JOURNAL OF BACTERIOLOGY, June 1985, p. 859–864
0021-9193/85/060859-06$02.00/0

Vol. 162, No. 3

Mechanism of Mutation by Thymine Starvation in *Escherichia coli*: Clues from Mutagenic Specificity

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Received 3 December 1984/Accepted 4 March 1985

To probe the mechanisms of mutagenesis induced by thymine starvation, we examined the mutational specificity of this treatment in strains of *Escherichia coli* that are wild type (Ung$^+$) or deficient in uracil-DNA-glycosylase (Ung$^-$). An analysis of Ung$^+$ *his-4* (ochre) revertants revealed that the majority of induced DNA base substitution events were A:T → G:C transitions. However, characterization of *lacI* nonsense mutations induced by thymine starvation demonstrated that G:C → A:T transitions and all four possible transversions also occurred. In addition, thymineless episodes led to reversion of the *trpE9777* frameshift allele. Although the defect in uracil-DNA-glycosylase did not appear to affect the frequency of total mutations induced in *lacI* by thymine deprivation, the frequency of nonsense mutations was reduced by 30%, and the spectrum of nonsense mutations was altered. Furthermore, the reversion of *trpE9777* was decreased by 90% in the Ung$^-$ strain. These findings demonstrate that in *E. coli*, thymine starvation can induce frameshift mutations and all types of base substitutions. The analysis of mutational specificity indicates that more than a single mechanism is involved in the induction of mutation by thymine depletion. We suggest that deoxyribonucleoside triphosphate pool imbalances, the removal of uracil incorporated into DNA during thymine starvation, and the induction of *recA*-dependent DNA repair functions all may play a role in thymineless mutagenesis.

Incubation of bacterial thymine auxotrophs in the absence of thymine, under conditions otherwise conducive to growth, has been found to provoke a number of thymineless phenomena including mutagenesis (for a review, see reference 19). The results of several studies led to the conclusion that thymine starvation causes primarily A:T → G:C transitions, as well as some frameshifts and deletions (2, 14, 26, 31). In these studies, the conclusions concerning the mutagenic specificity of thymineless mutagenesis are primarily based on the efficiency with which base analogs and chemical mutagens revert mutations induced by thymine starvation, the ability of the induced mutations to revert spontaneously, and the potentiation of thymineless mutagenesis by pretreatment with 2-aminopurine. To account for the induction of mutation by thymine deprivation, it has been proposed that base substitutions arise either by error-prone repair of DNA gaps generated during thymine starvation (3) or by misincorporation of cytosine in place of thymine as a consequence of an elevated dCMP/dTMP ratio (26). These proposals do not readily predict thymineless induction of frameshifts or deletions. It also had been found that thymine deprivation causes an increase in the pool of uracil deoxyribonucleotides (12). This led Breitman et al. (1) to suggest that uracil might be incorporated into DNA under conditions of thymine deprivation and become a substrate for a restriction-type nuclease. This would lead to the introduction of DNA strand breaks. The occurrence of DNA strand scissions resulting from uracil incorporation during thymine starvation subsequently has been confirmed (22, 32, 33). However, the relationship between uracil incorporation and thymineless mutagenesis has not been investigated.

In an attempt to understand more fully the molecular events responsible for the mutagenicity of thymine starvation, we carried out a rigorous analysis of the mutagenic specificity of thymineless mutagenesis. Specifically, we sought to characterize the base substitutions caused by thymine nucleotide depletion in *Escherichia coli*, to test the ability of thymine starvation to induce frameshift mutations, and to examine the influence of a defect in uracil-DNA-glycosylase on thymineless mutagensis.

MATERIALS AND METHODS

***E. coli lacI* system, strains, and media.** The *E. coli lacI* system permits the detection of nonsense mutations at numerous sites within the *lacI* gene (4). Because the location and DNA sequence of the nonsense mutations have been established, each mutation can be attributed to a specific base change (10, 24). Unless otherwise stated, suppressor and deletion strains and media and techniques for the *lacI* system were the same as described by Coulondre and Miller (4). Strain KMBL3835 [F′ *pro-lac/ara* Δ(*pro-lac*) *thi trpE9777*] has been described previously (11). A thymine-requiring derivative (NR8008) was selected after treatment with trimethoprim as described by Miller (23). Strain NR8009 [F′ *pro-lac/ara* Δ(*pro-lac*) *thi trpE9777 thyA* Tn*10*::*tyrA ung-1 nadB*] was constructed by P1 *vir*-mediated transduction with BD1467 [HfrPO45, *thi relA1* λ Tn*10*::*tyrA*, *ung-1*, *nadB*] (provided by B. K. Duncan) as the donor and NR8008 as the recipient. Tetracycline-resistant transductants were selected and screened for tyrosine and nicotinic acid auxotrophy because the *tyrA* and *nadB* genes flank the *ung-1* allele (7). To verify the presence of *ung-1*, suspected Ung$^-$ isolates were tested for the ability to support the growth of T2 bacteriophage containing uracil in their DNA (provided by D. Fix); such bacteriophage are unable to grow on Ung$^+$ bacteria (7). Viral strains, media, and techniques for suppression pattern analysis of *his-4* revertants have been described previously (29). Strain JC3890 [*his-4 trpE9777 uvrB301*, *argE3 thr-1 leu-6 proA2 thi-1 lacY1*, *galK2 ara-14 xyl-5 mtl-1 tsx-33 strA31 supE44*] was provided by T. Kato.

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A thymine-requiring derivative (NR8010) of this strain was selected as described above.

Spontaneous mutation. Spontaneous mutants were selected after overnight growth of low-titer inocula in 96-well microtiter plates as described previously (20).

Thymine starvation. Thymine-requiring strains were grown at 37°C to exponential phase (3×10^8 cells per ml) in L broth (23) supplemented with thymine (final thymine concentration for all media, 50 μg/ml). Cells were harvested by centrifugation; washed three times in VB solution ($MgSO_4 \cdot 7H_2O$ [0.2 g/liter], citrate [2 g/liter], K_2HPO_4 [10 g/liter], $Na(NH_3)HPO_4 \cdot 4H_2O$ [3.5 g/liter] [pH 7.0]); suspended in minimal medium, fully supplemented but lacking thymine; and incubated with shaking at 37°C. At regular intervals, samples were withdrawn, washed once in VB solution, diluted in VB solution when necessary, and spread on appropriately supplemented minimal media to detect survivors, *lacI* mutants, tryptophan prototrophs, or histidine prototrophs.

RESULTS

Thymineless death in Ung$^+$ and Ung$^-$ strains. Incubation of exponential phase cells of NR8008 (Ung$^+$) and NR8009 (Ung$^-$) in appropriately supplemented minimal medium lacking thymine led to cell death (data not shown). For both strains, killing was initiated within 2 h of cell transfer to thymine omission medium and occurred with virtually identical kinetics. This result is in agreement with previous findings for thymine deprivation of Ung$^-$ strains of *E. coli* (7). However, in the case of *Bacillus subtilis*, it has been reported that mutations in uracil-DNA-glycosylase confer a modest degree of resistance to thymineless death (22).

Thymineless mutagenesis in Ung$^+$ and Ung$^-$ strains. The appearance of *lacI* mutations coincided with the initiation of cell killing and occurred with similar kinetics in both the Ung$^+$ and Ung$^-$ strains (Fig. 1A). After 6 h of starvation, the frequencies of *lacI* mutants in both the Ung$^+$ and Ung$^-$ strains were similar and were considerably above that of the background (Table 1). The yield of *lacI* cells increased at least sevenfold in both strains, whereas the total cell population underwent less than one doubling. Thus, the increases reflect the induction of mutants and cannot be due to the selection of pre-existing mutants.

Thymine starvation of the Ung$^+$ strain also induced a 300-fold increase in the reversion frequency of *trpE9777* (Fig. 1B). This represents an 18-fold increase in the total yield of Trp$^+$ cells. The *trpE9777* frameshift mutation is the result of the addition of an A:T base pair to a run of five A:T base pairs, and so it is expected to revert by the loss of one of the six A:T base pairs (30). However, the induction of frameshifts by thymine starvation is not restricted to targets consisting of A:T runs. We have found that the *trpA21* and *trpA540* mutations, which are frameshifts in G:C-rich sequences (30), also revert by thymine starvation (B. A. Kunz and B. W. Glickman, unpublished data).

Although we observed no effect of the *ung-1* mutation on either thymineless killing or the induction kinetics of *lacI* mutants, the induction of tryptophan prototrophs was reduced by 90% in the Ung$^-$ strain (Fig. 1B). Whether those frameshift revertants recovered in the Ung$^-$ strain reflect the leakiness of the *ung-1* allele (21) or are the result of an alternate mutational pathway, e.g., the inaccurate repair of gaps in the DNA or *recA*-dependent error-prone repair processes (27, 34), remains unclear.

Mutational specificity: analysis of spontaneous background. Among the spontaneous *lacI* mutants, 2% of the total mutations in the Ung$^+$ strain and 8.8% in the Ung$^-$ strain were nonsense mutations (Table 1). Duncan and colleagues have demonstrated that this Ung$^-$ mutator effect is due to the specific enhancement of G:C → A:T transitions, presumably reflecting the retention of cytosine residues that have undergone spontaneous deamination to uracil (6, 8, 9). The mutational spectra for spontaneous *lacI* amber mutations in the Ung$^+$ and Ung$^-$ strains are shown in Fig. 2. Table 2 summarizes the mutational specificites of the base substitutions required to generate the amber mutations. From these results it can be seen that each spectrum has its own characteristics. As found previously by Coulondre and Miller (4), the spontaneous Ung$^+$ spectrum (Fig. 2A) has prominent hotspots at the G:C → A:T transition sites, ambers 6 and 15. There is a 5-methylcytosine residue at each of these sites, and it has been proposed that the hotspots are the result of spontaneous deamination of 5-methylcytosine to thymine (5). In the Ung$^-$ strain, 96% of the spontaneous amber mutations arose as a consequence of G:C → A:T transitions compared with only 72% of those in the Ung$^+$ strain (Table 2). The resultant 10-fold elevation in the spontaneous G:C → A:T transition frequency brings the level of this transition in the Ung$^-$ strain to that found at 5-methylcytosine sites in the Ung$^+$ strain (cf. Fig. 2A and C). Thus, the data confirm the Ung$^-$ *lacI* spectrum presented earlier by Duncan and Miller (6) and are consistent with the proposal that deamination accounts for the spontaneous G:C → A:T hotspots in the Ung$^+$ strain.

Mutational specificity: analysis of mutations induced by thymine deprivation. After thymine starvation for 6 h, similar increases in the frequencies of total *lacI* mutations were found for the Ung$^+$ and Ung$^-$ strains (Table 1). However,

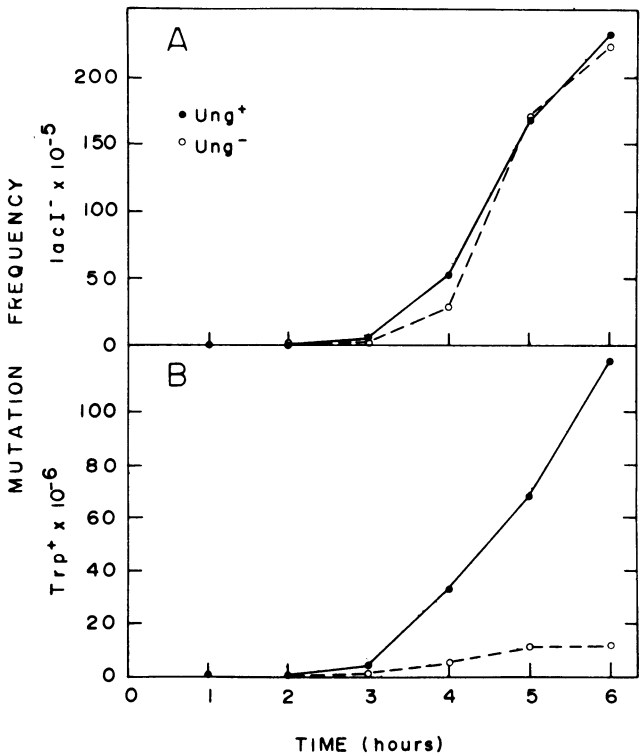

FIG. 1. (A) Induction of *lacI* mutations by thymine starvation. (B) Induction of tryptophan prototrophs by thymine starvation. Symbols: ●, NR8008 (Ung$^+$); ○, NR8009 (Ung$^-$).

TABLE 1. Induction of *lacI* mutations by thymine starvation

Strain	Period of starvation (h)	*lacI* frequency[a]	Amber mutants		Ochre mutants		Total nonsense mutants		Total *lacI* mutants screened
			%	Frequency[a]	%	Frequency[a]	%	Frequency[a]	
NR8008 (Ung^+)	0	5	1.6	0.08	0.4	0.02	2.0	0.1	1,000[b]
	6	2,312	1.5	34	0.6	13	2.1	47	16,600
NR8009 (Ung^-)	0	8	4.3	0.35	4.5	0.36	8.8	0.7	1,000[b]
	6	2,224	0.9	20	0.6	13	1.5	33	36,450

[a] Frequencies are per 10^6 viable cells.
[b] Only 1,000 isolates were screened for this control experiment. In excess of 50,000 isolates from each of the Ung^+ and Ung^- strains were screened to derive the spontaneous spectra.

among the *lacI* mutants examined in the Ung^- strain, the fraction of amber mutations was reduced significantly ($P < 0.005$), leading to a 30% decrease in the frequency of total nonsense mutations. The amber mutations were analyzed to determine which base substitutions had been induced. A critical fact for the *lacI* system is that the A:T → G:C transition cannot generate de novo nonsense codons. This fortuitously eliminates the masking of other base substitutions by A:T → G:C transitions that are expected to be predominant under conditions of thymine deprivation (see analysis of *his-4* revertants below).

The spectrum obtained after thymine depletion differs considerably from the spontaneous spectrum (Fig. 2). For example, the transition hotspot sites are absent from the thymine starvation spectrum (cf. Fig. 2A and B) and the relative proportion of transversion events increased from 28 to about 70% (Table 2). Increases in the relative fraction of mutations were found at 13 of the 28 sites at which adenine can be incorporated to yield amber mutations. Interestingly, 12 of the 13 sites showing increases are transversion sites. The predominance of transversions resulting from replacement by an A:T base pair is further illustrated by the finding that the fraction of mutations increased at only two of the eight sites at which transversions are the result of replacement by a G:C base pair. The prevalence of A:T base pair substitutions may reflect the heightened misincorporation of adenine because of the swelling of the dATP pool, an event that occurs during thymine deprivation (25). Alternatively, the induction of SOS functions during thymine starvation (35), a process which may favor the incorporation of adenine (28), may be responsible.

Thymine starvation of the Ung^- strain produced as many *lacI* mutants as were recovered in the Ung^+ strain (Table 1, Fig. 1). However, the fraction of amber mutants was reduced in the Ung^- background (Table 1). Indeed, when the spectra obtained after thymine starvation are compared, the level of thymineless mutagenesis is lower at 20 of the 29 amber sites recovered in both strains (cf. Fig. 2B and D). The base substitutions responsible for the amber mutants recovered in the Ung^- strain after thymine deprivation also are summarized in Table 2. Again, the G:C → A:T transition and all four possible transversion events are detected. Some differences are noted, however, between the induced spectra in the Ung^+ and Ung^- strains (cf. Fig. 2C and D). In particular, there is a substantial increase in the frequency of G:C → C:G transversions in the Ung^- strain.

Analysis of *his-4* revertants. Nonsense mutations constituted only a small fraction of the total mutations recovered in the *lacI* gene after thymine deprivation (Table 1). Because the A:T → G:C transition goes undetected in the *lacI* system, it was possible that missense mutations resulting from A:T → G:C transitions constituted a large fraction of the *lacI* mutants. To evaluate the relative prevalence of A:T → G:C transitions versus other base substitutions, we used a reversion analysis system described by Shinoura et al. (29). This system is based on evidence that locus revertants of the ochre allele, *his-4*, occur via A:T → G:C transitions, whereas other base substitutions give rise to suppressor mutations that suppress *his-4* (17). The base substitutions responsible for the *his-4* revertants can be characterized by screening the revertants against a series of amber and ochre mutants of bacteriophage T4. The pattern of suppression of the T4 nonsense mutations, obtained by infecting the revertants with the bacteriophage, reveals whether the *his-4* revertants are due to transitions or transversions.

For the purpose of this analysis, *his-4* revertants were selected after 6 h of thymine deprivation. This treatment increased the *his-4* reversion frequency more than 1,000-fold. Several hundred spontaneous and induced revertants were selected and characterized. Table 3 shows that spontaneous mutations arose as a consequence of all classes of base substitutions. However, all of the mutations induced by thymine starvation were the result of A:T → G:C transitions. This result argues strongly that thymine deprivation does induce primarily A:T → G:C transitions. Among the *lacI* mutants induced by thymine starvation, a large fraction could have been deletions or frameshift mutations. This seems unlikely, however, considering the lack of effect of the Ung^- mutation on the overall *lacI* induction frequency when under similar conditions the reversion of the *trpE9777* frameshift allele is effectively reduced in the Ung^- host.

DISCUSSION

Thymine starvation of *E. coli* induced forward mutation in the *lacI* gene and reversion of the *trpE9777* frameshift mutation (Fig. 1). An analysis of the *lacI* amber mutations (Fig. 2, Table 2) revealed that they arose via G:C → A:T transitions and all four possible transversions. (The A:T → G:C transition cannot be monitored in the *lacI* system.) However, these particular base substitutions do not appear to constitute the predominant mutational events, because nonsense mutations accounted for only 2% of the total *lacI* mutants that were induced (Table 1). The actual contribution of frameshift mutations to thymineless mutagenesis is difficult to ascertain, as we have found site-to-site variation among different *trp* frameshift alleles to be extensive, with the *trpE9777* allele being reverted the most effectively. If the contribution of frameshift events was very great, then one would expect the relative fraction of *lacI* nonsense mutations to increase and the overall $lacI^-$ frequency to decrease in the Ung^- strain in which thymineless frameshift mutagenesis appears to be limited (Fig. 1B). This is not the case

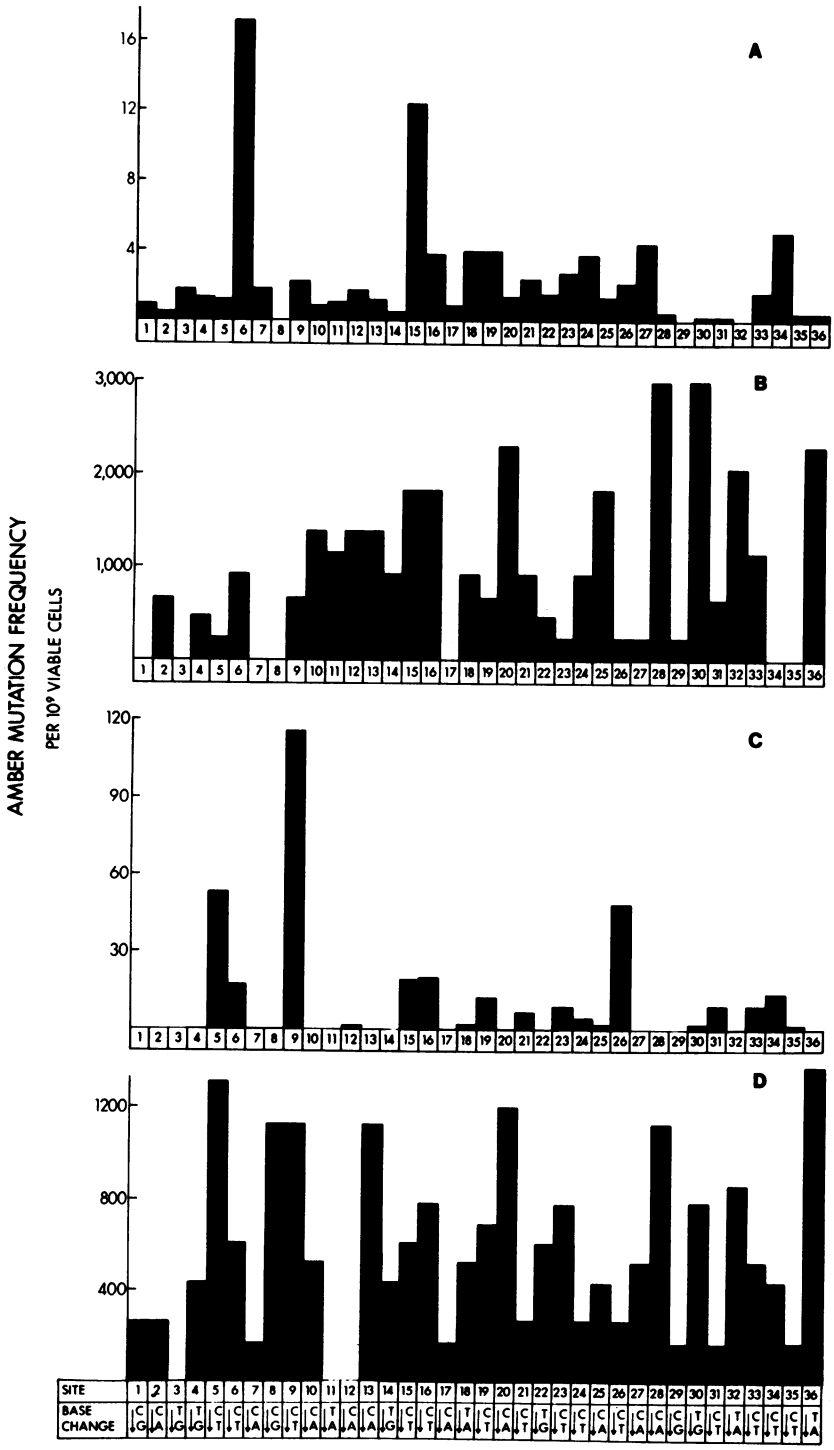

FIG. 2. Spectra for *lacI* amber mutations. (A) Bars represent spontaneous mutations obtained in the parental strain KMBL3835 (464 amber mutations were analyzed). (B) Bars represent mutations obtained after thymine starvation of strain NR8008 for 6 h (148 amber mutations were analyzed). (C) Bars represent spontaneous mutations obtained in strain NR8009 (182 amber mutations were analyzed). (D) Bars represent mutations obtained after thymine starvation of NR8009 for 6 h (232 amber mutations were analyzed).

(Fig. 1A, Table 1), and in fact, a slight reduction in the fraction of nonsense mutations was noted (Table 1). Characterization of 200 His^+ revertants induced by thymine starvation indicated that they all resulted from A:T → G:C transitions (Table 3), even though the *his-4* system used was capable of detecting a wide range of base substitution events (29). Taken collectively, these data indicate that in *E. coli*, thymine nucleotide depletion can induce frameshift mutations and all types of base substitutions, but the A:T → G:C transition event appears to predominate.

Although earlier studies did not elucidate the mechanism of thymineless mutagenesis, results of one part of the

investigation indicated the possibility that incorporation of uracil into DNA during thymine deprivation might be involved (1, 12, 22, 32, 33). Although we found no influence of a defect in uracil-DNA-glycosylase (*ung-1*) on the overall frequency of induced *lacI* mutants (Fig. 1), differences in the mutational spectra were observed. The fraction of amber mutants was reduced significantly in the Ung$^-$ strain ($P < 0.005$) and their distribution was altered (cf. Fig. 2B and D). The frequency of G:C → C:G transversions increased sevenfold, whereas the frequencies of the other three transversions were reduced uniformly by 55% (data from Tables 1 and 2). When DNA is synthesized during thymine deprivation, uracil incorporation occurs. The removal of uracil by uracil-DNA-glycosylase generates apyrimidinic (AP) intermediates that are highly mutagenic under conditions in which error-prone repair is induced, such as during thymine starvation. Studies of the mutational specificity of AP sites show that they often lead to transversion events (18, 28). Thus, the inability to excise uracil may explain the 55% reduction in the frequencies of three of the four possible transversions in the Ung$^-$ strain. The reason that the G:C → C:G transversion frequency is elevated is unclear. The most dramatic effect of the *ung-1* mutation on thymineless mutagenesis was the 90% reduction in the frequency of reversion of the frameshift allele *trpE9777* (Fig. 1B). In the case of frameshift mutations induced by thymine starvation, uracil-DNA-glycosylase, and hence uracil, in the DNA appears to play an important role. The mechanism by which *ung*-dependent frameshift mutations occur likely involves the repair of AP lesions.

On the basis of mutational specificity, it would appear that more than a single mechanism is involved in the induction of mutation by thymine deprivation. The predominant component of thymineless mutagenesis appears to be A:T → G:C transitions. It has been suggested that this transition event may reflect incorporation of cytosine into DNA in place of thymine under conditions in which thymine is unavailable (26). This "mass action" hypothesis is extremely attractive when the increased dCTP/dTTP ratio found during thymine deprivation is considered (25). It seems unlikely that this nucleotide pool alteration is solely responsible for thymineless mutagenesis because it does not readily account for the induction of G:C → A:T transitions, transversions, and frameshift mutations. Thymineless mutagenesis requires the proper functioning of the *recA*-dependent, error-prone SOS repair pathway (13, 27, 35, 36), and mutations blocking the induction of SOS repair, e.g., *lexA* (3) and *umcC* (B. A. Kunz and B. W. Glickman, unpublished data) prevent the induction of mutation by thymine deprivation. These observations suggest that both pool imbalances and the induction of error-prone repair are involved.

What remains to be resolved is the dependence of thymineless mutagenesis on error-prone repair. The transversion and frameshift events might be the consequences of a reduction in the fideltiy of DNA replication under conditions in which SOS is induced or they might result from intermediates such as AP sites, which require SOS induction for their mutagenic repair. But why should the major component of thymineless mutagenesis, the A:T → G:C transition, be *recA*-dependent? Possibly, a reduction of replicational fidelity, often accredited to the SOS-induced system, is required for the erroneous incorporation of dCTP opposite adenine under conditions of nucleotide pool imbalance. A second and perhaps more attractive hypothesis is that for the production of any mutations, DNA synthesis, which is under *recA* control, must occur during thymine depletion. It is intriguing that after thymine starvation, DNA synthesis continues for several hours without concomitant protein synthesis (15). This process, termed stable DNA replication, is semiconservative, does not occur in the absence of a functional *recA* gene, and is resistant to UV treatment that would inhibit normal DNA replication (16). This latter facet of stable DNA replication might be important with respect to mutation induction by thymine starvation. We propose that the *recA* requirement for thymineless mutagenesis reflects the need for stable DNA replication to be induced to have sufficient DNA synthesis during the period of starvation.

TABLE 2. Distribution of base substitutions leading to amber mutations[a]

Base substitution	Sites available	No. of sites found (% of total)			
		Spontaneous		Thymine starvation	
		Ung$^+$	Ung$^-$	Ung$^+$	Ung$^-$
G:C → A:T	14	14 (71.8)	14 (96.1)	12 (30.4)	14 (39.7)
G:C → T:A	10	10 (16.4)	2 (1.6)	7 (35.8)	9 (27.6)
A:T → T:A	4	3 (6.2)	1 (0.5)	4 (18.9)	3 (13.8)
A:T → C:G	5	5 (4.7)	1 (1.6)	4 (14.2)	4 (11.2)
G:C → C:G	3	1 (0.9)	0	1 (0.7)	3 (7.8)

[a] For spontaneous revertants the total number of amber mutations examined was 464 and 182 for Ung$^+$ and Ung$^-$, respectively. For conditions of thymine starvation, the total number of amber mutations examined was 148 and 232 for Ung$^+$ and Ung$^-$, respectively.

TABLE 3. Distribution of base substitutions leading to *his-4* reversion[a]

Base substitution	No. of sites found after period (h) of thymine starvation[b]	
	0	6
A:T → G:C	194 (74.0)[b]	200 (100)
G:C → A:T	37 (15.0)	0
Transversion	17 (11.0)	0

[a] Mutations were induced by thymine starvation of NR8010. Spontaneous events contribute to less than 1% of the total induced mutations.

[b] Numbers in parentheses are the percentages of total occurrences. The total number of revertants examined was 248 for those examined at 0 h of thymine starvation and 200 for those examined at 6 h.

ACKNOWLEDGMENTS

We thank J. W. Drake and T. A. Kunkel for critical reading of the manuscript; B. K. Duncan, D. Fix, and T. Kato for providing bacterial and bacteriophage strains; M. Skrzynski, R. Dunn, and D. Halderman for expert technical assistance; and C. Kelly for typing the manuscript.

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