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A specific role of MutT protein: To prevent dG·dA mispairing in DNA replication

(fidelity of DNA replication/DNA polymerase III/dGTPase/mutator)

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ABSTRACT **Occurrence of the transversion mutation A·T to C·G is specifically enhanced in *Escherichia coli mutT* mutants. With the aid of the cloned *mutT* gene, the MutT protein, which has a molecular mass of 15 kilodaltons, was overproduced and purified to near homogeneity. The protein catalyzes hydrolysis of dGTP to dGMP. dGDP and GTP were also hydrolyzed by the protein, but at a lower rate than seen with dGTP. No other deoxynucleoside triphosphates were hydrolyzed. Using poly(dA)·$(dT)_{20}$ as a template-primer, we investigated the misincorporation of dGMP, dCMP, and dAMP by the α subunit and the core of *E. coli* DNA polymerase III. When the polymerization reaction was performed with the α subunit, both dCMP and dGMP were misincorporated. The core, composed of α, ε, and θ subunits, misincorporated only dGMP. This would imply that the proofreading function of the ε subunit of DNA polymerase III may correct the dC·dA mispair but not the dG·dA mispair. Misincorporation of dAMP was not observed in reactions with the α subunit or core. The misincorporation of dGMP, but not dCMP, was almost completely suppressed by adding purified MutT protein to the reaction mixture. Under these conditions, only a portion of dGTP present in the reaction mixture was degraded. It is therefore likely that the MutT protein may prevent dGMP misincorporation by degrading a specific form of dGTP, probably the *syn* form, which can pair with deoxyadenosine.**

Organisms come equipped with elaborate mechanisms that keep mutation rates low. There are at least three steps required to prevent errors during DNA replication (1): (*i*) selection of a base complementary to the template by DNA polymerase, (*ii*) removal of a noncomplementary base by an editing nuclease, and (*iii*) correction of a misincorporated base by the methyl-directed postreplicational repair system (mismatch repair).

Mutators are a special class of mutations that render various genes unstable. It has been suggested that the products of mutator genes may be involved in the above-mentioned processes (1, 2). Indeed, certain mutants of *Escherichia coli* having mutations in the *dnaE* and the *dnaQ* (*mutD*) genes show a distinct mutator phenotype. The *dnaE* gene encodes the α subunit of DNA polymerase III (pol III), which carries polymerase activity, while the *dnaQ* gene encodes the ε subunit of pol III, which possesses 3′ → 5′ exonuclease activity. The *dam, mutS, mutR, mutL,* and *uvrD* genes are involved in the process of mismatch repair, and mutants defective in these genes show moderately high mutator activity.

Among the *E. coli* mutator genes, *mutT* warrants special attention. *mutT* mutants increase about 1000-fold the frequencies of a unidirectional A·T to C·G transversion (3, 4), and this type of transversion is apparently not enhanced in other mutator mutants (5, 6). DNA replication seems to be required for expression of the mutator effects of the *mutT* mutant (7). On the basis of the finding that the temperature-sensitive phenotype of *dnaE* mutations is partially suppressed by *mutT* mutation, it was suggested that the *mutT* gene product might be interacting with pol III (8). However, the mutT gene does not seem to be essential for cell growth (9).

To elucidate functions of *mutT*, we cloned the gene and determined the DNA sequence (10). The *mutT* gene was found to encode a polypeptide of 14,926 daltons. More recently, Bhatnagar and Bessman purified the MutT protein and showed that it possesses nucleoside triphosphatase activity (11). We report here evidence that the MutT protein suppresses the formation of dG·dA mispairs during the action of pol III *in vitro*.

MATERIALS AND METHODS

Materials. Poly(dA), $(dT)_{20}$, and dNTPs were purchased from Pharmacia-LKB. [α-^{32}P]dNTPs (800 Ci/mmol, 10 mCi/ml; 1 Ci = 37 GBq) were from Amersham. The α subunit and the core of pol III were purified, as described (12).

Purification of MutT Protein. The *E. coli* strain MA190 harboring plasmid pMA106, which carries the *mutT* gene, was used for the overproduction of MutT protein (10). Purification of the MutT protein was carried out at 4°C. Cells were grown in 4 liters of L broth (10), harvested (4.5 g, wet weight), and suspended in 35 ml of buffer A [20 mM Tris·HCl, pH 7.5/1 mM EDTA/1 mM 2-mercaptoethanol/5% (vol/vol) glycerol] containing 0.2 M NaCl. The cells were disrupted by an ultrasonic disintegrator and the supernatant was recovered (fraction I, 31 ml). Fraction I was mixed with an equal volume of DEAE-Sephacel (Pharmacia-LKB) slurry equilibrated with the same buffer. After stirring for several hours, the clear supernatant was dialyzed against buffer A (fraction II, 107 ml). Fraction II was applied to a DEAE-Sephadex A-25 (Pharmacia-LKB, 30 ml) column equilibrated with buffer A. The column was washed with 90 ml of buffer A and protein was eluted with buffer A containing a linear gradient (0–0.3 M) of NaCl. The samples eluted were monitored with an UV flow photometer and $NaDodSO_4$/PAGE. Fractions (0.15–0.2 M NaCl) containing the 15-kDa protein were pooled (fraction III, 55 ml) and subjected to precipitation with ammonium sulfate. The precipitate was collected by centrifugation and dissolved in 4 ml of buffer B (20 mM potassium phosphate, pH 6.8/1 mM 2-mercaptoethanol/5% glycerol) (fraction IV, 4.7 ml). Fraction IV was filtered through a column of Sephadex G-75 (Pharmacia-LKB; 2.5 × 100 cm) equilibrated with buffer B. The peak fractions were combined (fraction V, 48 ml), and applied to a column of hydroxylap-

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Abbreviation: pol III, DNA polymerase III.

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atite (Bio-Rad, 10-ml bed volume) equilibrated with buffer B. The protein was eluted with 100 ml of buffer B containing a linear gradient (20–200 mM) of potassium phosphate. The peak fractions were pooled and dialyzed against buffer A (fraction VI, 25 ml).

Assay of dGTPase. The reaction mixture (10 μl) contained 20 mM Tris·HCl at pH 7.5, bovine serum albumin at 80 μg/ml, 8 mM $MgCl_2$, 5 mM dithiothreitol, 4% glycerol, and 100 μM [α-^{32}P]dGTP (2 Ci/mmol). After incubation with MutT protein at 30°C for 5 min, the reaction was stopped by the addition of 10 μl of 100 mM EDTA. An aliquot of the mixture was spotted on a thin-layer sheet (PEI-cellulose, MERCK) and developed with 1 M LiCl. Regions containing dGTP and dGMP were cut out and the radioactivity was measured by Cerenkov counting. One unit of the enzyme forms 1 pmol of dGMP per min under these conditions.

Misincorporation of dGMP. $(dT)_{20}$ was annealed with the poly(dA) with an average gap size of 20 nucleotides. The reaction mixture (25 μl) was the same as that used for the dGTPase assay, except that 4.5 μg of poly(dA)·$(dT)_{20}$ (10 μM 3'-OH ends of primer) were added. After 90 units of either α subunit or core of pol III had been added, the mixture was incubated at 30°C for 10 min. When MutT protein was to be added, the reaction mixture was preincubated with the protein for 1 min at 30°C. The reaction was terminated by the addition of 75 μl of 68 mM EDTA and DNA was extracted with phenol/chloroform (1:1, vol/vol). The DNA was precipitated with ethanol, dissolved in 10 μl of 1 mM Tris·HCl, pH 8.0/2.5 mM EDTA/50% (vol/vol) formamide, boiled for 3 min, and quickly chilled on ice. Aliquots of the DNA were loaded onto 12% polyacrylamide gels containing 8 M urea and electrophoresed at 1800 V. An x-ray film (Fuji RX) was exposed against the gels at −70°C and developed. To measure rates of misincorporation of dCMP or dAMP, 100 μM (2 Ci/mmol) either [α-^{32}P]dCTP or [α-^{32}P]dATP was used.

Other Methods. $NaDodSO_4$/PAGE and staining of the gel were as described (13). Protein concentrations were determined by using the Bio-Rad protein assay dye reagent with bovine serum albumin as a standard. General methods used for DNA manipulation were as described (14). The N-terminal amino acid sequence of MutT protein was determined by an Applied Biosystems gas phase protein sequencer, model 470A.

RESULTS

Purification of MutT Protein. *E. coli* cells that harbor multicopy plasmids carrying the *mutT* gene overproduced MutT protein, with a molecular mass of about 15 kDa (10). Under the optimal conditions, about 2% of the cellular protein was MutT protein. A distinct band corresponding to the MutT protein was detected on $NaDodSO_4$/PAGE (Fig. 1), and we followed this band through purification of the protein. The most highly purified fraction (fraction VI) contained the 15-kDa polypeptide as a major component and a slightly larger one as a minor component. Both of these polypeptides seem to be encoded by the *mutT* gene, because the first 20 amino acid residues of the N-termini of the two proteins, determined by an automatic sequencer, were identical to those expected from the DNA sequence of the *mutT* gene (10). Changes in electrophoretic mobilities may be caused by post-translational modifications of the protein.

A dGTPase Activity of the MutT Protein. A search was made for an enzyme activity which might be associated with the MutT protein. No nuclease or ATPase activity was detected when the protein was incubated with double- or single-stranded DNA in the presence of $MgCl_2$ and ATP. The protein did not bind to a DNA-cellulose column in the presence of 20 mM NaCl, a finding indicative of a low affinity to DNA.

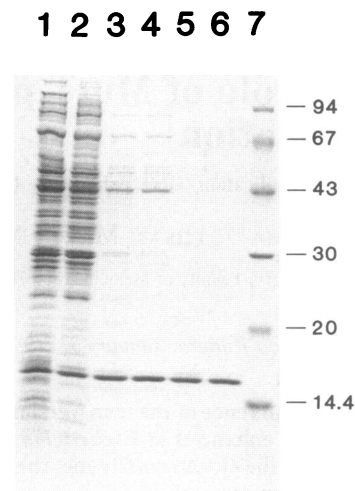

FIG. 1. $NaDodSO_4$/polyacrylamide gel electrophoretic analyses of MutT protein. Samples were applied to a $NaDodSO_4$/15% polyacrylamide gel, electrophoresed, and stained with Coomassie brilliant blue. Samples for various purification steps of MutT protein were analyzed. Lanes: 1, fraction I (50 μg); 2, fraction II (26 μg); 3, fraction III (9.1 μg); 4, fraction IV (12.3 μg); 5, fraction V (2.1 μg); and 6, fraction VI (1.9 μg). Marker proteins in lane 7 are phosphorylase b (94 kDa), bovine serum albumin (67 kDa), ovalbumin (43 kDa), carbonic anhydrase (30 kDa), soybean trypsin inhibitor (20 kDa), and α-lactalbumin (14.4 kDa).

The MutT protein possesses no DNA polymerase activity and does not affect the rate of polymerase reaction when added to the *E. coli* pol III core enzyme. However, on incubation of the MutT protein with DNA and dNTPs in the polymerase assay, formation of a small amount of dGMP was detected. No other nucleoside monophosphate was formed under these conditions. Subsequent experiments revealed that the MutT protein possesses an intrinsic dGTPase activity and that other components for the DNA polymerase reaction are not required for the activity.

Fig. 2*A* shows an elution profile of MutT protein from a Suparose-12 column. A peak of dGTPase activity coincided well with the peak of the 15-kDa protein. Mg^{2+} was required for the reaction. GTP and dGDP were also hydrolyzed, with lower rates as compared with the hydrolysis of dGTP.

Prevention of Misincorporation of dGMP by the MutT Protein. One explanation as to why dGTPase activity correlates with the function of MutT protein that is normally required to lower the frequency of A·T to C·G transversion in cell is that it acts to eliminate dG·dA mispairing, an event that could occur during DNA replication. The unfavorable pairing may be prevented by degrading a specific form of dGTP that can pair with the dA residue on the template.

To gain support for this notion, we tested effects of MutT protein on the misincorporation of dGMP onto a poly(dA) template during the *in vitro* DNA synthesis. In the absence of MutT protein, a small amount of dGMP (less than 1/10,000 of dTMP incorporated when dTTP was present) was incorporated onto poly(dA)·$(dT)_{20}$, the template-primer. When MutT protein was present in the reaction mixture, the rate of misincorporation was considerably reduced. This activity correlated well with the dGTPase activity, as shown in Fig. 2*B*.

Roles of Subunits of pol III for Preventing Misincorporation. We then examined whether other types of mispairing can also be prevented by the MutT protein. For this purpose, the polymerase reaction with the α subunit or the core of pol III, the latter being composed of α, ε, and θ subunits, was performed in the absence or presence of MutT protein (Fig. 3).

Misincorporation of dGMP onto the poly(dA)·$(dT)_{20}$ occurred when the reaction was performed with either form of

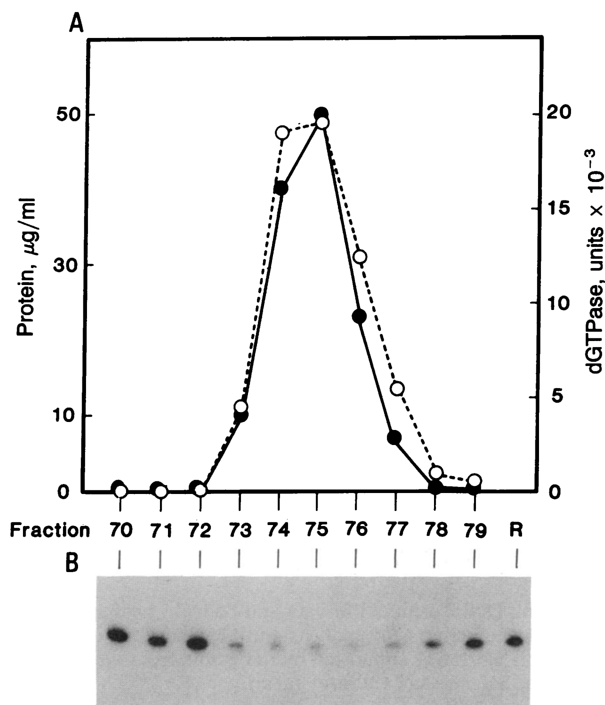

FIG. 2. Enzymatic activities coeluted with purified MutT protein from a Suparose-12 column. The MutT protein (fraction V, 0.3 μg) was filtered through a Suparose-12 column (Pharmacia-LKB) equilibrated with a buffer containing 50 mM Tris·HCl at pH 7.5, 1 mM EDTA, 5 mM dithiothreitol, 0.1 M NaCl, and 20% (vol/vol) glycerol. Fractions were assayed for protein, dGTPase activity, and an activity to suppress dGMP misincorporation by α subunit of pol III. (*A*) Elution profiles of protein and dGTPase. •, Protein; ○, dGTPase activity. (*B*) Autoradiogram for the misincorporation of dGMP. The MutT protein was not added to the sample of lane R.

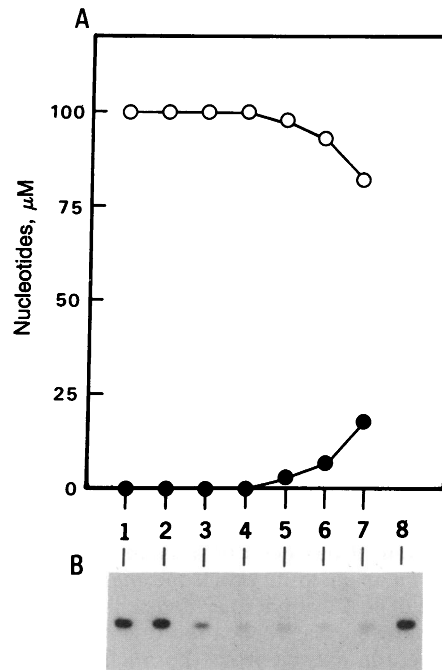

FIG. 4. Prevention of misincorporation of dGMP by MutT protein. Assays for the misincorporation of dGMP were carried out with 90 units of the α subunit and various amounts of the MutT protein. Aliquots were subjected to a PEI-cellulose chromatography to determine contents of dGTP and free dGMP, and the remainder was used for the misincorporation assay. Samples for lanes 1–7 contained 0, 0.33, 3.3, 33, 330, 1700, and 3300 fmol of MutT protein, respectively. To the sample for lane 8, no MutT protein was added. (*A*) Conversion of dGTP to dGMP by the MutT protein. ○, Amount of dGTP; •, amount of dGMP. (*B*) Autoradiogram showing the misincorporation of dGMP onto a poly(dA) template.

the polymerase (Fig. 3, lanes 1 and 2), and this misincorporation was almost completely suppressed by adding a small amount of MutT protein (lane 3). Thus, while dG·dA mispairing is not corrected by the subunits of pol III, the prevention is totally dependent on MutT protein.

In the case of dC·dA mispairing, a completely different result was obtained. Incorporation of dCMP onto the poly(dA)·$(dT)_{20}$ occurred with the α subunit but not with the pol III core (Fig. 3, lanes 4 and 5). Addition of the MutT protein to the reaction mixture with α subunit did not affect the misincorporation (lane 6). Thus, dC·dA mispairs may be corrected by the editing exonuclease activity associated with the ε subunit of pol III.

No dA·dA mispairs were produced by the α subunit or the pol III core (lanes 7 and 8). It seems likely that dA·dA mispair

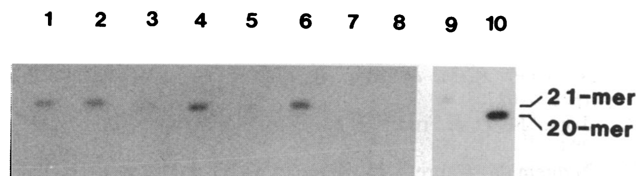

FIG. 3. Misincorporation of dGMP and other nucleotides with poly(dA) as a template. The reaction was carried out with 90 units of the α subunit (for lanes 1, 3, 4, 6, 7, and 9) or the pol III core (for lanes 2, 5, and 8). Samples for lanes 3 and 6 were with 25 ng of the purified MutT protein (fraction V). Lanes 1–3 and 9, dGMP misincorporation; lanes 4–6, dCMP misincorporation; lanes 7 and 8, dAMP misincorporation. The samples were run on a 12% polyacrylamide gels containing 8 M urea, and nucleotides misincorporated were detected on the autoradiogram. In lane 10, ^{32}P-labeled $(dT)_{20}$ was run, and the positions corresponding to 20-mer and 21-mer are shown.

is hardly formed or such mispairing is eliminated by the α subunit itself.

A Specific Role of MutT Protein to Prevent dGMP Misincorporation. The above described experiments revealed that the MutT protein possesses a distinct dGTPase activity and also an activity to prevent the misincorporation of dGMP onto the poly(dA) template. To correlate these two activities, we quantitatively measured both reactions catalyzed by the MutT protein.

On incubation with increasing amounts of MutT protein, the amount of dGTP in the reaction mixture decreased while that of dGMP increased (Fig. 4*A*). In parallel, the rate of misincorporation of dGMP decreased, but this suppression of misincorporation occurred when a large amount of dGTP was still present in the reaction mixture (Fig. 4*B*). Thus, it is unlikely that the misincorporation is prevented by loss of the dGTP substrate. Presumably some form of dGTP that can pair with dA is specifically degraded by the MutT protein.

DISCUSSION

To analyze mispairs formed upon DNA synthesis *in vitro*, we devised a procedure to detect amounts of the mispairs by gel electrophoresis. Using this procedure, we found that the α subunit of pol III, devoid of editing exonuclease activity (12), misincorporated dGMP and dCMP but not dAMP onto a poly(dA) template. When similar experiments were carried out with the core enzyme, a subassembly of pol III possessing both polymerase and 3′ → 5′ exonuclease activities, only the misincorporation of dGMP was evident. Thus, the formation of incorrect base pairs dA·dA and dC·dA is prevented by the pol III core itself; formation of dA·dA mispair may be prevented by a base selection of the α subunit, while dC·dA

mispairs may be eliminated by the proofreading function associated with the ε subunit.

Both of the error-preventing functions associated with pol III seem to be incompetent to avoid the formation of dG·dA mispairs. Using synthetic DNAs specially designed for a misincorporation assay, Sloane *et al.* (15) estimated the accuracy of DNA synthesis *in vitro* catalyzed by the α subunit; misinsertion frequencies of dG, dC, and dA opposite to dA were 5.2×10^{-4}, 7.1×10^{-5}, and 1.3×10^{-5}, respectively. Fersht and Knill-Jones (16) drew a similar conclusion by demonstrating that the relative frequencies of mispairs formed *in vitro* by the pol III holoenzyme were dG·dA > dA·dA = dC·dA = dT·dG > dT·dC. It has been shown that the dG·dA mispairs were not corrected either *in vivo* or *in vitro* by the mismatch repair system of *E. coli* (17).

If the dG·dA mispair is not corrected, it would result in an A·T to C·G transversion after the next round of DNA replication. However, the spontaneous mutation frequency of this type of transversion is extremely low in *E. coli* (18). Since the *mutT* mutant specifically increases the frequency of this type of transversion, it has been postulated that a special error-prevention mechanism in which the *mutT* gene is involved corrects the dG·dA mispair in *E. coli* cells (15). Schaaper and Dunn (19) showed that replication of single-stranded phage DNA in a crude extract from the *mutT* mutant cells proceeded with the specific induction of A·T to C·G transversion, thereby suggesting that the mutant lacks such an error-prevention mechanism.

Here we demonstrated that the MutT protein suppressed *in vitro* misincorporation of dGMP onto the poly(dA) template. Since preformed dG·dA mispairs were not corrected by the MutT protein (data not shown), the suppression could not be ascribed to a postreplicative repair of the mispair. A possible mechanism for the prevention of dG·dA mispairing may be related to an enzyme activity of the MutT protein that degrades dGTP.

Existence of specific deoxynucleoside triphosphatase activity in purified preparations of MutT protein was reported by Bhatnager and Bessman (11). They showed that the MutT protein hydrolyzed deoxynucleoside triphosphates, although the rates of hydrolysis for dATP, dCTP, and dTTP were 2–30% of the rate for dGTP. We found that the MutT protein degrades dGTP but not other deoxynucleoside triphosphates. The discrepancy may be due to the assay conditions; they used a buffer at pH 9.0 for the assay while we let the reaction run at pH 7.5.

With regard to the manner in which the dGTPase activity relates to the function of MutT protein in preventing formation of dG·dA mispair, we propose that this can be achieved if the MutT protein hydrolyzes a specific form of dGTP that can pair with dA on the template. The following observations would support this hypothesis. (*i*) Under conditions in which the MutT protein could completely suppress dGMP misincorporation onto the poly(dA) template, only a small portion of dGTP present in the reaction mixture was degraded. (*ii*) The apparent K_m for the dGTPase activity was extremely high (> 10 mM), thereby suggesting that the proper substrate of the MutT protein might not be the major component, the *anti* form of dGTP. (*iii*) There is a small amount of *syn*-form isomer in dGTP preparations.

According to a model proposed by Topal and Fresco (20), the *syn* form of dGMP can pair with a tautomer of dAMP or dGMP within the Watson–Crick geometry. If the MutT protein preferentially hydrolyzes the *syn*-form dGTP, it would explain prevention of formation of the dG·dA mispair. Since alteration between the *anti* and the *syn* forms seems to be nearly free (20), we assume that such isomerization occurs at the site of DNA replication. Since the occurrence of G·C to C·G transversion, which is also predictable from the Topal–Fresco model (20), is not stimulated in *mutT* mutants, the dG·dG mispairs might be excluded by other mechanisms, such as base selection by the α subunit.

Recently, another type of mutator mutant, *mutY*, was isolated. This mutant specifically produces G·C to T·A transversions (21). Although biochemical activity related to the *mutY* gene product has not been reported, it is conceivable that the MutY protein prevents the formation of dA·dG mispair in a manner opposite the function of the MutT protein. However, there is the possibility that the MutY protein acts in a step of postreplicational mismatch correction. *E. coli* possesses a unique endonuclease activity that recognizes dA·dG mismatch and excises a dG-containing region from the DNA (22).

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