ON THE SPECIFICITY OF DNA POLYMERASE

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When Watson and Crick[1] proposed the structure of DNA, they suggested that the faithful replication of the base sequence involves hydrogen bonding between old and new bases. A more specific version of this model would assume that the DNA replicating enzyme(s) could connect the incoming deoxyribonucleotide triphosphates to the growing strand only if they formed the proper hydrogen bonds to the complementary bases of the parental strand; the new nucleotides would then automatically fit into the regular structure of DNA. This model would also explain the induction of mutations that occurred as a consequence of DNA base alterations.[2] The additional observation of a frequent recurrence of both spontaneous and induced mutants at specific sites (hot spots)[2-4] could be attributed either to a specificity of chemical mutagens or to a specific effect of the DNA replicating system towards certain base sequences.[5, 6]

Conceivably, the DNA replicating enzyme(s) might have such a high specificity that complementary DNA bases could be matched regardless of their hydrogen-bonding properties. The DNA replicating system would then have to recognize the individual DNA bases and somehow (by an allosteric mechanism) bring them into apposition. This possibility has been explicitly proposed by Speyer,[7] who made the important discovery that a phage T4 mutant, which produces a temperature-sensitive DNA polymerase, exhibits a greatly increased frequency of spontaneous forward and reverse mutations. Although Speyer's finding shows that the DNA polymerase is somehow involved in the selection of nucleotides that are incorporated into DNA, it does not decide whether or not the polymerase can recognize individual bases.

This paper analyzes the specificity of DNA polymerase by comparing the rates of reversion induction in phage T4-rII strains which contain the gene for either the normal or a mutant polymerase. If the altered bases would pair according to their hydrogen-bonding properties, the rate of mutation induction would be the same for both strains. If, however, the normal polymerase would recognize the bases in detail, the mutant polymerase would be expected to have an altered recognition pattern, because it produces many "spontaneous" mutations. The two polymerase types should then show a different rate of mutation induction for at least some of the base alterations inflicted by different chemicals.

Materials and Methods.—T4 phages: The rII mutants[2] were AP72 and AP275, induced by 2-aminopurine; N17, N24, and N31, induced by bromouracil; and HA11, induced by hydroxylamine; these strains have the normal DNA polymerase (= pol^n). The temperature-sensitive DNA polymerase (= pol^{ts}) was produced by strain 43 L56 of Edgar.[8, 9] Double mutants, AP72 pol^{ts}, N31 pol^{ts}, etc., were isolated from crosses between the pol^{ts} and the different rII mutants.

Phage stocks: Phages were grown in minimal medium at 30°C;[10] 10^2 to 10^3 phages were added to 20 ml resting *E. coli* bacteria B (Berkeley) (= BB) of titer 0.5–1 × 10^8/ml, aerated vigorously overnight, shaken with chloroform, and centrifuged at 7000 rpm for 5 min.

Plating bacteria: Phages were plated together with either *E. coli* bacteria B-American (= BA) or K_{12} (λ) (= K) on tryptone (T) plates[3] and incubated at 30°C. In order to prevent multiplicity-

reactivation, bacteria K were grown to 5×10^8/ml, spun, and resuspended in smaller volumes of T-broth to ensure a ratio of phages to bacteria of at most 1:50.

Phage crosses of $r^+pol^{ts} \times rII\ pol^n$: Bacteria BA at titer 2×10^8/ml were infected with T4 phages pol^{ts} and rII at multiplicities of 2.5 and 0.2, respectively. The phages were adsorbed in T-broth[3] + 0.02 *M* KCN at 37°C for 5 min. The infected bacteria were spun, resuspended and diluted in broth, and incubated for 75 min at 30°C. The lysate was plated with bacteria BA on T-plates. After 10 hr growth at 30°C, rII plaques were picked and replated on bacteria BA and K to test for the rII property and for temperature sensitivity at 42 and 30°C. Mutants, which produced at 30°C rII plaques on bacteria BA but no plaques on K and which could not or only very slowly grow at 42°C, were called rII pol^{ts}. Stocks of these phages were grown in BB at 30°C. To check their double mutant character, the new strains were backcrossed to their parents. The backcross to the rII parent was done by spot tests for growth on bacteria K.[4] For the backcross to the pol^{ts} parent, *E. coli* BA were infected with both rII pol^{ts} and r^+ pol^{ts}, at a multiplicity of 2 each, in T-broth plus 0.02 *M* KCN. The lysates, obtained as above, were plated on BA and incubated at both 30 and 42°C.

Mutagenic treatment: (1) Phages were diluted tenfold in an ice-cold solution of 1 *M* hydroxylamine in 0.05 *M* sodium phosphate + 1 *M* NaCl + 10^{-3} *M* $MgCl_2$, pH 6.2. A control sample was diluted 50-fold into ice-cold stopping mixture (0.05 *M* Tris pH 7.5 + 1 *M* NaCl + 10% acetone + 10^{-3} *M* $MgCl_2$). The reaction tube was then incubated at 37°C, and at different times aliquots were diluted into the stopping mixture. (2) Treatment at pH 4.2, 45°C (see Freese[5]). (3) Phages of titer 1×10^{11} were diluted tenfold in 0.2 *M* ethylmethanesulfonate in 0.5 *M* Na phosphate, pH 7 + 10^{-3} *M* $MgCl_2$. A control sample was further diluted 50-fold in stopping mixture containing T-broth + 0.05 *M* $Na_2S_2O_3$. Further aliquots were taken after incubation at 45°C for different times. One ml of each sample was added to 1.5 ml growing bacteria BA (2×10^8/ml) in T-broth + 10^{-3} *M* KCN (multiplicity of total phage, dead + alive, was 0.6). After 10 min adsorption at 37°C, the phages were 200-fold diluted in T-broth, aerated for 3 hr at 30°C, shaken with chloroform, and plated. (4) Nitrous acid. Phages were diluted ninefold into 0.5 *M* sodium acetate, pH 4.2, at 25 or 15°C, as stated. Freshly prepared sodium nitrite of the same temperature, 1 *M*, was diluted tenfold into the phage-buffer mix. After different times, aliquots were diluted tenfold into ice-cold 1 *M* potassium phosphate buffer + 10^{-3} *M* $MgCl_2$, pH 8.1. (5) UV treatment.[11] (6) Treatment by 2-aminopurine (AP) and 5-bromodeoxyuridine (BD). An overnight culture of bacteria BB was aerated to 5×10^8/ml in minimal medium and then diluted fivefold in the same medium containing either 500 μg/ml AP or 50 μg/ml BD. After 15 min aeration, aliquots were distributed into bubbler tubes containing phages (multiplicity 2). After overnight growth at 30°C, chloroform was added and debris later removed by centrifugation. All experiments using BD were performed in the dark. Only lysates which had approximately the same final titer were accepted, because it had been observed that lysates with low titer had an exceptionally high frequency of revertants. The cause of this apparent selection (perhaps due to lysis inhibition) was not ascertained.

Results.—Several base analogue and hydroxylamine (HA)-induced rII transition mutants of phage T4[2] were used, whose properties with respect to reversion induction are shown in Table 1. Two mutants, AP72 and AP275, apparently carry a

TABLE 1

REVERSION INDUCIBILITY OF DIFFERENT rII TRANSITION MUTANTS BY SEVERAL MUTAGENS, AND BASE PAIRS ASSUMED FOR THE MUTANT SITES

rII mutant type	Inducing Agents: Nitrous acid	2-Amino-purine	5-Bromo-deoxyuridine	Hydroxyl-amine	Low pH (pH 4.2)	Ethyl-methane-sulfonate	Assumed base pairs at mutant site
AP 72	+	+	++	++	+	+	G-C
AP 275	+	+	++	++	+	+	G-C
N 24	+	+	+	−	−	−	A-T
N 17	+	+	+	−	−	−	A-T
N 31	+	+	+	−	−	−	A-T
HA 11	+	+	+	−			A-T
HA 25	+	+	+	−			A-T

TABLE 2

SPONTANEOUS REVERSION INDICES (=AVERAGE OF REVERSION FREQUENCIES FOR SEVERAL STOCKS GROWN FROM A SMALL INOCULUM) OF SEVERAL rII MUTANTS HAVING THE GENE FOR THE NORMAL (n) OR THE MUTANT (ts) DNA POLYMERASE

rII Mutant type	Spontaneous Reversion Index in Units of 10^{-6} for Polymerase Type n	ts	Factor of increase, ts/n
AP72	1.2	5.6	4.7
AP275	1.7	14.3	8.4
N24	0.23	49.5	215
N17	0.020	2.2	110
N31	0.024	4.4	183
HA11	0.033	1.45	44
HA25	0.003	0.31	103

G-C pair at their mutant site, because they can be efficiently induced to revert by HA; the other five mutants apparently have an A-T pair at their mutant site because they cannot be induced to revert by HA but do respond to base analogues or nitrous acid. These rII markers were crossed into the temperature-sensitive (ts) mutant polymerase strain L-56. The double mutants were checked by backcrosses for both the correct ts and rII markers, as described in *Materials and Methods.*

Spontaneous reversion frequencies: Phage stocks were grown from a small inoculum. The frequencies of spontaneous revertants per phage were measured by plating on bacteria K (only revertants grow) and BA (all viable phages grow). These frequencies are expressed as K/B in units 10^{-6} throughout this paper. Table 2 shows that the mutants having a G-C pair, AP72 and AP275, have a high spontaneous reversion frequency already in the normal polymerase strain and show an increase of a factor of 5 or 8, respectively, in the mutant polymerase strain. In contrast, the mutants having an A-T pair, i.e., N and HA mutants, have a low spontaneous reversion frequency in the normal polymerase strain which is highly increased, up to a factor of 200, in the mutant strain. More important, however, than the factor of increase is the absolute value of the spontaneous mutation frequency. For the strains having the normal polymerase, the G-C mutants had a much higher spontaneous reversion frequency than the A-T mutants. In contrast, for the mutant polymerase (ts) mutants, most reversion rates were of the same order of magnitude, 1.5–14 $\times$ 10^{-6}, except for markers N24 and HA25. Marker N24 reverted in the normal polymerase strain at an exceptionally high frequency when this was compared to the frequency of other rII mutants containing A-T pairs.[2] (N24 had the highest spontaneous reversion frequency among 11 base analogue induced rII mutant strains which could not be induced to revert by HA.) The DNA site of N24 apparently is especially vulnerable to pairing mistakes leading to transitions, because forward mutations, induced by 5-bromouracil, 5-bromodeoxyuridine, and 2-aminopurine, also recurred especially frequently at this site.[2–4] Marker HA25 represents another extreme of a site which is particularly resistant to mutagenesis, because it mutated at a very low rate in both the normal and the mutant polymerase strains.[2]

Apart from the exceptions, G-C pairs of transition mutants seem to mutate spontaneously about 50 times more frequently than A-T pairs in the normal polymerase strains, whereas they mutate at a much more equal frequency in the mutant polymerase strains.

Treatment by hydroxylamine (HA): When free phages of the rII mutant types

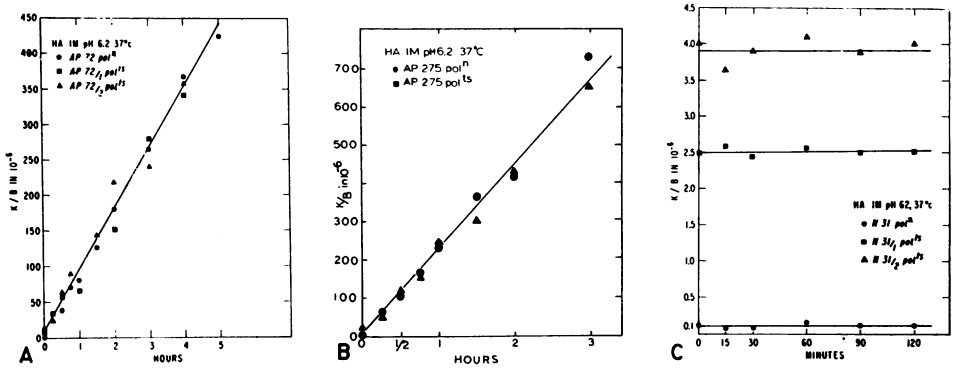

FIG. 1.—rII reversion induction of normal (n) and mutant (ts) polymerase T4 strains by 1 *M* hydroxylamine; rII mutant types: (*A*) AP72, (*B*) AP275, (*C*) N31.

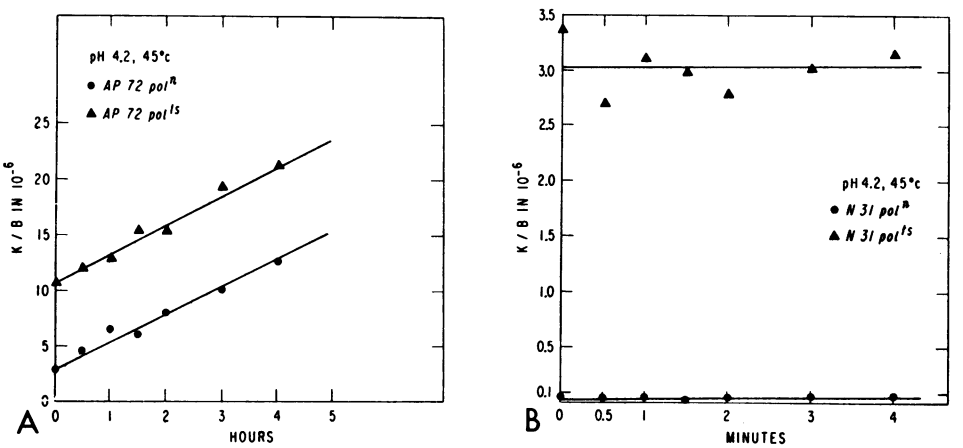

FIG. 2.—Reversion induction by pH 4.2, 45°C; rII mutant types: (*A*) AP72, (*B*) N31.

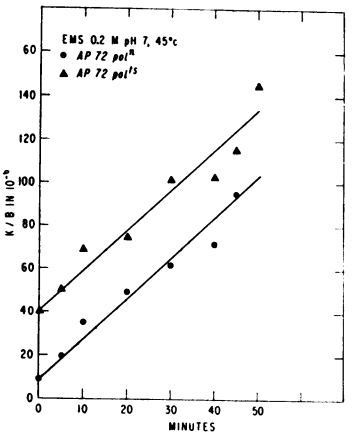

FIG. 3.—Reversion induction of AP72 by 0.5 *M* ethylmethanesulfonate.

AP72 and AP275 were treated by 1 *M* HA, pH 6.2, at 37°C, the frequency of revertants per viable phage increased linearly with the time of HA treatment (Fig. 1*A*, *B*). The rate of increase was the same, irrespective of whether the phages carried markers for the normal or the mutant polymerase. No increase of revertants was observed for all strains containing the rII mutations N17, N24, N31, HA11, or HA75 as shown for the case of N31 in Figure 1*C*.

Treatment by pH 4.2: Low pH treatment of phages at 45°C slowly induced revertants in AP72 but not in N31 containing mutants (Fig. 2*A*, *B*). No difference in rate was found between the n- and the ts-strains.

Treatment by ethylmethanesulfonate (EMS): When phages containing the AP72 marker were

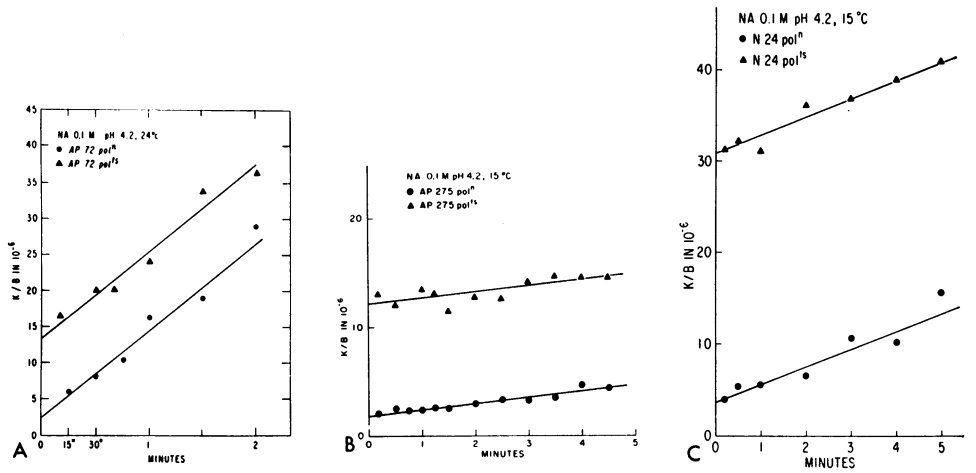

FIG. 4.—Reversion induction by 0.1 *M* nitrous acid; rII mutant types: (*A*) AP72, (*B*) AP275, (*C*) N24.

treated by 0.2 *M* EMS, pH 7.0, at 45°C, they showed the same rate of reversion induction for both polymerase strains (see Fig. 3). The N31 strains did not show any significant reversion induction.

Treatment by UV: No significant mutation induction was found in any of the strains containing markers AP72 or N31 after UV treatment at pH 6.2, 0°.

Treatment by nitrous acid: Since nitrous acid exerts in addition to its mutagenic effect a strong inactivating effect, it was necessary to control multiplicity reactivation carefully. This was achieved by using survivals $> 10^{-2}$ and a very low multiplicity of plating ($\leq 1/50$) of all (dead + alive) phages. Figure 4 shows that during the treatment of phages by 0.1 *M* nitrous acid, pH 4.2, the frequency of revertants increased at the same rate for the n- and ts-strains of all three rII markers tested, i.e., the two AP markers, corresponding to a G-C pair, and the mutant N24 having an A-T pair at the mutant site. The induced reversion rates (per lethal hit) of the other A-T pair mutants were so low that multiplicity reactivation could not be kept sufficiently low (as shown by an increase in the frequency of revertants with the square of time and a dependence on the multiplicity of infection).

Induction by 2-aminopurine (AP): Phages were grown at 30°C through essentially one lytic cycle (two phages per bacterium added) in the presence and absence of AP (500 μg/ml). Both G-C and A-T pair mutants showed reversion induction. The average frequencies of revertants, averaged over four to six lysates, are graphically represented in Figure 5. The frequency of revertants increased above the spontaneous level by about the same value for both the n- and the ts-strains of a given rII marker.

Induction by 5-bromodeoxyuridine (BD): Phages were grown at 30°C in the dark through essentially one lytic cycle in the presence and absence of BD (50 μg/ml). The average reversion frequencies are given in Figure 6. Again, essentially the same increases above the spontaneous level were found for the n- and the ts-strains.

Discussion.—As had been noted earlier,[2] rII-type transition mutants, having the normal polymerase, reverted more frequently (about 50 times) when they contained a

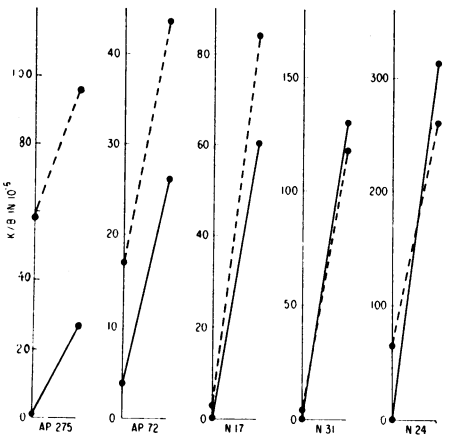

FIG. 5.—Average reversion frequencies of stocks grown for one cycle in the presence and absence of 500 μg/ml 2-aminopurine. Solid lines, strains with normal polymerase; dashed lines, strains with mutant polymerase.

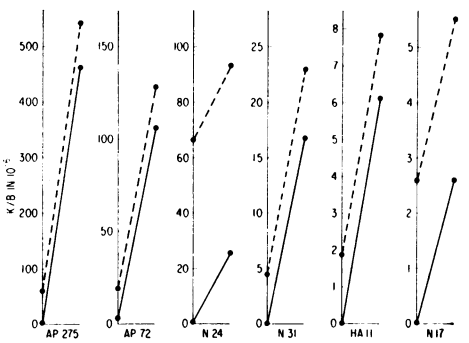

FIG. 6.—Average reversion frequencies of stocks grown for one cycle in the presence and absence of 50 μg/ml 5-bromodeoxyuridine. Solid lines, strains with normal polymerase; dashed lines, strains with mutant polymerase.

G-C rather than an A-T pair at their mutant site. This result shows that non-complementary base pairs, such as G-T, are rarely accepted by the normal polymerase, because they would mutate A-T pairs at the same frequency as G-C pairs (assuming that pairing between G-T were as frequent as that between T-G). In contrast, most rII mutants having the mutant (ts) polymerase marker exhibited spontaneous reversion indices of the same order of magnitude for both G-C and A-T pairs. Exceptions can be attributed to hot and cold spots of mutation (wh ch retain this property in the ts-strains). Such an equality of reversion indices would be expected if the mutant polymerase could frequently accept a combination of two bases belonging to different base pairs, such as G-T.

The increase in the frequency of rII-revertants, induced by different mutagens, occurred at the same rate irrespective of the type of polymerase marker (n or ts) present in the phage strains. Some of these strains contained a G-C and some an A-T pair at the mutant site and the employed agents affect these bases differently:[2, 12] hydroxylamine alters C; ethylmethanesulfonate G (and A,C), low pH G, C (and A); UV mainly forms thymine (T) dimers; nitrous acid deaminates G, C, and A; 2-aminopurine is an analogue of adenine (A), which frequently behaves like guanine (G); and 5-bromodeoxyuridine (BD) is an analogue of thymine (T), which frequently behaves like cytosine (C).

The observed equality of the rates of mutation induction for the normal and the mutant polymerase strains would be expected if the polymerase would not recognize the individual bases. In this case each altered base would itself determine its base-pairing property according to its hydrogen-bonding capacity. Deaminated or hydroxylaminated C would (nearly) always form two hydrogen bonds with A; deaminated A would similarly bind to C, whereas deaminated G would still bind to C. Ethylated G would occasionally form two hydrogen bonds to T, 5-bromouracil to G, and AP to C—each after having undergone a tautomeric shift or inducing one during the process of pairing. For both the frequently and the rarely occurring

base pairs, the correct angle and distance of the deoxyribose-phosphate moiety would be established by the two (or three) hydrogen bonds formed between the deoxynucleotide triphosphate and a base in the preexisting DNA strand.

It is thus not necessary to assume a model in which the DNA polymerase can specifically recognize individual bases in order to explain the observations on mutation induction. In fact, such a model would be difficult to reconcile with our findings: One would expect that the higher frequency of spontaneous mutations in the mutant polymerase strains were caused by some alteration in the specificity of base recognition. The specificity of the normal polymerase would not be known and one could not predict with which probability an altered base, say an altered A, would be regarded as A or G. One could not predict, therefore, which base alteration would be mutagenic (except for the extreme case of change from one normal base to another normal base). However, one would expect that the mutant polymerase would often produce a different rate of mutation induction (higher or lower) than the normal polymerase, because it should regard some of the altered bases differently. Such a difference in mutation rates has not been found.

Both earlier and present results on the induction of mutations and the specificity of DNA polymerase can be explained by the following hypothesis: The incoming deoxynucleotide triphosphate normally attaches by two or three hydrogen bonds to a base in the parental DNA strand. Subsequently the DNA replicating enzyme(s) link to the growing DNA strand those sugar-phosphate groups that are correctly located and have the correct (deoxyribose) structure. Sugar-phosphate groups which are incorrectly located (e.g., owing to hydrogen bonding between wrong groups on the bases or between noncomplementary bases) or which have the wrong structure (e.g., ribose) are rejected. The weak hydrogen bonds to a single base are formed only transiently and separate readily. Mutations may come about by spontaneous or induced alterations in the hydrogen-bonding properties of the bases, and by mistakes of the replicating system in occasionally accepting unusually hydrogen-bonded or even non-hydrogen-bonded base combinations. Many such combinations would fit into the somewhat flexible structure of DNA but apparently are usually rejected by the DNA replicating system.

A mutated polymerase may have, e.g., a different specificity for the sugar phosphate portion of the incoming deoxynucleotide triphosphate and accept for it unusual locations or structures. Depending on the particular polymerase it may thus favor either transitions (by accepting, e.g., a G-T pair which has one hydrogen bond), or transversions (by accepting, e.g., an A-G pair which has formed hydrogen bonds between the 6- and 7-positions), or deletions or insertions (by looping out or copying twice certain nucleotides). The polymerase can also be altered in its specificity by chemicals added to the cell, because Mn^{++} is known to cause the incorporation of ribonucleotides into DNA[13] and to induce mutations.[14] Other compounds influencing the polymerase will undoubtedly be found. It is even conceivable that DNA and RNA polymerase are related by evolution.

Summary.—For phage T4 rII transition mutants, containing either a G-C or an A-T pair at their mutant site, the spontaneous reversion indices were generally much higher for G-C than for A-T pairs. When a temperature-sensitive marker for DNA polymerase was crossed into these strains, the spontaneous reversion indices increased to about the same order of magnitude for both base pairs. This

indicates that the normal polymerase rejects noncomplementary base pairs (of normal DNA bases) much more effectively than the mutant polymerase.

Both the normal and the mutant polymerase strains showed the same rate of rII reversion induction by all agents used. This result agrees with a model in which the DNA polymerase recognizes the position and structure of the sugar phosphate groups of the incoming deoxynucleotide triphosphate but does not recognize the structure of the individual bases: the correct base pairing seems to be achieved by first hydrogen bonding of the bases and then rejection of incorrectly paired deoxynucleotide triphosphates by the DNA polymerizing enzyme(s).

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[1] Watson, J. D., and F. H. C. Crick, in *Cold Spring Harbor Symposia on Quantitative Biology*, vol. 18 (1953), p. 123.

[2] Freese, E., in *Molecular Genetics*, ed. J. H. Taylor (New York: Academic Press, 1963), part 1, p. 207.

[3] Benzer, S., and E. Freese, these PROCEEDINGS, **44**, 112 (1958).

[4] Benzer, S., these PROCEEDINGS, **47**, 403 (1961).

[5] Freese, E., in *Structure and Function of Genetic Elements*, Brookhaven Symposia in Biology, No. 12 (1959), p. 63.

[6] Koch, A. L., and C. Miller, *J. Theoret. Biol.*, **8**, 71 (1965).

[7] Speyer, J. F., *Biochem. Biophys. Res. Commun.*, **21**, 6 (1965).

[8] Epstein, R. H., A. Bollé, C. M. Steinberg, E. Kellenberger, E. Boy de la Tour, R. Chevalley, R. S. Edgar, M. Susman, G. Denhardt, and A. Leilansis, in *Cold Spring Harbor Symposia on Quantitative Biology*, vol. 28 (1963), p. 375.

[9] de Waard, A., A. V. Paul, and I. R. Lehman, these PROCEEDINGS, **54**, 1241 (1965).

[10] Adams, M. H., *Bacteriophages* (New York: Interscience Publishers, Inc., 1959), p. 446.

[11] For details see Freese, E. B., and E. Freese, *Genetics*, **54**, 1055 (1966).

[12] Freese, E., and E. B. Freese, *Radiation Res.*, Suppl. 6, **91** (1966).

[13] Berg, P., H. Fancher, and M. Chamberlin, in *Informational Macromolecules, A Symposium*, ed. H. J. Vogel, V. Bryson, and J. O. Lampen (New York: Academic Press, 1963), p. 467.

[14] Demerec, M., and J. Hanson, in *Cold Spring Harbor Symposia on Quantitative Biology*, vol. 16 (1951), p. 215.