*ALTERED BASE RATIOS IN THE DNA OF AN ESCHERICHIA COLI MUTATOR STRAIN**

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A strain of *E. coli* isolated by Treffers *et al.*[1] harbors a mutator gene (*mut T*) that increases the mutation frequency of a number of bacterial markers from *ca.* 10^{-8} to *ca.* 10^{-5}. The preferred *mut T*-induced mutational event has been identified as a transversion from an AT to a CG pair, the transversion event occurring with strong unidirectional preference.[2] The observation that the *mut T* gene has a unidirectional preference led us to predict that a strain carrying this gene would have an altered GC content related in some way to the number of generations that a strain carrying the gene had undergone.[2]

This communication provides evidence that the DNA of *mut T* stocks has an increased buoyant density, indicating an increased GC/AT ratio, and that the number of thymidylate runs in such stocks has been reduced. These changes occur during repeated subculturing of *mut T* strains. This paper also furnishes additional evidence strongly supporting the conclusion that the *mut T* gene has a unidirectional mutational preference.

Materials and Methods.—Bacterial stocks: *E. coli* T$^-$ 198 *mut T* was isolated by Treffers as a recombinant from the original *mut T* stock of Treffers *et al.*[1] and used in a previous study from this laboratory.[2]

E. coli mut T 24 is a *lac*$^-$ *thr*$^-$ A_z^R recombinant strain isolated by Skaar[3] from a cross between CS19, a derivative of the Treffers' mutator stock, and W677.

E. coli K12 strain 58 is the progenitor of the *mut T* strain isolated by Treffers *et al.*[1]

Reversion analyses: Reversion analyses with mutants A46, A88, A3, A11, and A487 of the tryptophan synthetase A gene were carried out exactly as described in a previous paper.[2] In the experiments designed to examine the stability of *mut T*-induced mutational changes in a *mut T* background, *tryp*$^-$ mutants were isolated nonselectively by replication from minimal agar supplemented with tryptophan. Stocks were then constructed with and without the *mut T* gene by transducing the *tryp*$^-$ *mut T*-induced mutant marker into an isogenic *mut T*$^+$ *tryp* deletion stock in which the *mut T* gene had been replaced by its wild allele.[4]

Transfer experiments: *E. coli* T$^-$198 *mut T* was grown to stationary phase at 37° with aeration (2.10^9 cells/ml) from an inoculum of 10^4 cells in minimal salts medium containing 0.2% glycerol and 20 μg/ml of threonine and of leucine. This procedure was repeated each day using inocula from successive cultures. Permanent stocks were made after each of ten such subcultures. Each stock was checked for the presence of the *mut T* gene by examining an overnight L-broth culture for the high level of *str-r* cells characteristic of *mut T* stocks.[1]

DNA isolation: DNA was isolated by the procedure of Marmur.[5]

Equilibrium sedimentation: The procedure used was essentially that of Schildkraut *et al.*[6] Control and experimental samples were run at the same time in a two-cell rotor using plane and wedge windows. The sensitivity of the method was increased somewhat by using two markers closely bracketing the density of *E. coli* DNA and by conducting the runs at a lower speed (31,410 rpm).

Thymidylate runs: Overnight cultures grown on minimal medium containing 0.2% glucose and 0.1% acid-hydrolyzed casein were diluted 20-fold into 10 ml of the same medium. The cells were then grown with aeration to a density of 1×10^8/ml, and 2.5 mg guanosine deoxyriboside was added to each culture followed by the appropriate amount of H3- or C14-thymidine labeled in the C-2 position.[7] The culture was grown to 2×10^9 cells/ml, harvested by centrifugation, and washed twice with 0.1 *M* Na_2SO_4, 0.05 *M* borate, 0.01 *M* ethylenediaminetetraacetate (EDTA), pH 9.0 (SEB buffer). The cell pellets were resuspended in 2.5 ml of SEB buffer, and the appro-

priate H3 culture was mixed with a C14-labeled preparation. The cells were pelleted, washed once more, and resuspended in 5 ml of SEB containing 27% sucrose. The DNA was prepared in crude form by lysis with 0.2% sodium dodecyl sulphate at 55°, followed by 4 extractions with phenol at 55°. The resulting preparation was extracted with ether several times and dialyzed overnight against 2 liters of SEB buffer at 4°. To this preparation was added 100 mg calf thymus DNA as carrier. Thymidylate runs were then isolated according to the procedure of Shapiro and Chargaff,[8] using formic acid-diphenylamine hydrolysis,[9] and DEAE-urea column chromatography.[10] Pooled column fractions were concentrated on DEAE-carbonate columns and freed of Cl^- with 0.02 M $(NH_4)_2$ CO_3.[11] After washing the column well with water, each fraction was eluted with 1 M triethyl-ammonium bicarbonate and taken to dryness three times in a flash evaporator. The fractions were taken up in 5 ml of H_2O, lyophilized, and dissolved in 1 ml of H_2O. A portion of each fraction (0.50 ml) was then counted in 20.0 ml of dioxane phosphor[12] in polyethylene vials. The absence of variable quenching in this system was verified with internal standards. The samples were counted until the counting error was reduced to a standard deviation of 0.5% (for runs of 1–4) or 2.5% (runs ≥ 5).

Results.—Mutator-induced reversion of A3, A11, A88, and A487: The results of a reversion analysis of A3, A11, A88, and A487 in a *mut T* genetic background are summarized in Table 1. Mutant A3 (position 48 in the α subunit) in nonmutator strains[13] reverts to a full-revertant (FR) type spontaneously at a frequency of about 10^{-8}. The change involved is *val* → *glu* in the protein and GTG → GAG in the DNA. As shown in Table 1, in a *mut T* stock the reversion frequency of A3 was not increased measurably.

TABLE 1

MUTATOR-INDUCED REVERSIONS OF A MUTANTS

Strain	Tube	Cells plated	Tryp$^+$ colonies* per plate	Characteristics
A46 *mut T*	1	7×10^4	1.7×10^3	All FR
A88 *mut T*	1	1.5×10^5	140	All FR
	2	1×10^5	160	"
	3	1.6×10^5	270	"
A 3 *mut T*	1	5×10^4	0	
	2	3×10^4	0	
	3	2×10^4	0	
A11 *mut T*	1	5×10^4	0	
	2	3×10^4	0	
	3	2×10^4	0	
A487 *mut T*	1	1×10^5	440	All PR†
	2	1.2×10^5	350	"
	3	1.5×10^5	410	"

Cells from an overnight culture of each strain were inoculated into 5-ml L-broth tubes (50–100 cells/tube) and grown for 5–10 hr. Cells were harvested by centrifugation, resuspended in saline, and plated on minimal and minimal + indole agar plates. All inocula were checked for the presence of the mutator gene and contained less than 1 *tryp*$^+$ revertant. A46 *mut T* has been included from a previous study.[2]

* The observed reversion frequencies reported in this table and in a previous paper[2] are approximately 100 times higher than that calculated from the reversion rate.[4] This excess number of revertants we attribute to reversion events that occur on the Petri plate after the cells have been spread.[4]

† These were very slow growing, and fast-growing FR types could be distinguished easily against this background.

Mutant A11, a strain with a *glu* → *gln* (GAG → CAG) change at position 48,[13] is also not reverted with greatly increased frequency in the *mut T* background (see Table 1). The failure to find full revertants in this and other studies with these strains make it unlikely that CG → GC transversions are promoted by the *mut T* gene product.

Strain A88, a mutant with an amber codon specifying position 48 in the protein, spontaneously gives but a single FR type (glutamic acid) when the *mut*$^+$ allele is present.[13] Since the A protein with serine at position 48 is an A cross-reacting material (CRM),[13] and since only glu (GAG) and ser (UCG) can arise by AT → CG

transversions from an amber codon, we conclude that the FR class observed with high frequency when A88 is in the mutator background contains glutamic acid. This represents another example of an AT → CG transversion.

Table 1 includes a reversion analysis of A487 (*leu* → *arg* at position 176). Two classes of revertants are observed in a *mut T* stock. The larger class is composed of very slow-growing partial revertants and has not been examined. Infrequently a large colony type was encountered that does not accumulate indole glycerol phosphate and is only slightly inhibited by 5-methyl tryptophan. We have attempted to purify the A protein from two of these apparent FR types. Both isolates, however, yield A proteins that are very labile. Thus the reversion events in these strains did not restore the wild-type amino acid. The importance of this finding is examined in the *Discussion*.

Mutator-induced tryp⁻ mutants: A more sensitive test of *mut T* reversibility of the AT → CG change has been carried out by examining the revertability of non-selectively isolated *tryp⁻ mut T*-induced mutants. The results are presented in Table 2. It is quite clear from the results presented in Table 2 that these classes of *mut T*-induced auxotrophs do not revert to wild type at increased frequency in a mutator background.

TABLE 2

REVERSION OF *mut T*-INDUCED TRYP⁻ MUTANTS

Strain	Tube	Cells plated	Tryp⁺ colonies per plate		*str-r* Colonies
AA1 *mut T*	1	5×10^8	6		100
" *mut T*⁺	1	8×10^7	11		0
AA2 *mut T*	1	5×10^8	10		150
" *mut T*⁺	1	7×10^7	9		0
In A *mut T*	1	5×10^8	6		150
" *mut T*⁺	1	5×10^8	9		0
In B *mu T*	1	5×10^8	6	10^3 PR's	100
" *mu T*⁺	1	5×10^8	9		0

Mutator-induced *tryp⁻* auxotrophs were isolated and isogenic *mut T* and *mut T*⁺ stocks prepared as described in *Materials and Methods*. Mutant AA1 and AA2 were isolated by replication of about 2×10^4 colonies and may not be independent isolates. Mutants In A and In B were independently isolated by replication of 700 colonies. L-broth cultures grown overnight from a revertant-free inoculum were harvested, resuspended in saline, and plated on minimal and minimal plus indole plates. Each culture was checked at the same time for the *mut T* allele by plating 0.1 ml of the overnight culture on streptomycin-nutrient agar plates.

The mut T gene product causes a change in the buoyant density of mut T DNA: Because of the marked unidirectional mutational preference of the *mut T* gene, we have examined the buoyant density of DNA isolated from several *mut T* stocks for changes in AT/GC ratios. In one such stock, *mut T* 24, we have consistently observed a small increase in the buoyant density of the DNA when compared to another K12 strain in our laboratory, *Ymel*. This is shown in Figure 1*A*. *B. subtilis* SB19 and *A. aeroqenes* DNA were run in the same cell as reference density markers. The tracings in Figure 1*A* have been presented with the *E. coli* peaks superimposed (upper) and with the reference peaks superimposed (lower). Both presentations show that the *mut T* 24 DNA has a higher buoyant density, equivalent to approximately a 0.3 per cent increase in the GC content. Figure 1*B* compares in the same manner the pattern of DNA from *E. coli* K12 strain *Ymel* with *E. coli* K12 strain 58, the strain in which the *mut T* gene was isolated. The buoyant densities of the DNA's from these two strains do not differ.

The differences observed in Figure 1*A* are undoubtedly the consequences of *mut T* action, since subculturing the T⁻198 mutator stock also results in a DNA of altered buoyant density (Fig. 1*C*). T⁻198 *mut T* was chosen as the starting culture for these experiments since preliminary buoyant density studies failed to show any difference between this strain and strain 58. The tracings presented in Figure 1*C* compare the second subculture of *E. coli* T⁻198 *mut T* (Msc2) with the sixtieth subculture (Msc60). Sixty subcultures represents approximately 1000 generations under our conditions and has resulted in a small change in the buoyant density of the DNA. Densitometer tracings of *S. typhimurum* LT7 and *E. coli Ymel* (Fig. 1*D*) DNA's illustrate how a 1 per cent difference in GC content appears under the same conditions.

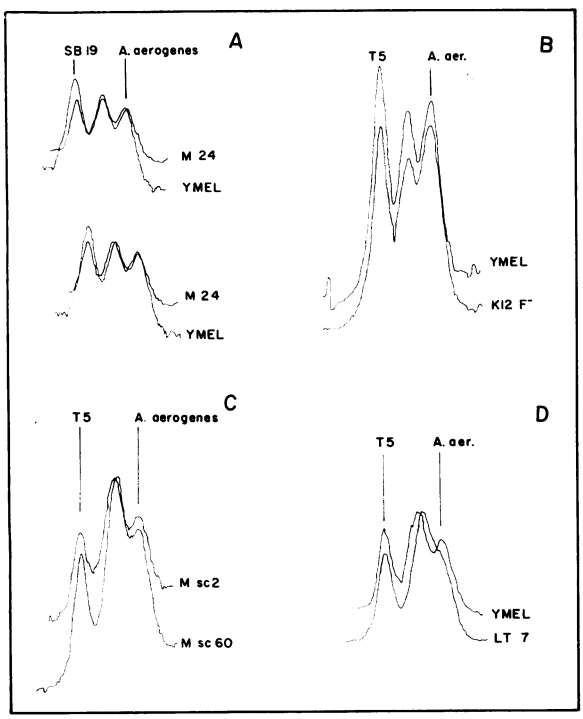

FIG. 1.—Microdensitometer tracings of ultraviolet absorption photographs of *mut T* DNA. Each photograph was taken after 72 hr of density-gradient centrifugation in 7.7 molal CsCl at 31,410 rpm and 25° in a Beckman model E analytical ultracentrifuge. *A. aerogenes* (57% GC) and *B. subtilis* SB19 (42% GC) or T5 bacteriophage DNA (43% GC) have been added as reference markers to each sample. (*A*) *E. coli* Ymel and *mut T* 24 (M24) DNA; (*B*) Ymel and K12 strain 58; (*C*) *E. coli* K12 T⁻198 *mut T* after two subcultures (Msc2) compared to the same strain after 60 subcultures (Msc60); (*D*) Ymel and *S. typhimurium* LT7 DNA.

The frequency of thymidylate runs is decreased in mut T stocks: A test of the significance of the buoyant density change that we attribute to mutator action was conducted by examining the thymidylate runs in *mut T* DNA before and after many generations of growth. We will assume that *mut T*-induced mutations (T → G events) are random and therefore should occur in long runs of thymidylate as well as at positions where T is adjacent to A, G, or C. Such an assumption leads to the prediction that long runs of thymidylate residues will be reduced in direct proportion to the number of residues in the run. For example, a 0.1 per cent GC increment in the total DNA would result in a 0.5 per cent decrement in runs of 5 and a 1.0 per cent change in runs of 10; a 0.2 per cent GC increment would reduce runs of 5 by 1 per cent; and runs of 10 by 2 per cent; and so on. This is because a single

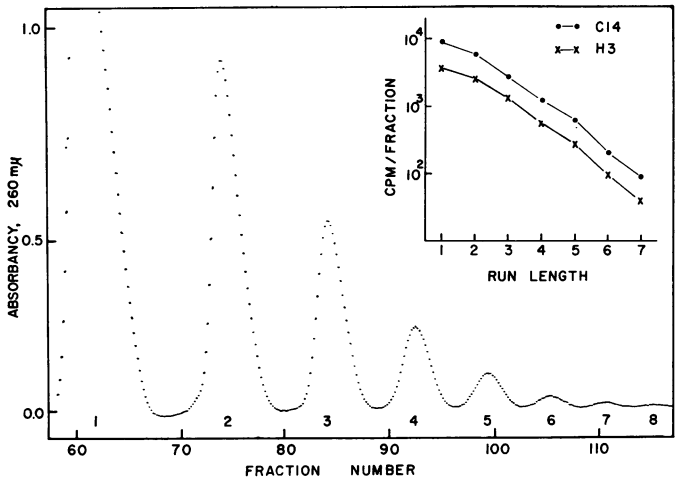

FIG. 2.—The separation of thymidylate runs by DEAE-chromatography. A formic acid-diphenylamine hydrolysate of hydroxylamine-treated deamino DNA was chromatographed on a 1 × 10-cm DEAE-Cl^- column using a 1500-ml linear gradient from 0 to 0.15 *M* NaCl in 7 *M* urea–0.003 *M* TRIS, pH 7.8. Ten-ml fractions were collected. The insert shows the number of counts obtained in each pooled and concentrated fraction.

T → G event in a run of 2 and in a run of 10 reduces that run by 1; but the runs of 10 can be reduced 10 different ways, while the runs of 2 can be reduced in only 2 different ways. It is therefore clear that for an equal number of random T → G events the *number of runs* in our example will differ by a factor of 5. This argument is quite general. It becomes limiting when the total number of accumulated T → G events is such that the probability of altering a single T in the very rare and very long runs becomes less than 1. We might expect then that a search for the longest n-mer that *has not been changed* should also yield information on the absolute increase in GC content, provided that there is not strong selection against changes in very long runs.

The results of double-labeling experiments designed to detect alterations in thymidylate runs are shown in Figures 2 and 3. The quality of the separation of thymidylate runs is shown in Figure 2. Runs from $n = 1$ to $n = 8$ are clearly separated, and it has been possible to identify runs of $n = 11$ in other samples. The counts obtained from the pooled fractions are shown in the insert and represent the minimum levels of radioactivity that we have worked with. The agreement between our results for calf thymus DNA (absorbancy) and *E. coli* (cpm, insert) and those of Chargaff and co-workers is good.[8, 14]

Mutator gene action alters the frequency of thymidylate runs in the expected direction (Fig. 3), i.e., there is a decrease in thymidylate runs. Since the H3/C14

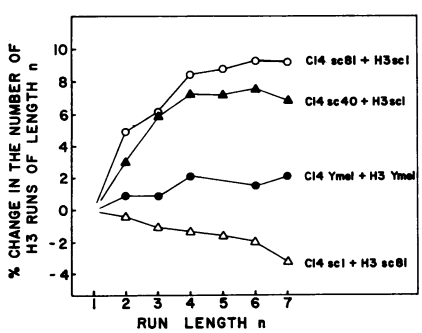

FIG. 3.—Decreased thymidylate run frequencies in the DNA of *mut T* stocks. The per cent change in the number of H3-labeled thymidylate runs (*ordinate*) is plotted against the length of the run (*abscissa*). C14 or H3 thymidine-labeled cultures were mixed and the DNA isolated. Thymidylate runs were separated by chromatography and counted as described in *Materials and Methods* and in Fig. 2. Ymel is our standard *E. coli* K12 strain; scl, sc40, and sc81 represent the 1st, 40th, and 81st subculture of *E. coli* T^- 198 *mut T* on minimal medium.

ratio *decreases* as a function of run length when C14 scl is mixed with H3 sc81, this must mean that the eighty-first subculture has fewer runs of a given length than the starting culture. This is the expected result, since the reversion analyses tell us that an AT → CG transversion is the preferred *mut T*-induced event. The data in Figure 3 show that the change is not a counting artifact since reversing the label in the two strains (C14 sc81 mixed with H3 scl) reverses the effect. As an additional control, Figure 3 includes data obtained when C14 *Ymel* is mixed with H3 *Ymel*.

The fortieth subculture was also examined by this method. The results are shown in Figure 3 and they show the expected change in runs of thymidylate, although of a somewhat larger magnitude than anticipated.

Discussion.—Mutational specificity of the mut T gene: The data presented here extend our knowledge of the specificity of the *mut T* gene product. Three of the mutants studied in this report have alterations that affect position 48 of the A protein (A3, A11, A88). The wild-type amino acid at this position is glutamic, and therefore the relevant codon is either GAG or GAA. Since a single base change gives rise to an amber codon in the sequence specifying position 48, the glu codon must be GAG.[13] It follows that the codons for A3 (val) and A11 (gln) are GUG and CAG, respectively. The failure to find a mutator-induced increase in the frequency of appearance of full revertants with A3 and A11 provides additional evidence for the unique specificity of the *mut T* gene product.

Mutant A487 (arg) does not revert to wild type (leu) in the *mut T* background with an increased frequency. The codon for leu at this position is CUG since (*i*) arg is mutant and (*ii*) the third position has been identified as G in a study of frameshift mutations in this region by Brammar *et al.*[15] The failure to find *mut T*-induced full revertants at this site is important, because it is the second specific example that we have studied (the other is mutant A23) in which an increase in the CG → AT transversion frequency would be detectable. Thus, if the mutator induced AT → CG event can be reversed by the action of the mutator gene, it must do so at a frequency several orders of magnitude lower in the CG → AT direction. This conclusion is also supported by the observation that three *mut T*-induced *tryp*$^-$ mutants revert at similar rates in both *mut T* and *mut T*$^+$ stocks. This is in agreement with our earlier studies with *mut T*-induced mutations,[2] but is more rigorous since the mutants were isolated nonselectively and reversion studies were performed in isogenic stocks.

Mutator gene action and altered base ratios: We can calculate from enlarged densitometer tracings that the buoyant density differences between mutator and nonmutator DNA observed in Figures 1*A* and *C* correspond to an 0.2–0.5 per cent increase in the GC composition of the DNA. These differences agree approximately with the differences estimated from the altered frequencies of thymidylate runs.[16] The actual values calculated from the data in Figure 3 give a net GC difference of 0.4–0.7 per cent for the eighty-first subculture compared to the starting mutator stock.

Does this estimate of the GC change in the DNA agree with the expected value based on known mutation rates? We have estimated that the mutation rate for a given AT pair in a *mut T* strain is 3.5×10^{-6}, expressed as the probability of such an event occurring per genome per generation.[4] Eighty subcultures under our conditions (15–20 generations per subculture) would involve 1200 to 1600 generations

and would correspond to about a 0.42–0.56 per cent change in the number of AT pairs, if the AT/GC ratio changed linearly with the number of generations. This agreement between the experimental findings and the calculated expectation is good, but cannot, however, be regarded too seriously, since both the estimate of the reversion rate and the quantitation of the thymidylate runs are subject to some error, and the effect of selection has not been ascertained.

Where do the mutational events occur? The microdensitometer tracings indicate that the altered bases are distributed evenly over the chromosome. If they were not, we would expect to see a skewed peak, in which case a small difference would probably be missed. Our data on the thymidylate run frequency is not accurate enough to decide if each AT → CG event has occurred at random. However, a completely random series of such events would give a line of constant slope, similar to the data in Figure 3 for C14 scl + H3 sc81. The fact that the experimental curve does not deviate grossly from a straight line (as it does when identical experiments are carried out with *Salmonella* and *E. coli* DNA's[4]), suggests that each mutation that hits a thymidylate run between $n = 1$ and $n = 7$ has occurred at random.

The mutation rate attributed to the *mut T* gene corresponds to approximately seven mutations per genome in each generation. The transfer experiments reported in this paper were carried out in minimal medium. It is worth asking, therefore, how the strain manages to survive and grow at other than drastically reduced rates under these conditions. If we consider the A gene of tryptophan synthetase as an *E. coli* gene with a representative nucleotide distribution, we can show, using the amino acid composition of the enzyme and the genetic code, that 153 of the 801 base pairs that code for the A protein code for leu, val, ser, pro, thr, ala, arg, and gly, the amino acids that have at least four codons varying only in the third position. Of these, 86 will have AT pairs in the third position if the GC content of the A gene is 50 per cent. Thus, if through selection only third position changes in these codons survived the mutation process in a *mut T* strain, the net composition of the A gene could change from 50 to 61 per cent GC without any alterations in the amino acid sequence.

However, we would expect only one third of the approximately seven mutations that occur at each replication to be third-position changes, and therefore two of every three events should lead to an altered amino acid sequence. Of these *mut T*-induced amino acid changes, some will interconvert like amino acids (ile → leu; leu, phe → val), and AGG (arg) → CGG (arg) leaves the protein unaltered. These changes should not subject the cell to strong selective pressures. The remainder of the *mut T*-induced events must either be selected against or must occasionally confer selective advantage. We can not at present quantitate the former. The latter, mutations which confer selective advantage, may seem unlikely but would be analogous to mutations that lead to periodic selection in microorganisms.[17]

Several theories have been advanced to account for the wide variation among species and small heterogeneity within the same species in the GC content of microbial DNA.[18–22] In particular, Sueoka has formulated a quantitative argument that the environment, operating on the cell phenotype, selects for base-pair interconversions of a given class that arise as a consequence of mutational pressures.[22] Our results offer experimental evidence for this view. We postulate that the DNA

replication apparatus of the cell generates a net number of mistakes and may, in certain cases, favor a particular class of base-pair interconversions. We think it likely that we are studying one of the possible sources of mutational bias that normally operate in the cell, magnified several orders of magnitude in a *mut T* stock. If it can be shown that the wild allele at the *mut T* locus results in errors in a preferential direction, such an argument would have considerable force. Indirect evidence that the replicating apparatus of T4 has a definite bias in the expected direction (toward high AT content of the DNA) may be found in the results of Freese and Freese.[23] These authors have observed that GC → AT events in the *r*II region of T4 are 10–100 times more frequent than AT → GC mutations. Moreover, a likely physical basis for this bias is replication errors caused by the T4-induced DNA polymerase, an enzyme which, when mutationally altered, can cause greatly elevated mutation rates.[24]

It is clear from these various examples that mutator genes, operating by any one of several mechanisms, may play an important evolutionary role, and may be partly responsible for the great variation in AT/GC ratios evident in the DNA's of different organisms.

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