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Genetics

Mutator tRNAs are encoded by the *Escherichia coli* mutator genes *mutA* and *mutC*: A novel pathway for mutagenesis

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ABSTRACT **We have previously described the mutator alleles *mutA* and *mutC*, which map at 95 minutes and 42 minutes, respectively, on the *Escherichia coli* genetic map and which stimulate transversions; the A·T → T·A and G·C → T·A substitutions are the most prominent. In this study we show that both *mutA* and *mutC* result from changes in the anticodon in one of four copies of the same glycine tRNA, at either the *glyV* or the *glyW* locus. This change results in a tRNA that inserts glycine at aspartic acid codons. In view of previous studies of missense suppressor tRNAs, the mistranslation of aspartic acid codons is assumed to occur at ≈1–2%. We postulate that the mutator tRNA effect is exerted by generating a mutator polymerase and suggest that the ε subunit of DNA polymerase, which provides a proofreading function, is the most likely target. The implications of these findings for the contribution of mistranslation to observed spontaneous mutation rates in wild-type strains, as well as other cellular phenomena such as aging, are discussed.**

Mutator genes, which confer an increased mutation rate on cells, have helped to define pathways of mutagenesis and repair in both bacteria and higher cells (1–3). Defects in repair genes often lead to a mutator phenotype. For instance, the *mutH*, *-L*, *-S*, and *uvrD* genes all encode proteins involved in the methylation-directed mismatch repair system in *Escherichia coli*, and mutators resulting from defects in each of these genes helped to define the mechanism of repair of mismatched bases arising during replication (1–4). Defects in the human counterpart to the *mutL* or *mutS* gene lead to both a higher mutation rate, as seen by microsatellite instability, and also an increased susceptibility to nonpolyposis colorectal cancer (3, 5–7). Moreover, mutations in the *E. coli mutY*, *mutM*, or *mutT* genes inactivate components of a system that repairs or prevents the incorporation of 7,8-dihydro-8-oxoguanine in the DNA, leading to a higher mutation rate (8–10). As an additional example, mutations in the *dnaQ* gene (*mutD*) that result in an inactive ε subunit, the editing function of *E. coli* DNA polymerase III, produce a mutator phenotype (11–15).

We previously reported two mutator loci that result in an increase in transversions, particularly the A·T → T·A and the G·C → T·A changes, in an otherwise wild-type strain (16). These loci, *mutA* and *mutC*, map at 95 minutes and 42 minutes, respectively, on the *E. coli* genetic map. Here we report the cloning, sequencing, and characterization of the *mutA* and *mutC* genes. Both *mutA* and *mutC* encode different copies of the identical glycine tRNA, which normally reads the GGU and GGC codons. The mutations that cause the mutator phenotype change the anticodon of the respective copy of the glycine tRNA so that it now reads the aspartic acid codons GAU and GAC. We propose a mechanism for the generation of inherited mutations by this translational misreading and discuss the implications of this finding.

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MATERIALS AND METHODS

Bacterial Strains and Methods. Strain CC105 and its *mutA* and *mutC* derivatives have been described (16, 17). All genetic methods are as in Miller (18).

Cloning the *mutA* Gene. The *mutA* gene was cloned using Kohara phage no. 652 (19). Originally, we cloned a 4.5-kb *Bam*HI fragment next to *miaA* and *mutL*, into the *Bam*HI site of pACYC184. The pRLA 8 plasmid formed in this way complemented *mutA* according to the blue papillation test we devised (20). The *Bam*HI fragment was further shortened to 2 kb [an *Eco*RV (2039 bp) fragment] and finally to a 663-bp *Eco*RV–*Bsu*36I fragment. Both constructs complemented *mutA*. DNA segments obtained by PCR amplification were blunt-end cloned into the *Pvu* II site of pBR329.

Plasmids were isolated by using QIAGEN (Qiagen, Chatsworth, CA) protocols and chemicals. Digestion and ligation steps were done as indicated by manufacturers (New England Biolabs and Pharmacia).

PCR Amplification of *mutA* and *mutC* DNA. The conditions for PCR were as follows: 30 cycles 94°C for 30 s, 50°C for 30 s, 72°C for 1 min. The reaction was initiated by 5-min incubation at 94°C and terminated by 3 min at 72°C. We used 10 pmol of specific primers and 100 ng of genomic DNA, isolated by standard procedures, and 1 unit of *Taq* DNA polymerase employed with reaction buffer supplied by the manufacturer (GIBCO/BRL, Life Technologies). For amplification of the open reading frame in the region near the *mutA* gene we used the following primers based on the sequence from GenBank (accession no. U14003): 5′-GATACGCCGAACGACACACCTGGAA-3′ and 5′-AAAGAAACTTCGCACGGTGAATAGT-3′; for PCR amplification of the *glyV* locus (primers based on the sequence from GenBank accession no. U14003): 5′-ACAGCGCTGGAAAGTCGTAAAGGTG-3′ and 5′-TACTACCGCGAGCATTTTATCAAGC-3′; for PCR amplification of the *glyW* locus (primers based on the sequence from the GenBank accession no. X52789, X03239, and M12299): 5′-CACGACACTGCTTATTGCTTTGATT-3′ and 5′-TGAGCCGGAAGGTGAGGGATTTAGT-3′.

Nucleotide Sequence Analysis. Sequencing was done by using [α-^{32}P]dATP and SequiTerm cycle sequencing kit (Epicentre Technologies, Madison, WI) with reagents and protocols supplied by the manufacturer.

RESULTS

Construction of Double Mutant. The mutators *mutA* and *mutC* were defined by several independently derived mutations after treatment with ethyl methanesulfonate (16; this work). When they were tested for the ability to revert a set of six Lac$^-$ strains to Lac$^+$, each of which requires a specific base substitution, it was clear that transversions but not transitions were stimulated, with the A·T → T·A and the G·C → T·A transversion the most active (16). We constructed the double mutant *mutA mutC* and compared the level of A·T → T·A transversions with the wild-type and each single mutant, as depicted in Table 1. It can be seen that the double mutant has

Table 1. Comparison of *mutA* and *mutC* with *mutA,C*

Strain (CC105)	Lac$^+$ revertants per 10^8 cells, no.
+	2.3 ± 0.65
mutA	26 ± 3.0
mutC	22 ± 3.8
mutA,C	67 ± 13

Mutant frequencies in *mutA*, *mutC*, and *mutA,C* strains. In general, five or more single colonies of each strain were inoculated into LB media and grown overnight. Samples were plated on lactose minimal medium. The average number of Lac$^+$ revertants of strain CC105, resulting from a transversion from A·T → T·A, is shown.

an increased level of transversions relative to either single mutant, but this increase is only between 2.5- and 3-fold.

Cloning the *mutA* and *mutC* Genes. We cloned segments of DNA near each mapped locus (95 min for *mutA* and 42 min for *mutC*) and tested for complementation of a *mutA* or *mutC* strain. For *mutA*, starting with a λ phage, Kohara phage no. 652 (19), carrying a large segment of the chromosome near the *mutL* and *miaA* genes at 95 minutes, we subcloned fragments onto a low copy number pACYC plasmid and looked for complementation. Plasmids conferring a restoration of the wild-type level of mutation to *mutA* could be detected. Fig. 1 summarizes the subcloned fragments.

Initially, the plasmid with the shortest subcloned fragment that complemented *mutA*$^-$ strains contained one open reading frame, which we sequenced. However, after PCR amplification of this DNA segment from two different *mutA* mutations, we failed to detect a single base change, and partial deletion of this gene did not affect complementation. Virtually the only remaining chromosomal DNA on the complementing plasmid was a segment containing the *glyV* locus, which encodes three identical tandem copies of the same glycine tRNA, which reads the GGU/C codon. Surprisingly, a plasmid made by PCR amplification of this set of tRNA genes did complement *mutA* (Fig. 2 and Table 2)! The mutation was also complemented by a fragment cut from the original complementing plasmid that contained only the tRNA genes. (The tandem set of three genes is unstable in such constructs, and usually only one or two copies of the tandem set of three are present on the plasmid.)

The *mutC* gene, which is 98% cotransducible with *uvrC*, must be within 2 kb of *uvrC*. Within this short distance is the fourth copy of the same glycine tRNA gene, the *glyW* locus. We found that the plasmid that carries the *glyV* gene and that complements *mutA*, also complements *mutC* (Table 3). We therefore PCR amplified and cloned the *glyW* locus and tested for complementation. As Table 3 also shows, the plasmid containing the *glyW* region complements *mutC*. Moreover, the plasmid containing the *glyW* locus complements the *mutA mutC* double mutant!

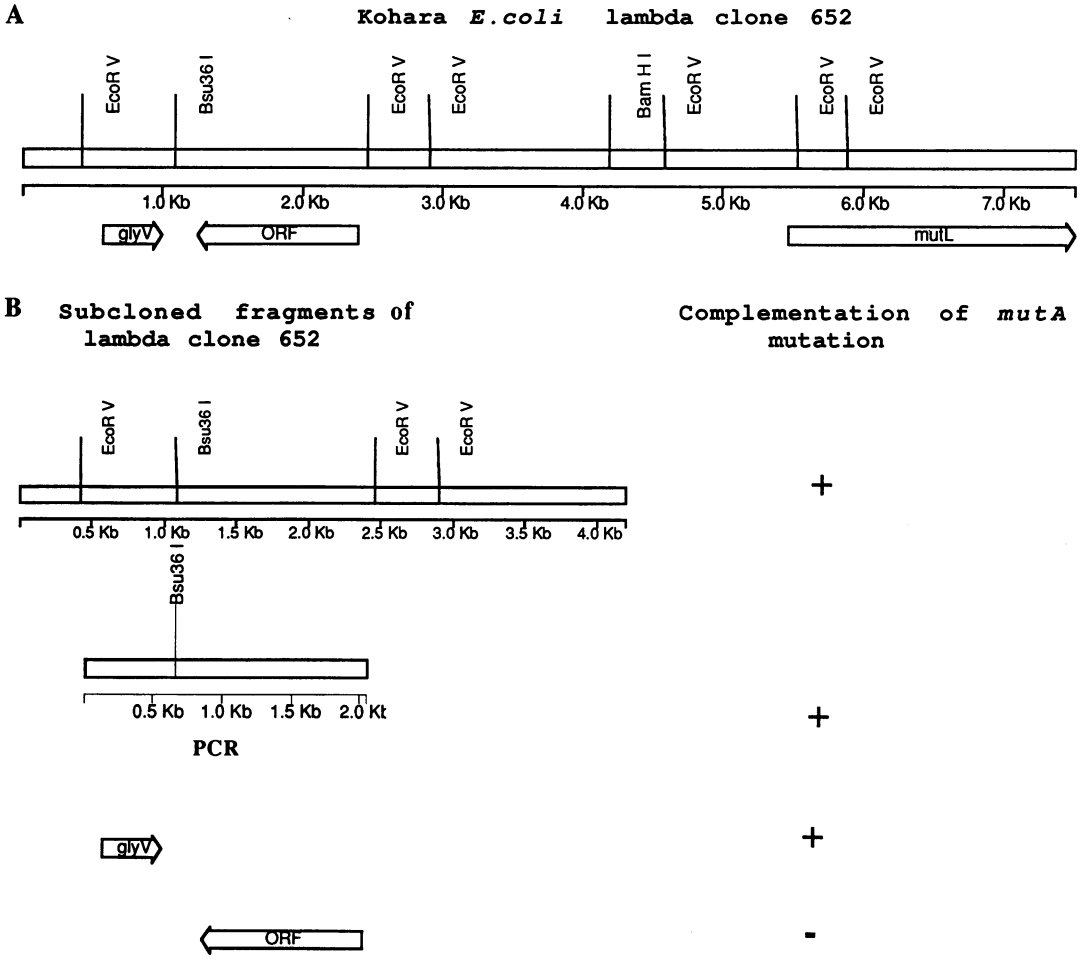

FIG. 1. Cloning of the *mutA* gene. The cloning strategy is summarized above. We have cloned a 4.3-kb *Bam*HI fragment of the Kohara library λ no. 652 (19) which complemented the *mutA* mutation in the blue papillae test (20). The fragment was cut down to a 2-kb *Eco*RV fragment that contained one open reading frame and the *glyV* locus. Both fragments were cloned into a pACYC184 vector. We then PCR-amplified both the open reading frame and the *glyV* gene and blunt-end cloned them into the *Pvu* II site of pBR329. We found that only the *glyV* complements *mutA* mutation in the blue papillae test, as well as in direct Lac$^+$ revertant determination on lactose minimal medium. (*A*) Diagram of Kohara library λ clone no. 652. (*B*) Fragments of λ clone 652 used for subcloning of the *mutA* mutation.

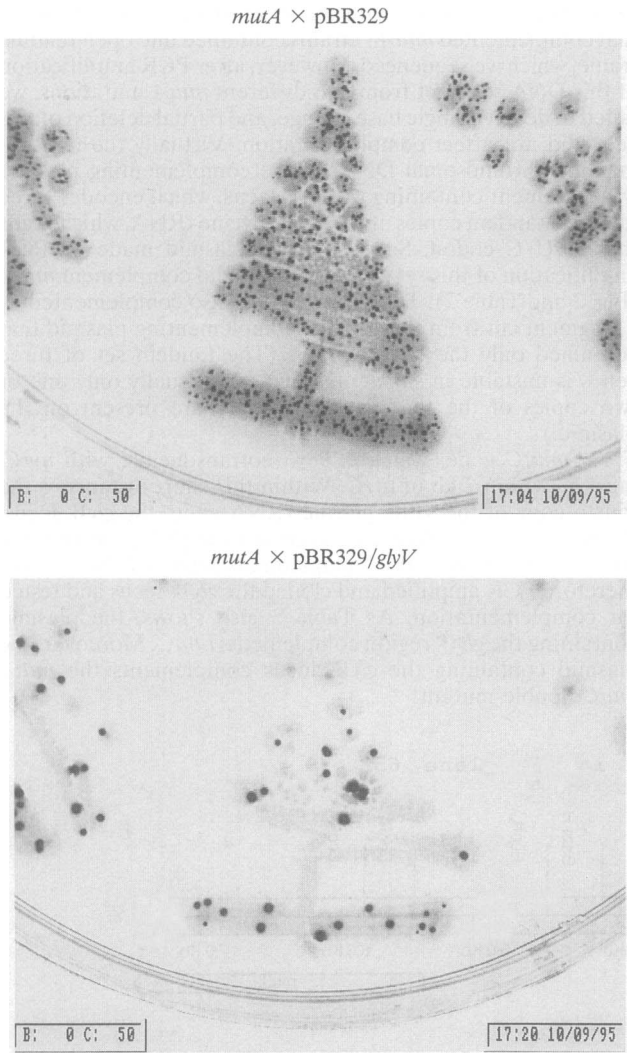

FIG. 2. Complementation of *mutA1* with the *glyV* gene in the blue papillae test. (*Upper*) *mutA1* strain with plasmid pBR329 growing on minimal glucose, P-Gal (phenyl β-D-galactoside), X-Gal (5-bromo-4-chloro-3-indolyl β-D-galactoside) plates. (*Lower*) Same *mutA1* strain with the *glyV* gene cloned into pBR329, growing also on minimal glucose, P-Gal, X-Gal plates. *glyV* on the pBR329 plasmid complements the *mutA1* mutation because single colonies contain only few, if any, blue papillae, whereas colonies of *mutA1* with pBR329 only contain many papillae.

The *mutA* and *mutC* alleles are dominant when introduced on a plasmid into an otherwise wild-type strain. Table 4 shows the negative complementation resulting from a multicopy plasmid carrying the altered *glyW* (*mutC*) allele. The level of mutagenesis is significantly increased, up to the level of the double mutant carrying *mutA mutC*. However, introduction of the same plasmid into the *mutA mutC* double mutant does not cause an additional increase in the mutator effect.

Determination of Sequence Change Responsible for Mutator Phenotype. To determine whether the mutation responsible for the mutator phenotype in *mutA* strains was, in fact, at the *glyV* locus, we relied on direct PCR amplification of the *glyV* region from both *mutA* and wild-type strains for comparative sequencing. The sequence change resulting from each of two different alleles of *mutA* is depicted in Fig. 3. It can be seen that in each case the sequence specifying the anticodon of one of the three copies of the $tRNA^{Gly}$ gene is altered. In one case the first of the three copies is affected, and in the other case the second of the three copies is changed. However, in both cases the mutational change is a G·C → A·T transition that alters the anticodon so that the glycine tRNA now reads the GAU/C (aspartic acid) codon instead of the GGA/U (glycine codon).

We also sequenced the *glyW* gene from wild-type and a *mutC* strain. As Fig. 4 shows, the only change in this fourth copy of the same glycine tRNA is in the anticodon and again results in a tRNA that reads GAU/C instead of GGU/C.

Table 2. Complementation of *mutA* with *glyV*

Strain (CC105)	Plasmid	Lac^+ revertants per 10^8 cells, no.
+	pBR	2.5 ± 0.7
mutA	pBR	9.4 ± 2.9
mutA	*glyV*pBR	2.4 ± 2.2

Complementation of *mutA* with *glyV*.

Table 3. Complementation of *mutC* and *mutA,C* with *glyV* and *glyW*

Strain (CC105)	Plasmid	Lac^+ revertants per 10^8 cells, no.
+	pBR	2.5 ± 0.7
mutC	pBR	17 ± 5.0
mutC	*glyV*pBR	3.7 ± 1.2
mutC	*glyW*pBR	1.4 ± 1.3
mutA,C		67 ± 13
mutA,C	pBR	65 ± 34
mutA,C	*glyW*pBR	3.5 ± 1.5

Complementation of *mutC* and *mutA,C* with *glyV* and *glyW*.

DISCUSSION

We have previously reported the detection of two new mutator loci, *mutA* and *mutC*, which map at ≈95 minutes and 42 minutes, respectively, on the *E. coli* genetic map (16). Strains defective in either locus have an increased level of transversions, with the A·T → T·A and the G·C → T·A transversions the most frequent, followed by the A·T → C·G transversion. Although transitions are not increased in otherwise wild-type backgrounds, in combinations with either a *mutH* or *mutL* mutation, which inactivates the mismatch repair system, the A·T → G·C transition is increased 12-fold over the level of the *mutH* or *mutL* strain at the one site tested. This latter result suggests that the defect in *mutA* or *mutC* strains involves replication errors. It should be noted that the detailed specificity of mutations that occur in a *mutA* or a *mutC* background is strikingly similar, suggesting that they affect the same pathway. The *mutA mutC* double mutant has a 2.5- to 3-fold higher rate of transversion than either single mutant (Table 1).

Our efforts to clone the *mutA* gene, and also the *mutC* gene, had been frustrated by our inability, despite exhaustive attempts, to isolate mutants with transposon inserts in these genes. Mutations at either locus are infrequent as it is, and so far have been detected only with ethylmethane sulfonate, which causes G·C → A·T transitions. ICR-191, a potent frameshift mutagen, does not generate either *mutA* or *mutC* strains. Because detailed mapping pinpointed each gene to a point 93% and 98% cotransducible with known genes, respec-

Table 4. Negative complementation with *glyW* from *mutC*

Strain (CC105)	Plasmid	Lac^+ revertants per 10^8 cells, no.
+	pBR	2.5 ± 0.7
mutA,C		67 ± 13
mutA,C	pBR	65 ± 34
+	*glyW*(*mutC*)pBR	55 ± 19
mutA,C	*glyW*(*mutC*)pBR	78 ± 35

Negative complementation with *glyW* from *mutC*.

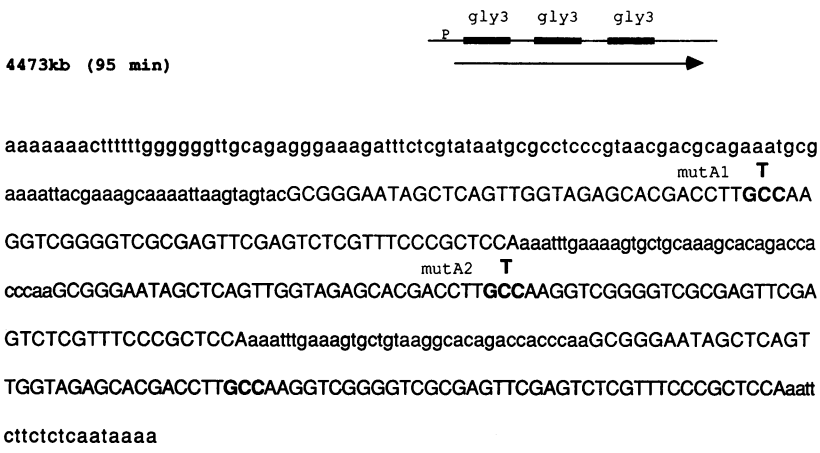

FIG. 3. The *glyV* locus and changes found in two of the *mutA* mutations. The diagram of the sequence of the *glyV* locus is adapted from ref. 21. The bases of the anticodon are boldfaced. The changes of C → T in the anticodon of the first glycine tRNA (gly3) found in *mutA1* and of the second glycine tRNA (gly3) found in *mutA2* are indicated.

tively, we decided to clone and sequence a region of the chromosome near each locus, which resulted in complementation of the respective mutation. Surprisingly, both *mutA* and *mutC* turned out to result from mutations affecting the anticodon of a different copy of the same tRNA gene. In each case, the mutation converts a glycine tRNA that normally reads the GGU/C codon to one than reads the GAU/C (aspartic acid) codon. The *mutA* mutations are at the *glyV* locus, which consists of a tandem set of three identical tRNA genes, whereas the *mutC* mutations are at the *glyW* locus, which encodes a single, fourth copy of the same glycine tRNA. The *mutA* and *mutC* alleles described here result in "mutator tRNAs" and represent a novel mechanism of spontaneous mutagenesis.

What are the molecular consequences of the *mutA* and *mutC* mutations? Mutations in the anticodon of both *glyV* and *glyW* have been reported that lead to missense suppressors that insert glycine in response to either the GAU/C, AGU/C, or UGU/C, among others (22), although no mutator effect was noted. These missense suppressors usually operate at ≈1%–2% efficiency and have been detected by restoration of Trp^- cells with specific mutations in *trpA* to the Trp^+ phenotype. The direct effects of the *mutA* or the *mutC* mutation is to allow the misincorporation of glycine at aspartic acid codons, at ≈1%–2% of the efficiency of normal translation. Recall that with four copies of the same tRNA gene in the cell, when one is mutated, the other three are still operating normally on GGU/C glycine codons. The lowered level of mistranslation by the altered tRNA is a reflection of poor charging by the glycine tRNA synthetase in response to the mutated anticodon. Thus, the pool of glycine-charged tRNAs with the altered anticodon is reduced. In the double mutant *mutA mutC*, which grows noticeably more slowly on rich medium than either single mutant, two of the four genes synthesize tRNAs with an altered anticodon, which increases the pool of the glycyl-tRNAs that recognize the GAU/C aspartic acid codon. A higher mutation rate is observed for the double mutant than for either single mutant. Complementation, which restores the wild-type phenotype occurs when an additional wild-type glycine tRNA gene is introduced on a multicopy plasmid, significantly lowering the proportion of the altered species of the mutant tRNA. Presumably, the increased number of wild-type tRNA molecules compete with the mutationally altered tRNAs for the cognate charging enzyme, reducing the pool of glycine-charged tRNAs that recognize the GAU/C codon. Thus, when introduced on a multicopy plasmid, the wild-type *glyV* can complement either *mutA* or *mutC* (Tables 2 and 3), and *glyW* can also complement *mutA* and *mutC* (Table 3, data not shown), because *glyV* and *glyW* encode the same tRNA molecule. Also, the wild-type *glyW* on a plasmid can complement the *mutA mutC* double mutant (Table 3).

How can an altered tRNA that mistranslates 1%–2% of the aspartic codons by inserting glycine result in a mutator phe-

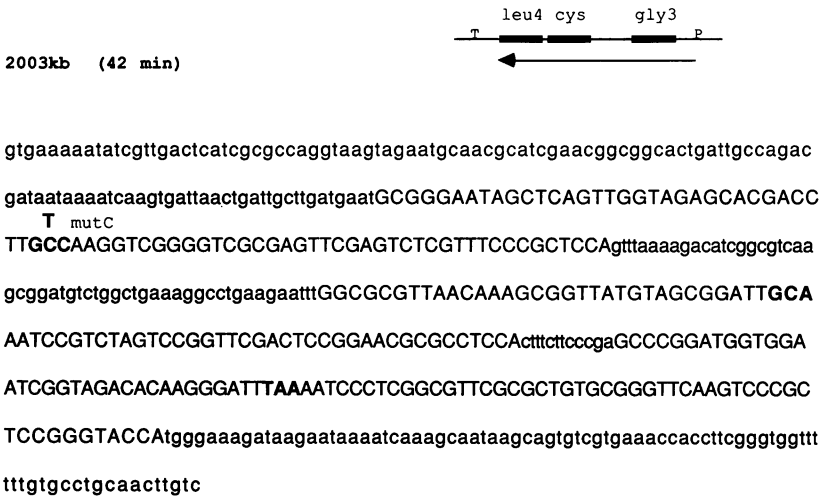

FIG. 4. The *glyW* locus and the change found in a *mutC* mutation. The diagram of the sequence of the *glyW* locus is adopted from ref. 21. The bases of the anticodon are boldfaced. The change of C→T in the anticodon of glycine tRNA (gly3) found in *mutC* is indicated.

notype? The indication is that one or more specific altered proteins produced at only a few percent of the wild-type counterpart(s) somehow generate an increased level of mutagenesis. One possibility is an error-prone polymerase. An excellent candidate is the product of the *dnaQ* gene, the polymerase III editing function (the ε subunit), as depicted in Fig. 5. Strong mutator strains, *mutD*, result from defects in the editing function (11–15, 23). The *mutD* mutations are dominant to wild-type (12), since it appears that one epsilon subunit remains bound to a polymerase molecule for an entire replication cycle (24). Thus, even a small percentage of a mutant ε subunit would give a mutator phenotype, although at a lower level. The mutational specificity of *mutA* and *mutC* is not unlike that seen for *mutD* under certain conditions (25). Often, strong *mutD* mutations saturate the mismatch repair system, resulting in observed transitions (23). When the mismatch repair system is not saturated, as expected with *mutA* and *mutC*, the transitions are eliminated by this system, yielding the observed spectrum. The increase in the A·T → G·C transition observed in a *mutA mutL* or a *mutC mutH* background can be explained as the transitions generated by replication errors not corrected by the altered ε subunit that are also not repaired by the mismatch repair system. We note with interest that two of the sequenced dominant *mutD* mutations represent the loss of an aspartic acid, in one case aspartate is replaced by alanine (R. M. Schaaper, personal communication), and in a second case a different aspartate is replaced by asparagine (D. L. Horner and E. C. Cox, personal communication).

The finding that a missense suppressor tRNA confers a mutator phenotype on the cell is unexpected and provocative. The proposed mechanism of generating a mutator polymerase that then causes increased mutations has implications for aging and degenerative diseases, since it has been argued that a buildup of damage to DNA, proteins, and other molecules in the cell accumulates with age, leading to increased errors in protein synthesis, which coupled with an increased somatic mutation rate might play a role in aging, a so-called "error catastrophe" (26–30), although this idea has been challenged [refs. 31–34; C. G. Kurland, 1986 lecture to Kungl. Vetenskapssocieteten, Uppsala (April 4)]. In the work reported here we describe an example of how mistranslation by a tRNA may lead to a higher mutation rate, which then leads to more errors. In this case the translational infidelity is caused by a mutation in the tRNA anticodon, but loss of fidelity can also occur by accumulated damage to ribosomes and other components of the translational apparatus.

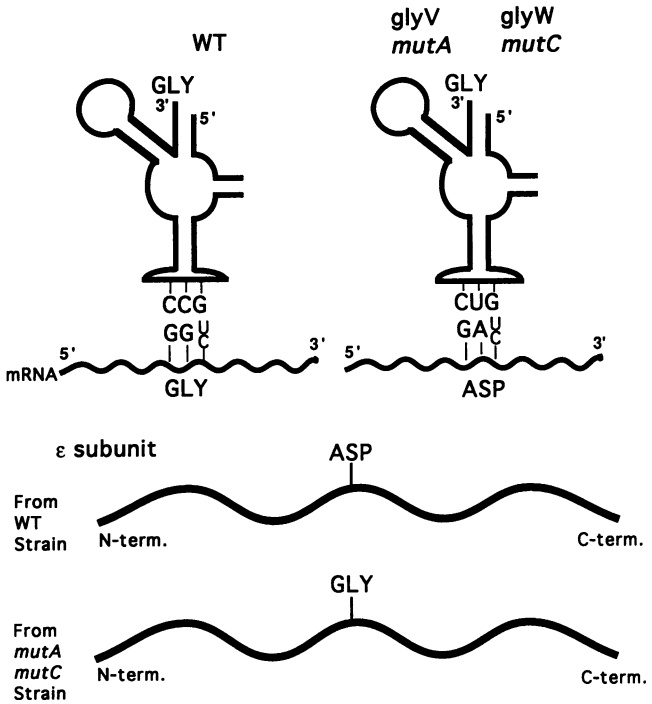

FIG. 5. Model for the mutator effect of *mutA* and *mutC*. Both *mutA* and *mutC* result in the insertion of glycine at aspartic acid codons, ≈1% of the time. It is proposed that the substitution of glycine for aspartate generates a mutator polymerase in 1% of the population due to an altered ε subunit.

Note that the *mutA* and *mutC* mutations are distinct from the *miaA* locus that is involved with tRNA modification. Loss of the *miaA* gene product results in loss of the ms^2i^6A[2-methylthio-N6-(Δ2-isopentenyl)-adenosine] tRNA modification adjacent to the anticodon of several tRNAs, results in *less* translation errors than normal, and gives a weak mutator effect that is specific for G·C → T·A transversions (35).

The finding that mutations are increased when mistranslation occurs in *mutA* or *mutC* strains allows us to ask what role, if any, translation (and transcription) errors play in generating some of the spontaneous mutations observed in otherwise wild-type strains. This question has been addressed previously; Ninio (36) argued that if the average error of mistranslation per amino acid is 10^{-4} (a commonly accepted average; ref. 37), then the number of mutant ε subunits might be sufficient to account for 10% of the spontaneous single mutations in bacteria, but most of the double mutations (36). Assuming that the mutator tRNAs described here are really operating by generating defective DNA polymerase ε subunits, then we can estimate the level of spontaneous mutation in a wild-type strain that might result from the rare mistranslated ε proteins, based on our empirical findings. Let us assume that the *mutA* and *mutC* tRNAs are mistranslating aspartic acid codons at 1%. We note that they increase the frequency of transversions ≈10-fold (16). Because almost 10 sites in the *dnaQ* gene have already been sequenced, which result in dominant *mutD* mutators, there are probably at least 20 such sites. The two above-mentioned aspartic acid codons would then represent 10% of such sites. A general level of mistranslation of 10^{-4} would be 100-fold less frequent per site than *mutA* but operate on 10-fold more sites, to give an effect ≈10% of that seen for *mutA* or *mutC*. However, a level of transversions of 10% that seen for *mutA* or *mutC* equals the spontaneous transversions seen in a wild-type strain. Thus, it is not inconceivable that a sizable fraction of the spontaneous transversions observed in a wild-type strain are due to the transient effect of altered ε subunits resulting from mistranslation. Such effects have been termed "transient mutators" since the mutator effect in each case lasts for one round of replication (36).

We might ask whether specifically altered tRNAs can play a role in mutagenesis in human cells and whether any of the spontaneous mutations that lead to cancer might arise from transient mutator effects due to translation (and/or transcription) errors.

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