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*THE UNUSUAL MUTAGENIC SPECIFICITY OF AN E. COLI MUTATOR GENE**

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Genes which lead to an appreciable increase in the rate of mutation of other genes have been recognized in several organisms.[1–6] On the basis of our current understanding of mutagenesis, the following mechanisms of mutator gene action could be envisaged: a polymerase alteration which results in errors during the replication of normal nucleotide sequences; production of mutagenic base analogues which lead to replication or incorporation errors; modification of one or more of the different bases in DNA, thereby leading to replication errors. Whether all of these mechanisms are operative *in vivo* is not known. Recently, however, Speyer has shown[7] that an alteration in the gene controlling T4 DNA polymerase results in a mutator effect on other genes.

The present investigation was initiated as the first stage in an attempt to determine the mechanism of mutagenesis of the *Escherichia coli* mutator gene described by Treffers *et al.*[3] The experiments that are reported were designed to examine the mutagenic specificity of this mutator gene. As will be seen, the mutator gene is highly specific, unidirectional in its action, and it causes an unexpected base-pair change.

The system selected for study of mutator-gene specificity is the tryptophan synthetase A gene-A protein system of *E. coli*. Extensive mutational studies with specific A protein mutants and a variety of mutagenic agents have led to a reasonably complete survey of the amino acids that can replace one another as a result of single mutational events.[8–12] Since multiple amino acid changes are observed at several positions in the A protein, the mutator gene can be tested to determine whether it favors any one of the alternative amino acid changes. With this information, and knowledge of the RNA codons that probably correspond to the relevant amino acids, it is possible to designate the base-pair change that is favored by the mutagenic agent. This approach was employed in an examination of the mutagenic specificity of the mutator gene.

Materials and Methods.—The tryptophan synthetase A protein mutants studied were strains A23, A46, A58, A78, and A223. Each of these mutants produces an inactive A protein which has a single amino acid difference from the wild-type A protein. A *tryp* deletion mutant (lacking the tryptophan synthetase A gene) was isolated in the mutator stock and this deletion was replaced by each altered A gene by transduction with phage Plkc. Reversion experiments were performed with each stock carrying a mutant A gene in the mutator background. To ensure that

many independent reverse mutational events were being examined, several independent cultures were sampled in each experiment, and a small revertant-free inoculum was employed. In addition, a short growth period was used so that differential growth rates and differential survival of revertant types would not be complicating factors. If both fast- and slow-growing revertant colonies were observed, representatives of both types were picked for further testing. After purification by streaking, the revertants were characterized by previously described procedures.[13] Revertants selected for protein primary structure studies were transduced out of the mutator background into a T3 *tryp* $ABC^{deletion}$ stock and T3-"A reversion" stocks were selected. Each T3-"A reversion" stock carried the reverted A gene from the corresponding mutator stock. The T3 marker was introduced into these stocks to permit derepression of the *tryp* enzymes and the formation of high levels of A protein. Mutant T3 lacks anthranilate synthetase.

Characterization of revertants: Revertants were classified[13] as full revertants (FR) or partial revertants (PR). Full revertants are strains which have approximately the same growth rate as the parental wild-type strain on minimal medium, do not accumulate indoleglycerol, and are as sensitive to DL-5-methyl tryptophan inhibition as the wild-type strain. Partial revertants form a less-active A protein and are distinguishable from the wild type and FR's on the basis of the characteristics mentioned above. In some cases genetic tests were performed to distinguish between "true" reversions and suppressor mutations.

Examination of the A proteins of revertants: The A protein of each revertant examined was purified as described elsewhere.[14] The purified proteins were digested with the appropriate proteolytic enzyme(s) to release the peptide presumed to have an amino acid change. Tryptic digestion was used with the A proteins of revertants of mutants A58 and A78, and peptide TP6 was isolated by chromatography on Dowex 1-X2 columns as previously described.[10] Chymotryptic digests were employed for the isolation of peptide CP2 from the A proteins of revertants of mutants A23 and A46. The peptide was separated and eluted from two-dimensional peptide patterns as described elsewhere.[11] Digestion with trypsin and chymotrypsin was employed for the release of peptide TP4 of the A proteins of revertants of mutant A223.[12] This peptide was also isolated from peptide patterns. All eluted peptides were hydrolyzed in 5.7 *N* HCl for 48 hr, and the amino acid composition was determined with a Beckman/Spinco amino acid analyzer.

Results.—Mutator-induced reversion of mutant A223: The results of two typical reversion experiments with the A223 A gene in a mutator background are summarized in Table 1. It can be seen that the reversion frequency is very high;

TABLE 1

MUTATOR-INDUCED REVERSION OF MUTANT A223

	Tube	Approximate number of viable cells per plate	Number of tryp+ colonies per plate	Colonies picked and examined	Characteristics	Amino acid change in peptide TP4
Expt. 1	1	1040	1	1	All FR	(1)* Ileu → Ser
	2	1520	3	3	"	
	3	10^3	0	0	—	
	4	"	1	1	All FR	
	5	"	8	8	"	(1) Ileu → Ser
	6	"	2	2	"	
	7	"	5	5	"	(1) Ileu → Ser
	8	"	0	0	"	
	9	"	0	0	"	
	10	"	2	2	"	(1) Ileu → Ser
Expt. 2	1	2000	2,2	4	All FR	
	2	2790	2,2	4	"	
	3	3510	3,1	4	"	
	4	1970	2,1	3	"	
	5	1950	3,4	4	"	
	6	2360	3,2	3	"	

In all tables, M* = presence of the active mutator gene.

Five ml L-broth tubes were inoculated with 50–100 cells each of strain A223 M* and grown at 37° with shaking for 4–5 hr. The cells were centrifuged, washed with saline, and resuspended in saline. Aliquots were plated on minimal and minimal + indole agar plates. The inoculum for each experiment contained < 1 tryp+ cell/10^3 viable cells.

* The number in parentheses refers to the number of revertant proteins examined for amino acid changes.

approximately one revertant colony was observed per 10^3 cells plated. The spontaneous reversion frequency of mutant A223 (in a nonmutator background) is about $1/10^8$ cells plated. In view of the high frequencies observed and the fact that microcolonies probably could develop from each plated cell in the absence of a tryptophan supplement, many of the reversion events probably occurred after plating. For this reason it is felt that the values reported represent maximum values and are not reliable for estimating mutation rates. It can also be seen in Table 1 that only FR colonies were recovered in mutator experiments although mutant A223 is known to give PR types spontaneously as well.[12] Four revertants selected at random were all found to form functional A proteins with Ser at the position in peptide TP4 occupied by Ileu in mutant A223. The Ileu → Thr change was not observed, although it is a common spontaneous change and, in fact, is the only change favored by 2-aminopurine mutagenesis.[12]

Mutator-induced reversion of mutant A78: The results of a typical reversion experiment with mutant A78 in the mutator background are shown in Table 2.

TABLE 2

MUTATOR-INDUCED REVERSION OF MUTANT A78

Tube	Approximate number of viable cells per plate	Number of tryp$^+$ colonies per plate	Colonies picked and examined	Characteristics	Amino acid change in peptide TP6
1	1060	3	2	2FR	
2	1260	4	2	2FR	(1) Cys → Gly
3	10^3	2	2	1 FR, 1 su*	
4	"	1	1	su	
5	"	4	2	1 FR, 1 su	
6	"	8	2	2FR	(1) Cys → Gly
7	"	4	2	1 FR, 1 su	
8	"	11	2	1 FR, 1 su	

Eight tubes of L-broth, each containing 5 ml, were inoculated with approximately 50 cells of A78 M* each. The tubes were shaken at 37° for 4.5 hr and the cells collected by centrifugation. The cells were suspended in saline and aliquots were plated on minimal and minimal + indole agar plates. The inoculum contained approximately 1 tryp$^+$ cell/10^4 viable cells.

* su = suppressed mutant.

Both FR and su types are recovered, and at high frequency. The same types are observed in spontaneous mutation experiments but the frequency is no greater than 0.5 per 10^8 plated cells when the A78 gene is in a mutator-free background. When the mutator gene is present, the frequency of both types of changes is obviously increased considerably. Two of the FR strains were selected for protein primary structure studies. The Cys introduced in peptide TP6 by the A78 mutation was replaced by Gly, the amino acid occupying this position in the wild-type protein. Neither the Cys → Gly change, nor mutation to the FR type, was observed in mutagenesis experiments with 2-aminopurine.[12]

Mutator-induced reversion of mutant A58: The results of two reversion experiments with a mutator stock containing the A58 A gene are summarized in Table 3. FR types were not observed although, in the two experiments reported, over 1000 similar tryptophan-independent colonies were recovered. Primary structure studies were performed with the A proteins isolated from two revertants selected at random, and the analyses showed that the Asp residue introduced in peptide TP6 as a result of the A58 mutation was replaced by Ala. The change Asp → Ala occurs spontaneously, but only the Asp → Gly change is favored by 2-aminopurine treatment.[12] Since Gly is the amino acid at the relevant position in TP6 of the

TABLE 3

MUTATOR-INDUCED REVERSION OF MUTANT A58

Expt.	Number of tubes	Approximate number of viable cells plated from each tube	Number of tryp$^+$ colonies per plate	Characteristics	Amino acid change in peptide TP6
1*	10	10^4	30–90	All PR or su‡	—
2†	9	Not counted	Not counted	All PR or su‡	(2) Asp → Ala

* Cells of A58 M* (17 viable cells/tube) were inoculated into 5 ml L-broth tubes. The cultures were incubated with shaking at 37° for 5 hr. The cells were collected by centrifugation, suspended in saline, and aliquots plated on minimal and minimal + indole agar plates. The inoculum contained approximately 1 tryp$^+$ cell for every 10^3 tryp$^-$ cells.

† Procedure essentially the same as in experiment 1.

‡ All of the colonies, without exception, were slower growing than wild-type colonies on minimal agar. Twenty were tested for the accumulation of indoleglycerol and all accumulated this compound, indicating that they were either PR or su strains. Genetic tests were not performed to determine the relative proportion of PR and su types, but the two colonies selected for primary structure studies both proved to be PR types. Four prototrophs selected from other experiments also arose by reversion rather than suppression.

wild-type protein, the absence of FR types in the mutator reversion experiments with mutant A58 is particularly important and strongly suggests that the change Asp → Gly cannot be brought about by the action of the mutator gene.

Mutator-induced reversion of mutants A23 and A46: The A23 and A46 mutations are in the same codon since the same Gly residue in peptide CP2 is replaced by Arg (A23) or Glu (A46). Both mutants spontaneously give FR and PR types at low frequency, approximately $1/10^8$. Experiments with these mutant alleles in the mutator background are summarized in Table 4. It can be seen that the reversion frequency of both mutant alleles is increased considerably and only FR types are obtained. Six mutator-induced revertants of A23 and 8 of A46 were examined in primary structure studies. Only the changes Arg → Ser and Glu → Ala were observed (Table 4). These changes were at the same position in peptide CP2 that was affected by the A23 and A46 mutations. A23 also reverts spontaneously to a second FR type in which Arg is replaced by Gly, but this change was not observed in the mutator experiments. The change Arg → Gly but not Arg → Ser is favored by 2-aminopurine treatment.[8, 9] Mutant A46 also spontaneously reverts to a second FR type, in which the amino acid change is Glu → Gly. This change was not observed in the mutator experiments, although this change is favored by 2-aminopurine treatment.[8, 9]

Examination of mutator-induced reversion of mutator-induced auxotrophs: The stability of mutants appearing in a mutator background was examined by determining the reversion frequencies of a group of mutator-induced auxotrophs isolated by penicillin selection. The frequency of appearance of mutants in mutator cultures was extremely high compared with nonmutator cultures, ensuring that

TABLE 4

MUTATOR-INDUCED REVERSION OF MUTANTS A23 AND A46

Expt.	Number of tubes	Approximate number of viable cells plated from each tube	Number of tryp$^+$ colonies per plate	Colonies picked and examined from each plate	Characteristics	Amino acid change in peptide CP2
1 (A23 M*)	10	2.5×10^4	10–100	4	All 40 FR	(4) Arg → Ser
2 (A23 M*)	10	10^4	10–50	5	All 50 FR	(2) Arg → Ser
1 (A46 M*)	10	4×10^4	10–200	4	All 40 FR	(5) Glu → Ala
2 (A46 M*)	10	10^4	10–70	5	All 50 FR	(3) Glu → Ala

Cells (50–100) of each parent strain (A23 M* and A46 M*) were inoculated into tubes containing 5 ml of L-broth, and the cultures were grown with shaking at 37° for 4–6 hr. The cells were collected by centrifugation, resuspended in saline, and aliquots were plated on minimal and minimal + indole agar plates. The inocula contained less than 1 tryp$^+$ cell/10^3 viable cells.

TABLE 5

REVERSION FREQUENCIES OF MUTATOR-INDUCED MUTANTS

Mutant	Reversion frequency*	Mutant	Reversion frequency*	Mutant	Reversion frequency*
1	1×10^{-5}	9	7×10^{-5}	17	1×10^{-8}
2	2×10^{-8}	10	1×10^{-5}	18	2×10^{-7}
3	3×10^{-8}	11	5×10^{-5}	19†	$<2 \times 10^{-9}$
4†	$<2 \times 10^{-9}$	12†	$<2 \times 10^{-9}$	20	1×10^{-7}
5	1×10^{-7}	13	2×10^{-6}	21†	$<2 \times 10^{-9}$
6	3×10^{-7}	14	4×10^{-8}	22	8×10^{-8}
7	2×10^{-7}	15	9×10^{-9}	23	$<1 \times 10^{-8}$
8	8×10^{-5}	16	2×10^{-7}		

Mutants are grouped according to their nutritional requirements. Mutant 1 responds to a mixture of purines and pyrimidines; mutants 2–5 to a B vitamin mixture; mutants 6 and 7 to a mixture of phenylalanine, tyrosine, and tryptophan; mutants 8–11 to a mixture of arginine, methionine, and proline; mutants 12–16 to isoleucine plus valine; mutant 17 to a mixture of cysteine, lysine, and histidine; mutants 18–22 to acid-hydrolyzed casein plus tryptophan; and mutant 23 to tryptophan. The specific nutritional requirements were not determined.

* Ratio of the number of colonies found on minimal plates to the total number of cells plated as determined by plate counts on nutrient agar.

† Not determined whether a single or double mutant.

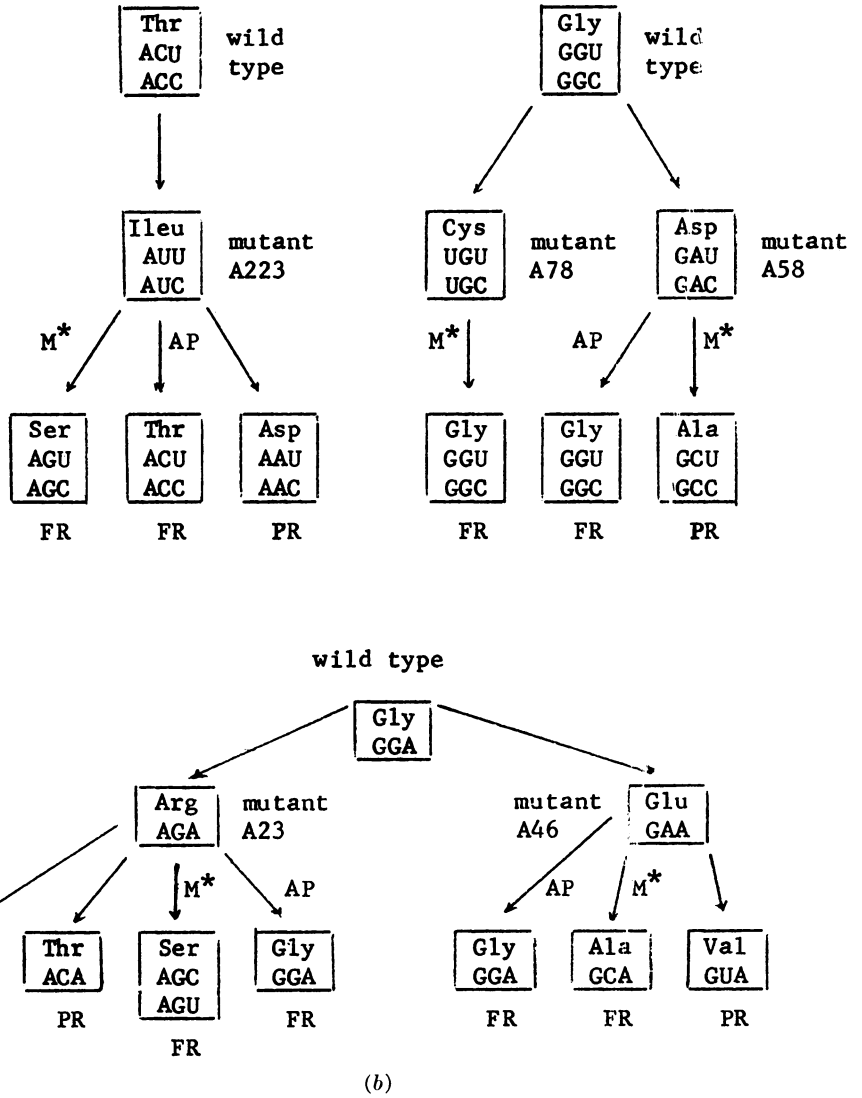
the mutants examined were mutator-induced. Five cultures, started from different single colonies, were treated with penicillin. Aliquots of each culture were plated on a complete medium, and mutants were detected and classified by replication to differently supplemented media. Twenty-three mutants were selected, representing different nutritional classes from each culture. Throughout the isolation and classification procedure, care was exercised not to discard mutants which appeared

	2nd nucleotide				
1st	U	C	A	G	3rd
U	Phe	Ser	Tyr	Cys	U
	Phe	Ser	Tyr	Cys	C
		Ser			A
	Leu	Ser		Try	G
C		Pro	His	Arg	U
	Leu	Pro	His	Arg	C
		Pro	Gln	Arg	A
	Leu	Pro	Gln	Arg	G
A	Ileu	Thr	Asn	Ser	U
	Ileu	Thr	Asn	Ser	C
		Thr	Lys	Arg	A
	Met	Thr	Lys		G
G	Val	Ala	Asp	Gly	U
	Val	Ala	Asp	Gly	C
	Val	Ala	Glu	Gly	A
	Val	Ala	Glu	Gly	G

(*a*)

FIG. 1.—(*a*) Ordered RNA codons described by Nirenberg *et al.*[15] and Khorana *et al.*[16] This method of presentation was suggested by Dr. F. H. C. Crick.

to be unstable. Each mutant was grown overnight in L-broth. Suitable aliquots were then spread on minimal and on completely supplemented plates, and the frequency of reversion to prototrophy scored. The results obtained are presented in Table 5. It is evident that of the 23 isolates studied, only 4 revert with a frequency that can be considered to reflect a pronounced effect of the mutator gene. We have also examined the stability of the mutator gene itself, on a more limited scale, by testing colonies derived from single streptomycin-sensitive cells for the presence of appreciable numbers of resistant cells. Of approximately 4,600 colonies examined by this technique none had lost the mutator gene.



(*b*)

FIG. 1.—(*b*) Primary-site amino acid changes observed in mutation experiments[8-12] and the probable corresponding RNA codon changes. *FR*, full revertants; *PR*, partial revertants; *AP*, only this change of those possible was found following 2-aminopurine mutagenesis;[8, 9, 12] *M**, only this change was observed in stocks with the mutator gene.

Discussion.—The *E. coli* mutator gene studied appreciably increased the reversion frequencies of all five of the tryptophan synthetase A protein mutants examined. In each case, mutation to a particular revertant type was favored, although it was known from spontaneous mutation experiments that each mutant gives rise to several distinguishable revertant types. Of particular importance is the fact that the reversion events favored by the mutator gene were not the same ones observed following 2-aminopurine mutagenesis. The amino acid replacements resulting from mutator action, 2-aminopurine treatment, and spontaneous reversion are summarized in Figure 1*b*.

The RNA codons for all 20 amino acids, based on the studies of Nirenberg *et al.*[15] and Khorana *et al.*,[16] are presented in Figure 1*a*. RNA codons given in Figure 1*b* were selected on the basis of the assumption that each amino acid replacement results from a single base-pair change. The RNA codon for the Ileu of mutant A223 is taken as AUU or AUC, since single mutational events lead to the replacement of Ileu by Thr, Ser, or Asp. The fact that Asp is found in revertant proteins rather than the expected Asn cannot be explained, although it is conceivable that the particular Asn was deamidated during purification of the appropriate peptide. The Cys and Asp codon assignments (A78 and A58) are the only ones possible. Since the A23 Arg is replaced by Thr, Gly, Ser, or Ileu, the A23 Arg RNA codon must be AGA,[17] and AUA is probably the codon for the Ileu derived from the A23 Arg. As yet, *in vitro* studies have not led to the assignment of an amino acid to the AUA triplet. Since the A23 Arg is related to the A46 Glu by mutation from the same Gly, the corresponding Glu and Gly codons must be GAA and GGA. The other assignments follow from these. It is clear from the assignments presented in Figure 1*b* that the Treffers mutator gene caused the same base-pair change in the DNA of all five mutants—a change of AT → CG.[18] All other possible base-pair changes, but one (Table 6), are represented by the amino acid replacements which occur spontaneously at these positions; nevertheless, the AT → CG transversion appears to be the only change favored by the mutator gene. The reverse change, CG → AT, if it were caused by the mutator, should have given Arg → Ileu changes at the A23 position, but these were not observed. Thus, the mutator gene appears to be unidirectional in preference, a conclusion also supported by the finding that mutator-induced auxotrophs are generally not reverted by the mutator gene. The few cases in which the reversion frequencies of mutator-induced auxotrophs were high (Table 5) could be due to mutator-induced suppressor mutations or second-site reversions. This marked mutational specificity of the mutator gene was noted in earlier studies by Bacon and Treffers.[19]

We can postulate on the basis of these results that mutator genes with unidirectional preferences could have been responsible for the very different AT/GC ratios that are now evident in the DNA's of different organisms. The genetic code would appear to permit nucleotide changes in the third position of many codons without concomitant amino acid changes. Consequently, AT/GC ratios could vary considerably without any change in the amino acid content of the proteins of an organism. Furthermore, since amino acids with similar properties appear to be equally acceptable at many positions in many different proteins, changes in the amino acid content of specific proteins could also be tolerated. Thus, the result of the presence of a mutator gene analogous to the one studied here would be an

TABLE 6

BASE-PAIR CHANGES AND AMINO ACID REPLACEMENTS THAT WERE NOT FAVORED BY THE MUTATOR GENE

Base-pair change in DNA	Amino acid changes expected and not observed[8-12]
A → G / T → C	Arg → Gly (A23); Asp → Gly (A58) Glu → Gly (A46); Ileu → Thr (A223)
A → T / T → A	Ileu → Asp (A223); Gly → Val (A46)
C → G / G → C	Arg → Thr (A23)
C → A / G → T	Arg → Ileu (A23)
C → T / G → A	None expected

altered GC content in the DNA of the organism, accompanied by changes in the amino acid content of its proteins. Studies by Sueoka[20] have, in fact, shown that the amino acid content of the total protein of microorganisms does vary, in a definite manner, as the AT/GC ratio varies. On the basis of these considerations and the unidirectional mutagenic preference of the Treffers mutator gene, it might be expected that the AT/GC ratio of the DNA of the mutator stock would be abnormal and that the amino acid content of the tryptophan synthetase A protein of the mutator stock might be altered. These possibilities are presently under investigation.

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