T G A G 1083

Figure 3. DNA sequence of the *E. coli mutY* gene. The nucleotide sequence of the *mutY* gene is shown with the numbering relative to the A of the ATG translational start site (+1). Amino acid sequence is shown below in single-letter code and is also numbered in the margin. The underlined sequence corresponds to a potential ribosome binding site.

	10	20	30	40	50
mutY	MQASQFSAQVLDWYDKYGRKTLPMQIDKTPYKVMLEVMLEQQTQVATVPIYFERFMR				
endo III	MNAKRLLEILTRLRENNPHETTELNFSSPPELLIAVLLSAQATDVSVNKATAKLPV				
	10	20	30	40	50
	60	70	80	90	100
	FPTVTDLANAPLDEVLHMTGLGYY-ARARNLEKAAQVATLHGKFPETFEVVAALPGV				
	60	70	80	90	100
	ANTPAAMLELGVGVKTYIKTIGLYNSKAENIKTCRILLEQNGEVPEDRAALEALPGV				
	120	130	140	150	160
	GRSTAGAILSLSLGKHFFILDGNVKKVRLARCYAVSGWPGKKEVENKMLSLSEQVTPAVGV				
	120	130	140	150	160
	GRKTANVVLNTAFGMPETIAVDTHIFRCNRTQFAPG-KNVEQVEEKLKV---VPAEFKV				
	180	190	200	210	220
	ERFNOAMDLGAMICTRSKPKCSLPLNGCIAAANNMAYLPGRKPKQTLPTERTGY...				
	180	190	200	210	220
	DCHHWLILH-GRYTCIARKPCGSCIEDLCEYKEKVDIX				
	180	190	200	210	250
	260				

Figure 4. Comparison between the MutY protein and endonuclease III. The 181 amino acid overlap region is shown. The '.' indicates identical amino acids, the ':' indicates similar amino acids, and the '-' represents a frameshift that has been introduced to optimize the homology.

genetic map (24). The proteins are 66.3% similar and 23.8% identical in a 181 amino acid overlap (Fig. 4).

The significance of the homology between MutY and endonuclease III is strengthened by the fact that endonuclease III has *N*-glycosylase activity. The glycosylase is active on damaged DNA templates and releases a number of ring-saturated and ring-fragmented derivatives of thymine (25–28). In addition, endonuclease III also releases a cytosine photoproduct, cytosine hydrate (29). However, no other glycosylases in the database showed significant matches. The same was true for nucleases and other DNA-processing proteins.

Recently endonuclease III has been identified as an iron-sulfur protein with a $[4\text{Fe-4S}]^{2+}$ cluster (30). This enzyme is unique in being both a DNA repair enzyme and an iron-sulfur protein. Among the Fe-S proteins that have been sequenced, it is common to find cysteine-containing runs, where the cysteines function as ligands that coordinate with the cluster (31). In the alignment produced by TFASTA, MutY and endonuclease III share a set of four identically spaced cysteines within a run between amino acid 194–210 in MutY and 187–203 in endonuclease III (Fig. 4). We propose that these cysteines are coordinated with the prosthetic group, $[4\text{Fe-4S}]^{2+}$, and that MutY is the second example of a DNA repair enzyme that is also an iron-sulfur protein.

DISCUSSION

We have cloned and sequenced the *mutY* gene and verified its identity by sequencing the juncture between the gene and a mini-*tet* insertion and by comparison to the seven known amino acids in the N-terminal sequence of the MutY glycosylase. Several tests show that the clone genetically complements a *mutY* strain (Table 1). The gene encodes a 350 amino acid protein ($M_r=39,123$) that bears significant homology to endonuclease III, a 211 amino acid protein ($M_r=26,300$) previously shown to have glycosylase activity on damaged thymines and cytosines (25–28).

The homology between MutY and endonuclease III spans 181 amino acids with 66.3% similarity and 23.8% identity among amino acids in the overlap. Endonuclease III has recently been characterized as an iron-sulfur protein with a $[4\text{Fe-4S}]^{2+}$ cluster (30). Sequence analysis of other iron-sulfur proteins has revealed

characteristic runs of cysteine ligands. Among the six other iron sulfur proteins with one $[4\text{Fe-4S}]^{2+}$ cluster, there is a conserved spacing between cysteines, Cys- X_2 -Cys- X_2 -Cys; the fourth cysteine in the set of ligands is usually far removed from the other three (31). This spacing is not conserved in endonuclease III, which made it impossible to predict which of the 7 cysteines in endonuclease III were bound to iron atoms. Comparison to MutY has revealed four cysteines in the C-terminal end of endonuclease III (aa 187–203) whose spacing is identical to MutY (aa 194–210) (Fig. 4). The four cysteines in this run are spaced Cys- X_6 -Cys- X_2 -Cys- X_5 -Cys. These cysteines are most probably bound to the $[4\text{Fe-4S}]^{2+}$ cluster in endonuclease III, and their presence in MutY predicts that MutY is also an iron-sulfur protein. If this prediction is proven correct, MutY will be the second DNA repair enzyme that is also an iron sulfur protein.

The role of the cluster and the significance of the spacing of the ligands remain to be elucidated. It is interesting to note that *Bacillus subtilis* glutamine phosphoribosylpyrophosphate aminotransferase, which is involved in the first step of *de novo* purine nucleotide biosynthesis, also contains an iron-sulfur cluster. This cluster has been proposed to serve a structural or regulatory role (32), while other $[4\text{Fe-4S}]^{2+}$ clusters have been implicated in substrate binding (33).

MutY and endonuclease III differ from each other in several important ways. First, they recognize and remove entirely different substrates. Endonuclease III displays glycosylase activity against damaged nucleotides such as ring-saturated and ring-fragmented thymine derivatives and cytosine photoproducts. These nonplanar pyrimidines are the result of UV, X-ray and oxidative damage (25–29). On the other hand, MutY glycosylase removes an undamaged purine (adenine) that is part of a G-A mispair. The obvious common factor between the substrates is that they have unnatural base pairing.

A second difference between the enzymes is that endonuclease III is an AP endonuclease that cleaves the first phosphodiester 3' to the AP site via a β -elimination mechanism (34), whereas the purified MutY protein lacks an endonuclease activity. The products of the nicking reaction at AP sites are similar to those found by mild alkali treatment and it is likely that basic residues in endonuclease III are positioned near the glycosylase active site such that they are able to catalyze the β -elimination reaction subsequent to the glycosylase reaction (34). Perhaps MutY glycosylase lacks the nicking activity because key basic residues are not properly positioned in the protein. In support of this hypothesis, of the 20 basic amino acids present in endonuclease III in the 181 amino acid overlap region, only 9 are conserved in MutY, and 7 of the 30 amino acids in endonuclease III that are outside of the overlap region are basic residues.

While the purified MutY protein is strictly a glycosylase (14), Lu and Chang have partially purified an activity from *E. coli* that binds G-A mismatches and cleaves the first phosphodiester 3' to and the second phosphodiester 5' to the mismatched A (35,36). As yet there is no evidence that the binding and cleavage reactions are catalyzed by a single protein in the partially purified extract. Further work in *Salmonella typhimurium* has shown that similar activities are present in a wild type extract but absent from a *mutB* extract, the analog to *mutY* (37). It is of interest to note that in a search for mammalian DNA repair enzymes, investigators have identified extracts that have endonuclease III-like activities in terms of substrate specificity and 3' AP endonuclease activity. However, unlike endonuclease III, these

extracts also have a 5' AP endonuclease activity (28), similar to the endonuclease activity described by Lu and coworkers.

The significance of glycosylases that function in mismatch repair may well extend beyond bacterial systems. Wiebauer and Jiricny have found that nuclear extracts of human cells can process G-T mismatches to G-C base pairs (38). Incubation of a G-T heteroduplex with the extract results in incision 3' and 5' of the mismatched T. Evidence suggests that a glycosylase mediates the initial step in the correction by removing the mispaired thymine.

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