Proc. Nat. Acad. Sci. USA
Vol. 73, No. 4, pp. 1269–1273, April 1976
Genetics

Heat mutagenesis in bacteriophage T4: The transition pathway

(spontaneous mutation/acid mutagenesis/cytosine deamination/evolution rates)

RICHARD H. BALTZ*, PAUL M. BINGHAM, AND JOHN W. DRAKE†

Department of Microbiology, University of Illinois, Urbana, Ill. 61801

Communicated by Matthew Meselson, January 19, 1976

ABSTRACT **G·C → A·T transitions are induced by heat, and arise from the deamination of cytosine (5-hydroxymethylcytosine in the case of bacteriophage T4) generating uracil. The reaction is proton-catalyzed, and is also characteristic of acid mutagenesis. Mutation rates and activation energies of mutation are site-specific, and are presumably influenced by neighboring bases. Rates of heat-induced mutation in bacteriophage T4 under conditions of temperature, pH, and ionic strength similar to those prevailing in higher eukaryotic cells suggest that heat mutagenesis may present a serious challenge to organisms with large genomes, and may comprise an important determinant of the rates of spontaneous mutation.**

Heat mutagenesis has been described qualitatively in bacteria (1, 2) and more quantitatively in bacteriophage T4 (3–6), where it is also clearly related to acid mutagenesis. Most early studies attributed heat mutagenesis to depurination (1–4, 7), although, as discussed below, depurination is probably nonmutagenic, at least in bacteriophage T4.

Heat mutagenesis can be studied particularly effectively in bacteriophage T4: phage particles themselves are relatively resistant to heating compared to most cellular systems, efficient T4 DNA transformation systems are available, and the genetics of the T4*rII* system exhibit high resolving power. Heat may be an example of an important class of mutagens which are more important in evolution than are ordinary errors of DNA replication. Finally, as we shall show, rates of heat mutagenesis are unexpectedly high when extrapolated from T4 to higher organisms.

MATERIALS AND METHODS

Media. L and N broth and Drake top and bottom agar were described previously (8). Enriched H bottom agar contained 13 g of Gibco or Bacto tryptone, 8 g of NaCl, 2 g of sodium citrate dihydrate, 1.3 g of glucose, and 10 g of Gibco or Bacto agar per liter of distilled water. Enriched H top agar was identical to enriched H bottom agar except that it contained 6.5 g of agar and 3 g of glucose. Buffer L (low ionic strength) contained 10 mM sodium phosphate, acetate, or chloroacetate, depending upon the desired pH (and 0.5 mM Mg^{2+} after addition of phage); phosphate was used for pH 5.7–8.0, acetate for pH 4.0–5.6, and chloroacetate for pH 3.0–3.9. Buffer H (high ionic strength) contained 150 mM NaCl, 20 mM $MgCl_2$, and 10 mM sodium phosphate, acetate, or chloroacetate, as above.

Bacteria. *Escherichia coli* was used throughout. Strain BB was used to grow and titer most T4 stocks. Strain B was used to detect *r* mutant and revertant phenotypes. The lambda lysogen KB was used to score *rII*$^+$ revertants, using either Drake or enriched H agar at 37°. The ochre-suppressing lambda lysogen CA165 was used to score convertants of opal (UGA) *rII* mutants to the ochre (UAA) configuration, using enriched H agar at 32°.

Bacteriophages. T4B was used throughout. The *rII* mutants *rUV7*, *rUV13*, *rSM94*, *rNT88*, and *rHS8-153* are revertible by hydroxylamine. *rX655* is an opal (UGA) *rII* mutant obtained from J. Karam. Stocks were grown in BB cells in L or N broth, purified by differential centrifugation (8), and resuspended in 10 mM sodium phosphate buffer (pH 7.0) plus 10 mM $MgCl_2$ at 1 to 4 × 10^{12} particles per ml.

Mutagenesis. Purified phage suspensions were briefly prewarmed to the proper temperature, diluted 20-fold into prewarmed buffer, and incubated in a water bath at the desired temperature (± 0.5°). pH determinations were made at the incubation temperature after adding phage. Samples were withdrawn at various times, diluted into chilled L broth or 0.1 M phosphate buffer (pH 7.0), and plated on *E. coli* BB for total viable counts and on KB or CA165 for revertant or UGA → UAA convertant counts, respectively. (In the case of UAA convertants, plaques were picked from CA165 and spot tested on KB to exclude revertants.) The frequency of revertants among survivors increased linearly with time of heating for most mutants tested; exceptions will be noted below. These measurements were facilitated by the absence of multiplicity reactivation among heat-inactivated T4 particles.

Phage inactivation in buffer L displays strictly first-order kinetics with all phages tested except *rD19*, which displays a brief lag followed by exponential inactivation; the exponential portion of the *rD19* inactivation curve on a semilogarithmic plot back-extrapolates to about 2–3. In buffer H, both *rUV7* and *rD19* display brief lags followed by exponential inactivation, which also back-extrapolates to about 2–3. Inactivation rates were calculated within dose ranges yielding strictly exponential inactivation using the first-order expression $N_2/N_1 = \exp[-k(t_2 - t_1)]$, where N_2 and N_1 are viable phage titers at times t_2 and t_1 and k is the inactivation rate constant expressed in lethal hits per day.

RESULTS

Among the revertants of *rII* mutants which arise as *rII*/*r*$^+$ heteroduplex heterozygotes, only those containing a revertant DNA base on the transcribed strand are detected by direct plating on lambda lysogens such as KB cells; those containing a revertant base on the complementary strand must be grown for a single cycle on a permissive host such as BB before revertants become detectable on KB cells. The mutagen hydroxylamine specifically converts cytosine and 5-hydroxymethylcytosine residues to good thymine analogues (9). An *rII* mutant revertible by hydroxylamine (G·C → A·T) therefore contains the responding 5-hydroxymethylcytosine (hereafter called cytosine) residue on the transcribed strand if the induced revertants appear upon direct plating on KB cells, but on the complementary strand if the induced revertants require a prior cycle of growth in BB cells for de-

* Present address: Biochemical Development Division, Eli Lilly and Co., Indianapolis, Ind. 46206.
† To whom reprint requests should be addressed.

Table 1. Immediate versus delayed reversion of heated *rII* mutants

Reverting mutant	Transcribed base*	Heat†	Passage	Revertants per 10^7 survivors
rUV7	C	—	—	4.4
		—	+	4.0
		+	—	330
		+	+	180
rD19	C	—	—	1.1
		—	+	0.9
		+	—	69
		+	+	28
rX655UGA‡	C	—	—	7.2
		—	+	8.0
		+	—	36
		+	+	28
rNT88	G	—	—	0.2
		—	+	0.3
		+	—	0.2
		+	+	5.1
rHS8-153	G	—	—	0.1
		—	+	0.2
		+	—	0.1
		+	+	1.4

* Mutants producing the full yield of hydroxylamine-induced revertants upon immediate plating contain cytosine in the transcribed strand, while those producing the full yield only after a cycle of growth under permissive conditions contain guanine in the transcribed strand; data of Levisohn (26) and unpublished data of R. Baltz and G. Johnson.

† *rUV7* and *rHS8-153* were heated for 24.5 hr at 31° at pH 4.6 in buffer L; *rD19* for 114 hr at 31° at pH 4.6 in buffer H; *rX655UGA* for 6 days at 23° at pH 4.6 in buffer L; and *rNT88* for 30 min at 59° at pH 4.6 in buffer L (plated on enriched H agar).

‡ Convertants from UGA to UAA were scored on CA165 (UAA suppressor) on enriched H agar, and confirmed by spot tests on KB cells.

tection. Since heat induces mutations only at G·C base pairs in T4 (5, 6), we have determined whether the mutagenic target consists of cytosine or guanine by comparing hydroxylamine- and heat-induced reversion of *rII* mutants before and after a cycle of growth in permissive cells (Table 1). The complete congruence of results with these two mutagenic treatments identifies cytosine as the common mutagenic target. The approximately 2-fold reduction in revertent frequency observed when an immediately reverting mutant is passaged in BB cells is due to the resolution of rII/r^+ heteroduplex heterozygotes into homozygous mutant and wild-type progeny.

Cytosine deamination (10–12) is an attractive mechanism for the heat-induced transition G·C → A·T. We therefore anticipated, on the basis of studies of the deamination of cytidine in solution, that the rates of heat-induced reversion of appropriate *rII* mutants would show dependencies upon the hydrogen ion concentration consistent with the involvement of pre-equilibrium protonation of the N3 of cytosine in the mutagenic reaction. Specifically, the rate of mutation should show a positive dependence upon the hydrogen ion concentration in dilute buffer between pH 6.0 and 4.0, and should reach a half-maximal value in the pH region equivalent to the pK_a of cytosine (approximately 4.5). Typical results are shown in Fig. 1. The reversion rates of *rUV7* and *rD19* are directly proportional with unit slope to the hydrogen ion concentration over a considerable pH range and exhibit "genetic" pK_a values in dilute buffer (buffer L) of 4.6 and 4.9, respectively. At pH values above 6.5 the reversion rates of both mutants show a decreased dependence upon hydrogen ion concentration. This pattern is similar to that observed for the deamination of cytidine in solution (10). The rate of reversion of *rUV13* shows only a slight positive dependence on hydrogen ion concentration in the weakly acidic range. We will discuss the possible basis for the pH-dependence of reversion of *rUV13* below. Reversion rates for all three mutants were severalfold lower in the buffer of high ionic strength (buffer H), except for *rD19* at pH values below about 3.6. The "genetic" pK_a values were also reduced in buffer H, becoming unmeasurable in the case of *rD19* because of sharply increased rates of inactivation below pH 3.

The rate of inactivation of *rUV7* as a function of pH is shown in Fig. 2a. The rate of inactivation is directly proportional with unit slope to hydrogen ion concentration between about pH 3.0 and 6.5, and is 4- to 5-fold lower in buffer H than in buffer L. Essentially identical results were obtained with *rUV13* and *rD19* except for 2- to 3-fold variations around neutrality.

In order to test whether buffer anion concentrations influence mutation rates (10), the reversion of *rUV7*, *rUV13*, and

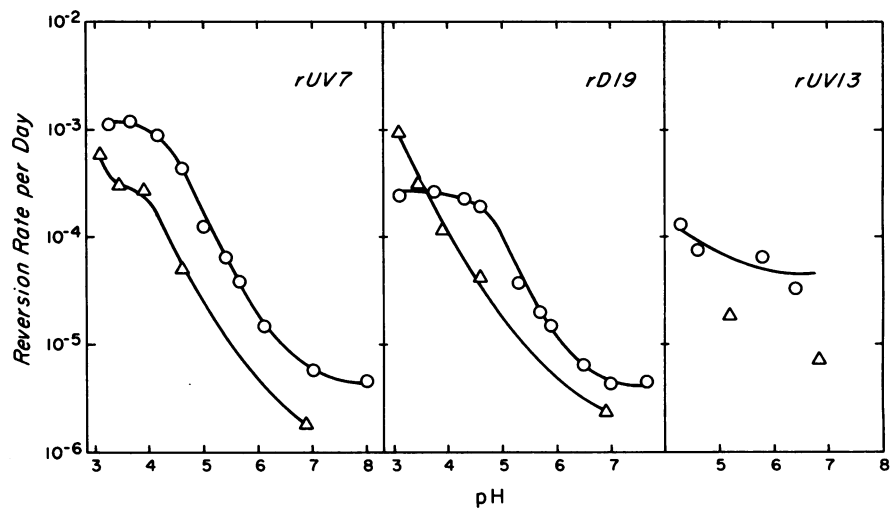

FIG. 1. Heat-induced reversion rates of *rII* mutants as functions of pH. Reversion rates were determined at 59° in buffer L (○) or buffer H (△).

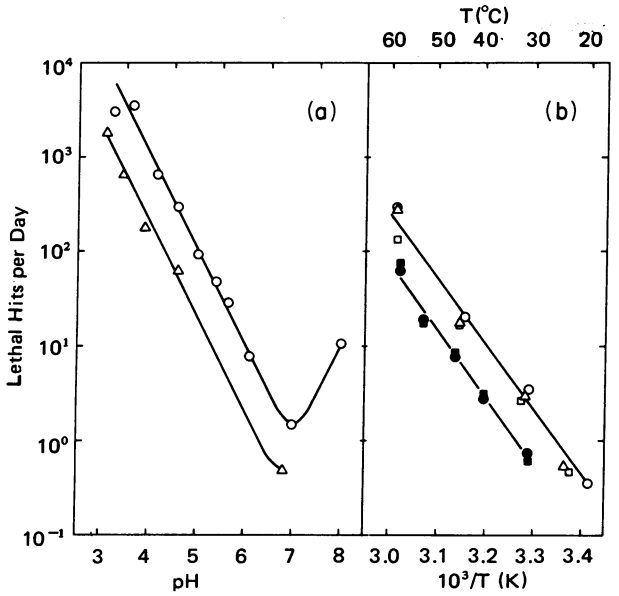

FIG. 2. Heat-induced inactivation rates as functions of pH and temperature. (a) Inactivation of *rUV7* at 59° as a function of pH in buffer L (○) and buffer H (△). (b) Inactivation of *rUV7* (circles), *rD19* (squares), and *rUV13* (triangles) at pH 4.6 in buffer L (open symbols) and buffer H (filled symbols) as a function of temperature.

rSM94 was examined at pH 5.2 at 59° at two different buffer concentrations, 1.0 and 10 mM acetate, with NaCl added in each case to maintain the total sodium ion concentration at 10 mM. No differences in mutation rates were observed.

Arrhenius plots of the temperature dependencies of the reversion rates of *rUV7*, *rD19*, and *rUV13* at pH 4.6 are shown in Fig. 3, and the corresponding activation energies are listed in Table 2. In addition to absolute mutation rates, the activation energies of mutation are site-specific, ranging from about 20 to 32 kcal/mol (84 to 134 kJ/mol) in buffer L. Both absolute mutation rates and activation energies of mutation were generally lower in buffer H than in buffer L.

Fig. 3 also shows an Arrhenius plot of the reversion rates

Table 2. Activation energies of mutation and inactivation

Phage	Buffer	pH	Activation energy*	
			Mutation	Inactivation
rUV7	L	4.6	20.5 ± 0.5	32.4 ± 1.8
	L	7.0	22.5 ± 1.7	32.1 ± 4.6
	H	4.6	17.0 ± 1.6	30.7 ± 0.5
rD19	L	4.6	32.2 ± 0.5	30.4 ± 0.6
	H	4.6	26.5 ± 1.0	33.4 ± 1.3
rUV13	L	4.6	26.7 ± 0.8	
r^+	L	4.85	26.2 ± 0.4	
	L	7.0	24.3 ± 2.0	

* Arrhenius activation energies (kcal/mol) were determined by least squares linear regression analysis; error limits represent 2 standard deviations.

of *rUV7* in buffer L at pH 7.0. Below 44° the activation energy remains essentially the same as at pH 4.6 (Table 2). The absolute reversion rates are 250-fold lower at pH 7.0 than at pH 4.6, indicating a direct proportionality between mutation rate and hydrogen ion concentration in these temperature and pH ranges. At 59°, however, the reversion rate of *rUV7* is only 80-fold lower at pH 7.0 than at pH 4.6. This deviation from linearity in the Arrhenius plot is due to the reversion of *rUV7* at two different sites. At least 50% of the revertants induced at 59° in buffer L at pH 7.0 were pseudorevertants (semi-*r* plaque morphology on B cells), whereas only about 10% of the revertants induced at pH 4.6 under otherwise identical conditions were pseudorevertants. Both types of revertants, however, arise by the same mechanism, since hydroxylamine-induced reversion of *rUV7* also generates both types. At high temperatures and neutral pH, therefore, reversion of *rUV7* occurs predominantly at the site exhibiting the higher mutational activation energy. Reversion of *rUV7* from two sites probably also accounts for the discontinuity (below pH 3.4) in the pH profile of reversion of this mutant in buffer H (Fig. 1).

The Arrhenius activation energies for inactivation (Fig. 2b and Table 2) are essentially identical in both buffers, about

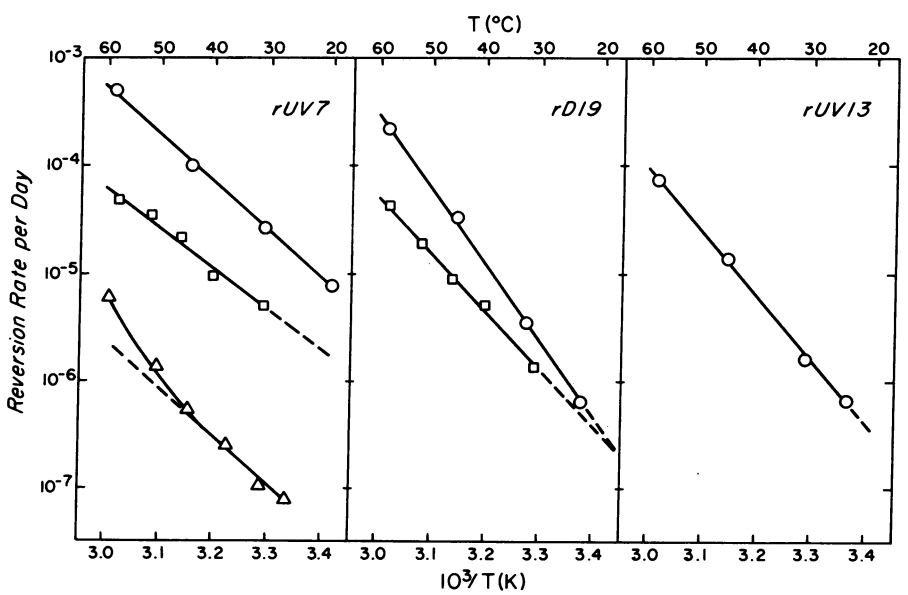

FIG. 3. Heat-induced reversion rates of *rII* mutants as functions of temperature. Reversion rates were determined at pH 4.6 in buffer L (○), at pH 4.6 in buffer H (□), and at pH 7.0 in buffer L (△).

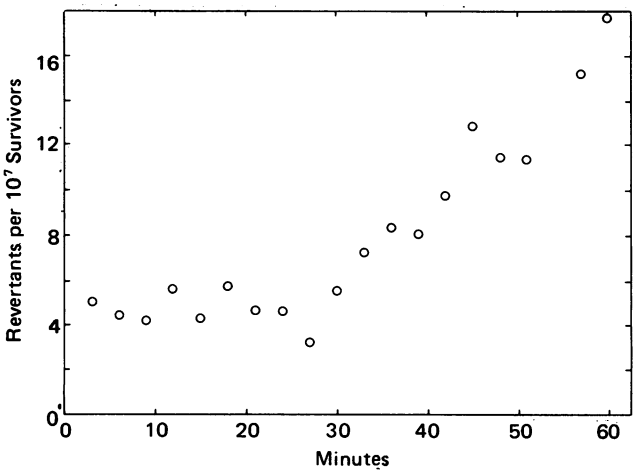

FIG. 4. Heat-induced reversion of *rSM94* in buffer L at 50° at pH 5.2.

32 kcal/mol. The absolute rates of inactivation, however, are 4- to 5-fold lower in the buffer of higher ionic strength.

Under certain conditions revertants of *rSM94* accumulate as a nonlinear function of time (Fig. 4), and similar kinetics were observed with *rUV13* at 59° at pH 7.6 in buffer L. These results, however, are not necessarily inconsistent with our hypothesis that the deamination of cytosine is the mutagenic reaction (see *Discussion*).

Arrhenius plots of total forward mutation rates ($r^+ \rightarrow r$) at pH 7.0 and 4.85 in buffer L are shown in Fig. 5. The corresponding activation energies are 26 and 24 kcal/mol at pH 4.85 and 7.0, respectively (Table 2). Upon extrapolation and correction for a different heating medium, the data of Fig. 5 are consistent with the forward mutation rates previously measured (6) at 20° and at 0°.

DISCUSSION

The characteristics of heat-induced reversion of T4*rII* mutants are in excellent agreement with the hypothesis that heat induces deamination of cytosine, thereby promoting the transition pathway G·C → A·T (10–12). All hydroxylamine-revertible *rII* mutants tested are also induced to revert by heat, and the conversion of an opal (UGA) codon to an ochre (UAA) codon occurs uniquely by this transition. The effect of passage on the response of *rII* mutants to heat and to hydroxylamine is identical, indicating that cytosine is the mutagenic target for the heat-induced transition. Both the deamination reaction and the transition pathway are promoted by hydrogen ions, and the two reactions show similar temperature dependences.

The mechanism of the deamination of cytosine has not been established (10–12). On the basis of studies on the deamination of cytidine in solution, Shapiro and Klein (10) proposed a mechanism involving pre-equilibrium protonation of the N3 position followed by a second pre-equilibrium step, buffer anion addition to the six position of the ring, and ultimately the nucleophilic displacement of the amino group. We were unable to detect a depression in the rate of the mutagenic reaction resulting from a reduction in buffer concentration. Similarly, no buffer concentration effect upon the rate of deamination of cytidylate residues in polynucleotides has been detected (11). If saturation of the pyrimidine ring system is an important step in the deamination of cytosine in polynucleotides, therefore, water may well be the saturating species (10, 11). Note also that the "genetic"

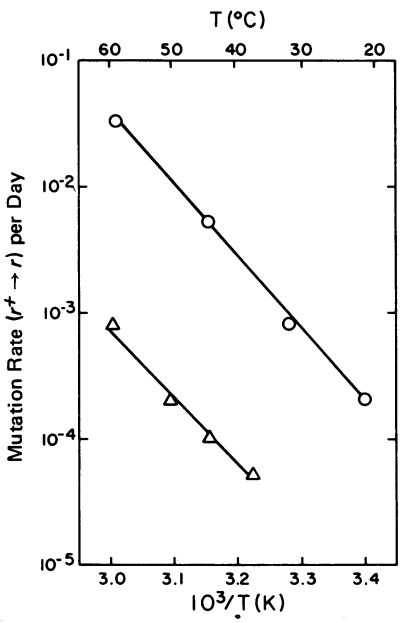

FIG. 5. Heat-induced forward ($r^+ \rightarrow r$) mutation rates. Mutation rates were determined in buffer L at pH 4.85 (○) and pH 7.0 (△).

pK_a was depressed in buffer of high ionic strength; a similar effect is observed for the ionization of bases in polynucleotides and is consistent with the proposed mutagenic reaction (13).

The rates of heat-induced reversion of different *rII* mutants are markedly site-specific, and are determined by the cytosine N3 pK_a values, the activation energies, and other factors influencing absolute mutation rates, all of which are apparently site-specific. Site-specific differences in mutation rates are generally attributed to the influence of neighboring bases or base pairs, although the mechanisms of such effects are still obscure.

Revertants of *rUV7* and *rD19* accumulate as linear functions of time, and their reversion rates show a simple dependence upon hydrogen ion concentration. Revertants of *rSM94* and *rUV13*, on the other hand, accumulate as nonlinear functions of time under some conditions, and the reversion rate of *rUV13* displays a dependence upon hydrogen ion concentration consistent with a complex mutagenic reaction mechanism. These results do not, however, necessarily indicate reversion of these mutants by different mechanisms. It seems likely that the nonlinear accumulation of revertants seen in the case of *rSM94* and *rUV13*, and the complex pH dependence of the reversion rate of *rUV13*, are manifestations of the same phenomenon. We interpret these results to indicate that the reaction leading to the deamination of cytosine is complex and involves multiple steps. Noting that the values for kinetic constants and activation parameters are site-specific, these data may be explained by assuming that the values for the rate constants corresponding to the individual steps in the reaction are such that at the *rUV7* and *rD19* sites only a single step is kinetically significant, whereas at the *rUV13* and *rSM94* sites more than a single step is kinetically significant under some conditions.

The lag period sometimes observed in the accumulation of revertants of *rSM94* and *rUV13* has not been observed in the course of chemical studies of the deamination of cytosine (10–12). The extent of reaction in our studies using a genetic assay is in the micromole-fraction range, whereas the extent

of reaction in chemical studies is typically at least four orders of magnitude greater. A reaction step that is kinetically significant on the time scale of our measurements might well be treated as a pre-equilibrium step in the context of chemical studies.

The question remains whether depurination (or depyrimidination), followed by the random insertion of a base into the progeny strand opposite the parental null-base site, could account for our results. Depurination has frequently been invoked as a mutagenic mechanism, not only in the case of heat and acid (1–4, 7), but also in the case of alkylation mutagenesis (14). This type of mechanism, however, seems unlikely for at least three reasons. First, average forward and reverse mutational activation energies are about 25 kcal/mol, whereas the average depurination activation energy under comparable conditions is about 31 kcal/mol (15), and perhaps even larger for depyrimidination (16). Second, the r^+ allele must be expressed in a lambda lysogen before DNA replication if an rII/r^+ heteroduplex is to survive (17). The random-base-insertion model requires at least one round of DNA replication to generate an r^+ allele regardless of the strand involved, whereas many *rII* mutants express the transition revertant phenotype upon immediate plating. Third, depurination mutagenesis should occur at both G·C and A·T base pairs, whereas A·T base pairs are refractory to heat-induced mutagenesis (5, 6). We cannot exclude the possibility that an error-prone excision repair process, initiated by depurinated sites, is responsible for the observed mutagenesis. There is, however, to our knowledge, no precedent for invoking such a repair process.

Phage inactivation, unlike the heat-induced transition, has many properties consistent with a depurination mechanism (15): the rate is proportional to hydrogen ion concentration below neutrality, inactivation displays an activation energy of about 32 kcal/mol, and the rate but not the activation energy is depressed at higher ionic strengths.

Heat mutagenesis must be significant well beyond the narrow confines of the *T4rII* cistrons. Despite some notable exceptions, rates of amino-acid substitution in eukaryotic evolution generally tend to be proportional to absolute time (years) rather than to biological time (generations) (18). In relatively rapidly growing microbial organisms, however, mutations accumulate in proportion to generations (17). This contradiction can be resolved if it is assumed that higher eukaryotic germ cells [and perhaps also microbial genomes in nature (19, 20)] spend relatively long times in nondividing states, and accumulate mutations by time-dependent mechanisms which on the whole outnumber mutations arising as errors of DNA replication. Heat mutagenesis is a good example of a time-dependent, generation-independent process.

Heat mutagenesis may also present a serious problem for prokaryotic organisms inhabiting hot or acid environments, such as hot springs (21), and for warm-blooded eukaryotes. At pH 7 at 37° in buffer L the forward mutation rate from r^+ to r is about 5×10^{-5} per day (Fig. 4). If we assume conservatively that this rate will decrease about 5-fold at physiological ionic strengths (Figs. 1 and 3), that 46% of the induced r mutants arise in the *rII* cistrons (6), that the efficiency of detection of base pair substitution mutants is about 10% (17), and that the *rII* cistrons contain about 1500 base pairs (22) (of which 34% are G·C base pairs), then the average T4 heat-induced mutation rate is about 4×10^{-8} per G·C base pair per day. Since the human haploid genome contains about 1.4×10^9 G·C base pairs, the endogenously heat-induced human mutation rate would be about 100 per diploid cell per day, providing that none of the mutations were repaired and making the reasonable assumption that rates of deamination are similar for cytosine and 5-hydroxymethylcytosine. Since conservative values were used in this calculation, the result is likely to be a minimal rate. It is also clearly an intolerable rate, and we must conclude that a very large fraction of these mutations either are prevented from occurring in the first place (perhaps by the binding of polyamines or chromosomal proteins to DNA) or are repaired. The transition mutations described in this report are in fact likely to be repaired (at least in *E. coli*) by the joint action of a deoxyuracil *N*-glycosidase (23) and a specific endonuclease (24), followed by gap repair.

Our results may help to explain the peculiar extraperitoneal location of the testes in most mammals. The (nude) human scrotal temperature is about 32.5° (25, 26), which would result in a 2-fold reduction in the heat-induced contribution to the spontaneous mutation rate in male germinal tissues compared to tissues at 37°.

We are grateful to Gary Johnson and Michael Saruk for their technical assistance and to Lynn Ripley for many helpful discussions. This work was supported by grants from the National Institute of Allergy and Infectious Diseases (AI04886), the National Science Foundation (GB30604 and BMS71-01243), and the American Cancer Society (VC5).

1. Zamenhof, S. & Greer, S. (1958) *Nature* **182**, 611–613.
2. Zamenhof, S. (1960) *Proc. Nat. Acad. Sci. USA* **46**, 101–105.
3. Freese, E. (1959) *Brookhaven Symp. Biol.* **12**, 63–75.
4. Freese, E. B. (1961) *Proc. Nat. Acad. Sci. USA* **47**, 540–545.
5. Drake, J. W. (1966) *Proc. Nat. Acad. Sci. USA* **55**, 738–743.
6. Drake, J. W. & McGuire, J. (1967) *Genetics* **55**, 387–398.
7. Greer, S. & Zamenhof, S. (1962) *J. Mol. Biol.* **4**, 123–141.
8. Baltz, R. H. & Drake, J. W. (1972) *Virology* **49**, 462–474.
9. Phillips, J. H., Brown, D. M. & Grossman, L. (1966) *J. Mol. Biol.* **21**, 405–419.
10. Shapiro, R. & Klein, R. S. (1966) *Biochemistry* **5**, 2358–2362.
11. Lindahl, T. & Nyberg, B. (1974) *Biochemistry* **13**, 3405–3410.
12. Garrett, E. R. & Tsau, J. (1972) *J. Pharm. Sci.* **61**, 1052–1061.
13. Michelson, A. M. (1963) *The Chemistry of Nucleosides and Nucleotides* (Academic Press, New York), chap. 8.
14. Bautz, E. & Freese, E. (1960) *Proc. Nat. Acad. Sci. USA* **46**, 1585–1594.
15. Lindahl, T. & Nyberg, B. (1972) *Biochemistry* **11**, 3610–3618.
16. Lindahl, T. & Karlström, O. (1973) *Biochemistry* **12**, 5151–5154.
17. Drake, J. W. (1970) *The Molecular Basis of Mutation* (Holden-Day, San Francisco).
18. Sarich, V. M. & Wilson, A. C. (1973) *Science* **179**, 1144–1147.
19. Koch, A. L. (1971) *Adv. Microb. Physiol.* **6**, 147–217.
20. Drake, J. W. (1974) *Symp. Soc. Gen. Microbiol.* **24**, 41–58.
21. Brock, T. D. & Darland, G. K. (1970) *Science* **169**, 1316–1318.
22. O'Farrell, P. Z., Gold, L. M. & Huang, W. M. (1973) *J. Biol. Chem.* **248**, 5499–5501.
23. Lindahl, T. (1974) *Proc. Nat. Acad. Sci. USA* **71**, 3649–3653.
24. Verly, W. G., Paquette, Y. & Thibodeau, F. (1973) *Nature New Biol.* **244**, 67–69.
25. Ehrenberg, L., von Ehrenstein, G. & Hedgran, A. (1957) *Nature* **180**, 1433–1434.
26. Levisohn, R. (1967) *Genetics* **55**, 345–348.