GENOTYPIC REVERSION BY HYDROXYLAMINE:
THE ORIENTATION OF GUANINE-HYDROXYMETHYLCYTOSINE
AT MUTATED SITES IN PHAGE T4 *rII*¹

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HYDROXYLAMINE (HA) is known to induce point mutations by reacting with cytosine and hydroxymethylcytosine (FREESE, BAUTZ-FREESE, and BAUTZ 1961; CHAMPE and BENZER 1962; PHILLIPS, BROWN, ADMAN, and GROS-MAN 1965; JANION and SHUGAR 1965). BRENNER, STRETTON, and KAPLAN (1965) used the mutagen to identify the orientation of bases of mutated sites for mutants resulting from a base pair transition: guanine-hydroxymethylcytosine to adenine-thymine (GC → AT). In the study described in this paper, HA was used to induce *in vitro* reversion of *rII* mutants of phage T4 back to the standard (wild) type, *r*⁺, as a means to identify for the first time the orientation of bases for mutants resulting from a transition AT → GC. In contrast to *r*⁺, *rII* mutants cannot grow on a restrictive host, *Escherichia coli* strain KB (λ) whereas, like *r*⁺, they can grow on a permissive host, strain B. If it is assumed that for mutants which occur by AT → GC mutations, HA-reacted C must be in the *transcribed* strand of the infecting phage DNA molecule in order for a revertant to grow on the restrictive host, two classes of AT → GC mutants can be expected with respect to their response to HA treatment: (1) Mutants that have C in the transcribed strand will grow as well on the restrictive host as on the permissive one. (2) Mutants that have C in the nontranscribed strand will not be able to grow on the restrictive host. However, when grown on the permissive host, the HA-reacted C will lead to the incorporation of A instead of G in the transcribed strand during DNA replication, yielding newly synthesized strands identical to those of *r*⁺. Therefore, the resulting phage particles will be able to grow on the restrictive host.

This paper will show that *in vitro* treatment of GC mutants with HA indeed permits distinction between two classes of mutants—those that exhibit a high reversion index when plated directly on the restrictive host and those that do so only after being allowed to replicate first on the permissive host.

MATERIALS AND METHODS

Phage and bacterial strains: *rII* mutants of phage T4B and *Escherichia coli* strains were obtained from Drs. S. P. CHAMPE and S. BENZER, (CHAMPE and BENZER 1962).

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Phage lysates were grown on *E. coli* BB in M9S medium (CHAMPE and BENZER 1962).

E. coli B and BB support growth of both the standard (wild) type of phage T4, r^+ , as well as the growth of *rII* mutants. Strain KB(λ) supports the growth of the standard type only.

Media and reagents: Broth contained 1.3% Difco Bacto-tryptone plus 0.7% NaCl. Plates contained in addition 0.7% agar in the top layer and 0.5% Difco Bacto-meat extract plus 1.2% agar in the bottom layer.

HA reaction mixture was prepared just prior to use according to FREESE *et al.* (1961): 1.25M $\text{NH}_2\text{OH}\cdot\text{HCl}$ (General Chemical Division) plus 1.0M NaCl, 0.001M MgSO_4 and 0.075M Na_2HPO_4 , adjusted to pH 7.5 with NaOH.

HA stopping mixture (FREESE *et al.* 1961) contained 0.5% Difco Bacto-tryptone plus 6% NaCl and 2% acetone.

Treatment with HA and adsorption to *E. coli* B: Ice-cold phage lysate containing about 2×10^{11} phage/ml was diluted fivefold into ice-cold HA reaction mixture. A sample taken im-

TABLE 1
Response of rII mutants to treatment with HA in vitro

Mutant No.	Previously assigned base pair*	Reversion index (KB/B) in units of 10^{-7}		4-hour sample		Indicated base in <i>rII</i> transcribed strand
		Zero sample Direct plating	After growth on B	Direct plating	After growth on B	
1 <i>r</i> AP129	GC	12	19	820	3400	C
2 <i>r</i> AP218	GC	0.16	0.8	880	1100	C
3 <i>r</i> H221	GC	0.05	0.9	<u>0.84</u>	<u>580</u>	G
4 <i>r</i> AP100	GC	0.5	1.0	2200	1300	C
5 <i>r</i> 607	GC	0.06	1.5	2400	4300	C
6 <i>r</i> EM114	GC	0.6	1.2	390	310	C
7 <i>r</i> NT88	GC	0.2	1.4	<u>2.4</u>	<u>320</u>	G
8 <i>r</i> DAP56	GC	3.2	1.8	1400	1700	C
9 <i>r</i> 380	GC	0.5	1.6	350	310	C
10 <i>r</i> SN103	GC	0.26	2.4	<u>26</u>	<u>1900</u>	G
11 <i>r</i> 263	GC	5.6	12	3200	9500	C
12 <i>r</i> F72	GC	1.2	6.5	490	1300	C
13 <i>r</i> J33	GC	1.0	6.2	<u>22</u>	<u>1200</u>	G
14 <i>r</i> BC35	GC	0.15	0.27	3400	1300	C
15 <i>r</i> AP275	GC	0.74	2.3	2500	3000	C
16 <i>r</i> UV363	GC	0.33	1.3	<u>16</u>	<u>680</u>	G
17 <i>r</i> UV181		0.2	0.5	0.2	0.7	
18 <i>r</i> G178		0.05	0.9	0.9	0.9	
19 <i>r</i> 2074		4.4	15	10	44	
20 <i>r</i> 609		0.6	3.5	0.9	6.6	
21 <i>r</i> 1221		1.4	1.1	1.8	11	
22 <i>r</i> HB118	AT	0.06	0.1	0.2	0.8	A*
23 <i>r</i> N74	AT	0.07	0.3	0.02	0.1	T*
24 <i>r</i> 1272	Deletion	0.00	0.00	0.00	0.00	

Phage stocks were treated with 1.25M HA at pH 7.5 and 37°C. Samples taken at time zero and 4 hours later were plated directly on *E. coli* KB and B and also plated after being allowed first to grow on *E. coli* B in broth. (See MATERIALS AND METHODS for details.)

* CHAMPE and BENZER (1962).

mediately after dilution and another taken after a 4 hour incubation at 37°C were diluted 50-fold into ice-cold HA stopping mixture. Part of each stopped phage mixture was plated on *E. coli* KB and *E. coli* B, without further treatment. Another part of each stopped phage mixture was added to a culture of exponentially growing *E. coli* B at a multiplicity of 0.2 to 0.3 phage/bacterium, incubated for 21 to 23 minutes at 37°C, shaken vigorously with chloroform—to lyse prematurely the infected cells—and plated on both bacterial strains for plaque counting. Tiny plaques on strain KB, representing mostly false revertants, were not counted.

RESULTS

Lysates of 24 *rII* mutants were treated with HA. Samples taken at time zero and after 4 hours exposure were diluted into stopping mixture. Logarithmically growing cultures of *E. coli* B were infected with portions of these samples and lysed prematurely. The samples were plated on *E. coli* B and *E. coli* KB and the resulting plaques were counted. (For details see MATERIALS and METHODS.)

The first 21 mutants in Table 1 are clearly induced to revert by 2-aminopurine and 5-bromodeoxyuridine, according to CHAMPE and BENZER (1962). They found that the first 16 of these, given approximately in the order of their position on the genetic map, also showed genotypic reversion due to exposure to HA. Thus CHAMPE and BENZER suggested that these 16 mutants have a GC base pair at the mutated site. Mutants *rHB118* and *rN74* have an A and a T respectively at the mutated site of the DNA transcribed strand, according to these authors. Mutant *r1272* is a deletion mutant lacking the entire *rII* region. Table 1 shows that none of the latter three mutants reverted owing to exposure to HA.

Among the 16 GC mutants, 11 mutants showed high reversion either when plated directly after the HA treatment on *E. coli* KB or when first allowed to grow on strain B. However, five GC mutants (*rH221* and *rNT88* in cistron A, *rSN103*, *rJ33*, and *rUV363* in cistron B) showed a large increase in reversion index only when the HA-treated phages were allowed first to grow on strain B. The plaque morphology of these revertants on strain B was like that of the standard type, i.e. there was no indication of false reversion.

The remaining five mutants which respond to 5-bromodeoxyuridine failed to show a large increase in reversion index by HA even after growth on the permissive host. Apparently they are GC → AT mutants. According to FREESE (1963) 5-bromodeoxyuridine induces both AT → GC and GC → AT transitions.

DISCUSSION

The assumption that the initial effect of the backward mutation has to be in the transcribed strand of the infecting DNA in order for a revertant to grow on the restrictive host seems to be only partially valid in view of the data presented above. Five mutants treated with HA showed a high reversion index only after growth on the permissive host. However, they did show a small, but significant, increase in reversion index when plated directly on the restrictive host. Following infection with *T4r⁺* or *T4rII* the DNA synthesized in infected cells contains hydroxymethylcytosine, i.e., it is phage-specific (NOMURA 1961). The synthesis of phage DNA starts only 5 to 10 minutes after infection at 37°C (COHEN 1948).

During the first 10 minutes of DNA production the rate of synthesis is about the same in T4 r^+ and T4 rII infected cells and 10 to 15 phage DNA units are synthesized per cell (GAREN 1961). The small increase in reversion index is apparently due to a minority of cells which is able to produce phage yield even when "standard" rII protein—" rII^+ protein"—appears only after DNA synthesis started.

It must be emphasized that, as in other mutagen studies, the base assignment depends on the assumption that the reversion involves a change at the original mutated site back to the original base pair.

The fact that there are mutants which show a high induced reversion index only when extensive DNA replication is allowed to take place first is an additional evidence that, *in vivo*, physiologically active messenger RNA is formed on the template of only one strand of the DNA molecule.

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SUMMARY

rII mutants of phage T4 were mutagenized with hydroxylamine. Out of 16 mutants that have a guanine-hydroxymethylcytosine base pair at the mutated site, 11 showed a high reversion index when plated directly on the restrictive host, whereas five did so only when allowed first to replicate on a permissive host following the exposure to hydroxylamine. It is suggested that the former class has hydroxymethylcytosine at the mutated site of the transcribed DNA strand whereas the latter class has guanine there.

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