Nonsense Mutants in the rII A Cistron of Bacteriophage T4¹

ROBERT D. SCHWARTZ² AND VERNON BRYSON

Institute of Microbiology, Rutgers University, The State University, New Brunswick, New Jersey 08903

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After *in vitro* treatment of bacteriophage T4 with hydroxylamine (HA), 54 nonsense mutants in the rII A cistron were isolated. These mutants were characterized by growth on suppressor strains of *Escherichia coli*, and the mutational sites were mapped in the rII A cistron. Twenty-five (9 sites) were amber (UAG), 20 (6 sites) were opal (UGA), and 9 (6 sites) were ochre (UAA). Mapping experiments further indicated that there were three closely linked pairs of amber and opal mutations, conceivably involving mutations occurring in adjacent nucleotides. Based on the specificity of HA mutagenesis (GC → AT), the amino acid codons in which the mutations occurred have been inferred. It is suggested that the three amber-opal pairs arose in tryptophan codons (UGG) and the six ochre mutants arose in glutamine codons (CAA). The six unpaired ambers and the three unpaired opals have been tentatively assigned to glutamine codons (CAG) and arginine codons (CGA), respectively, in the wild-type phage.

In 1961, Benzer and Champe (5) identified and described ambivalent rII mutants of phage T4. These were defined as mutants that did not grow in most strains of *Escherichia coli* K-12 (λ) but that could grow in certain mutants of the originally nonpermissive hosts.

It has since been demonstrated that each ambivalent mutant contains a nonsense mutation of which there are three types: amber (UAG), ochre (UAA), and opal (UGA). The hosts in which the phages can grow contain suppressor mutations which overcome, more or less effectively, the polypeptide chain-terminating effect of nonsense mutations during translation of messenger ribonucleic acid (mRNA) at the ribosome (10).

The work reported here aims to relate further the origin of nonsense codons to specific sense codons from which the former might be derived and to locate the corresponding mutations on the rII A cistron. Also, an attempt is made to estimate the degree of saturation of this short segment of the map, by means of hydroxylamine (HA)-induced nonsense mutations.

HA can act as an *in vitro* mutagen. The major

and probably exclusive effect is to cause cytosine → thymine transitions (9, 12). For a diagram of HA-induced mutations to amber, ochre, and opal and for an interpretation of the relation between the read (sense) and non-read (anti-sense) strand of deoxyribonucleic acid (DNA), reference may be made to Fig. 2 in the paper by Stretton (13).

MATERIALS AND METHODS

Organisms used. The bacterial and viral strains used in the isolation, identification, and characterization of rII mutants are listed in Table 1, with their significant characteristics and primary use. All of the T4 rII deletion mutants used have been listed previously by Benzer (4).

Media. PAGFe medium was used for the preparation of high-titered lysates. It contained, per 100 ml: 0.8 ml of 0.01% FeCl₃, 5 ml of 10% glucose, 1 ml of a solution containing 10% NH₄Cl plus 2.5% MgSO₄·7H₂O, and 5 ml of a solution containing 14.2% Na₂HPO₄ plus 6% KH₂PO₄. Bacterial strains were maintained on nutrient agar (Difco) slants. Hershey enriched nutrient agar was employed for phage assays and contained, per liter: 13 g of agar (Difco), 10 g of tryptone (Difco), 5 g of NaCl, 2 g of sodium citrate, and 1.3 g of glucose. This was used as the "bottom agar" in the agar overlay procedures. The "top agar" contained, per liter: 8 g of tryptone (Difco), 8 g of agar (Difco), and 5 g of NaCl. For the routine cultivation of all bacteria and for dilution of phage, we used T broth containing 10 g of tryptone (Difco) and 5 g of NaCl per liter.

Phage assays. Phage assays were performed by the

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² Present address: Nova University, Life Sciences Research Center, Fort Lauderdale, Fla. 33314.

TABLE 1. *Bacterial and virus strains*

Strain	Significant characteristics	Primary use
<i>E. coli</i> BB	Wild type	Lysate preparation
<i>E. coli</i> BA	Wild type	Visualization of r plaques
<i>E. coli</i> K-12 (λ)	Wild type, non-permissive for rII	Identification of rII mutants
<i>E. coli</i> KB3	Amber suppressor (probably same as su_{rII})	Identification of amber mutants
<i>E. coli</i> K-12 (λ) CA 165	Ochre suppressor su_B^+	Identification of ochre mutants
<i>E. coli</i> K-12 (λ) CAJ64	Opal suppressor su_{UGA}^+	Identification of opal mutants
T4D	Wild type	Source of rII mutants
T4 am	Amber mutant	Control
T4 oc	Ochre mutant	Control
T4r X655	Opal mutant	Control
T4r 1364	A cistron deletion	Complementation test to differentiate rIIA from rIIB cistron mutants
T4r 196	B cistron deletion	

agar overlay method (1). Plates overlaid with bacteria containing a suppressor gene were incubated at 28 to 30 C overnight. All other plates were incubated at 37 C overnight.

Spot test. For spot tests, agar overlay plates were prepared with the desired bacteria, but without phage, unless otherwise stated. The plates were permitted to solidify for 15 min and then were refrigerated for at least 15 min. Phage was deposited on the seeded plates by using strips of Whatman no. 1 filter paper.

Lysate preparation. Crude lysates were prepared by inoculating a test tube containing 3 ml of *E. coli* BB (grown in T broth to 2×10^8 cells/ml) with a single plaque and incubating the tube on a shaker for 10 hr at 37 C. We then added a few drops of chloroform to lyse bacteria, and the tubes were titrated for phage.

High-titer lysates were prepared in bubbler tubes in a water bath at 37 C by using *E. coli* BB grown to a concentration of 5×10^8 bacteria per ml in PAGFe medium. The bacterial cultures were inoculated with phage at a multiplicity of infection of 0.1. Immediately after the addition of phage, 0.1 ml of a solution of 5 μ g of DL-tryptophan per ml was added per 100 ml of lysate. Lysis was allowed to continue for 8 to 10 hr,

chloroform was added, debris was precipitated by centrifugation, and the supernatant fluid was removed to a sterile tube and titered.

HA mutagenesis. A high-titer lysate (2.5×10^{11} phage/ml) was prepared from a single plaque of T4D (wild type) isolated on *E. coli* K-12 (λ). The HA solution contained 5.25 g of hydroxylamine hydrochloride plus 2.9 g of NaCl diluted to 50 ml with 1 N NaOH. The pH was 5.95. This was prepared just before use. To induce mutation, 2 ml of HA solution was added to 0.2 ml of phage. The mixture was incubated for 12 hr at 30 C. The reaction was stopped by diluting 0.1 ml of the HA mixture into 10 ml of ice-cold stopping solution containing 10 g of tryptone (Difco), 58.5 g of NaCl, and 20 ml of a 2% solution of acetone, per liter. The dilution was kept in an ice bath for 10 min and then diluted another 10^3 - and 10^4 -fold (Bautz, *personal communication*). This procedure was carried out only once, all mutants being isolated from the last two dilutions.

Isolation and identification of rII mutants. Agar overlay plates were prepared by using *E. coli* BA and enough phage from the HA-treated sample to yield about 100 plaques per plate. After overnight incubation of plates, those plaques which appeared to contain rapid lysis mutants (large, clear plaques) were cut from the agar and added to about 1 ml of T broth. We added a few drops of chloroform, and the contents of the tube were mixed. Samples were spotted on two plates, one seeded with *E. coli* BA and the other with *E. coli* K-12 (λ). The isolates which grew on BA but not on K-12 (λ) were presumed to be rII mutants.

Characterization of rII mutants as to type and map location. Characterization of mutants as amber, ochre, or opal and localization in the A or B cistron were determined by spot test. Agar overlay plates containing one of the three suppressor strains were prepared. Dilutions of the crude rII lysates containing 10^7 to 10^8 phage/ml were spotted on each plate. Growth in the presence of the amber suppressor or in the presence of both amber and ochre suppressors indicated an amber mutant. Growth only in the presence of the ochre suppressor was indicative of an ochre mutant. Growth restricted to the opal suppressor indicated an opal mutant.

Complementation tests were used to distinguish between A and B cistron mutants. Two agar overlay plates were prepared: one contained *E. coli* K-12 (λ) plus 10^8 to 2×10^8 particles of T4 r1364 (A deletion); the other contained *E. coli* K-12 (λ) plus 10^8 to 2×10^8 particles of T4 r196 (B deletion). The mutants being tested were spotted on each plate.

Crosses for mapping mutants. Mapping was done by the method of Benzer (3). About 10^9 phage of the mutant to be mapped, and about 10^9 phage of a known deletion mutant were added to a test tube to which we then added 0.5 ml of *E. coli* BB titering 10^8 to 2×10^8 ml. The mixture was incubated at 37 C for 7 to 8 min to allow adsorption to occur, followed by spotting a sample of the mixture on a plate previously seeded with *E. coli* K-12 (λ). The plates were incubated overnight at 37 C. All mutants found to map within the same "segment" (5) were crossed to each other to

determine whether the mutants were different or were repeats of each other. Failure to recover wild-type recombinants in crosses between nonsense mutants belonging to different classes and found to map within the same segment was taken to indicate that the mutations were closely linked, possibly occurring in adjacent nucleotides.

RESULTS

After *in vitro* treatment of phage with HA, 239 T4 rII mutants were isolated. The mutants were characterized by growth on suppressor strains and mapped by the deletion method, phage crosses, and complementation tests.

Fifty-four of the mutants are of the nonsense type in the rII A cistron. Twenty-five are amber (UAG) and are distributed among 9 sites, 20 are opal (UGA) and are distributed among 6 sites, and 9 are ochre (UAA) and are distributed among 6 sites.

Table 2 lists the amber, opal, and ochre mu-

TABLE 2. *Nonsense mutants, segment location, and number of sites previously found within the segment*

Mutant type	Mutant no.	Segment ^a	No. of sites ^a
Amber	58, 100, 125, 184, 200, 206 ^b	A2h1b1	1
	4, 41, 101, 117, 193	A3a	4
	40	A4d	8
	67	A5a	3
	119, 148	A5c2a2	8
	13, 15, 57, 190	A6a1 ^c	13
	48, 129	A6a1 ^c	13
	74, 147, 201	A6c1 ^c	21
	170	A6c1 ^c	21
Opal	28, 86, 135, 136, 194, 204	A3a	4
	43, 87, 219	A3d	6
	111, 114, 140, 216	A3g2	1
	5, 115	A4c	4
	35, 42	A5c2a2	8
	49, 144, 183	A6c1	21
Ochre	20	A1a	6
	2, 177, 202	A1b2	17
	3	A2a ^d	5
	88, 94	A2a ^d	5
	7	A3e	4
	22	A4e	8

^a From Benzer (4).

^b Mutants listed on each horizontal line represent duplicate isolates of the same mutation.

^c Segments in which two different amber mutants have been found.

^d Segments in which two different ochre mutants have been found.

tants isolated, along with their segment location and the number of sites previously found to be within the segment (4). Each site represents a different point mutation. Therefore, the number of sites known to exist within a given segment gives a rough estimate of the minimal size of the segment. The number of sites and the segment designations are from Benzer (4). Figure 1 shows the location of the mutants according to segment.

As can be seen from the map in Fig. 1, 21 different nonsense mutants have been found. Segment A2a contains two different ochre mutants. Segments A6a1 and A6c1 each contain two different amber mutations.

Three segments have been found which contain both amber and opal mutants: A3a, A5c2a2, and A6c1. Phage crosses, with representative mutants from each site, indicate that *am* 170 and *op* 49 are paired and distinguishable from *am* 147 found in the same segment (A6c1). In the other two examples, the crosses gave similar evidence for the existence of amber-opal pairs.

DISCUSSION

Twenty-one sites are involved in the distribution of the 54 different nonsense mutants that have been isolated and mapped in the rII A cistron of phage T4 after HA mutagenesis. Nine sites contain amber (UAG), six contain ochre (UAA), and six contain opal (UGA) mutations. Mapping experiments further indicate that there are three closely linked pairs of amber and opal mutations.

As a result of HA mutagenesis, a cytosine (C) in the sense strand of the DNA can undergo transition to a thymine (T). The corresponding change in the mRNA would be guanine (G) to adenine (A). If the C occurs in the anti-sense strand of the DNA, the transition will be C to T in the anti-sense strand but G to A in the sense strand. The corresponding change in the mRNA would be C to uracil (U). The latter change would require replication, whereas the former would not (7, 13, 15). In the present studies, replication was permitted to occur before isolation of the mutants; therefore, mutants resulting from transitions in both the sense and anti-sense strands have been isolated.

The sensitivity of the spot test crosses is such that recombination between mutants which give 0.01% recombinants in standard crosses is easily detected. The separation of adjacent nucleotides corresponds tentatively to about 0.003% recombination (2). From this, it follows that recombination between two nucleotides, separated by two intervening nucleotides, might be detected unless excluded by the nonuniformity of recombination over small distances. In the

FIG. 1. Map of the A cistron showing the location of the nonsense mutants by segment. The size of a given segment is proportional to the number of sites known to exist within the segment. The position of a mutant within a given segment is not known. Segment designation from Benzer (4).

present investigation, it is assumed that failure to detect recombination between mutants of different nonsense type indicates that the mutations probably occur in adjacent nucleotides (amber-opal pairs).

The existence of amber-opal pairs may be explained if both mutations occur in the same tryptophan codon (UGG; 8, 16). Whether an amber or opal mutant is produced depends on which C, in the sense strand of the DNA, is the target of HA mutagenesis. Mutation of the internal C will result in an amber mutant (UAG); mutation in the terminal C will result in an opal mutant (UGA). The ratio of ambers to opals isolated at the three amber-opal sites is 8:11 (i.e., segment A3a, five amber and six opal; segment A5c2a2, two amber and two opal; segment A6c1, one amber and three opal). Although the numbers are too small for statistical significance, this suggests that the internal and terminal C bases are equally susceptible to the action of HA.

The remaining six amber mutants (unpaired) may arise from either a tryptophan codon (UGG) or a glutamine codon (CAG). The former would result from a mutation in the sense strand of the DNA, and the latter from mutation in the anti-sense strand. Because no opal mutants have been found paired with these ambers, it may tentatively be concluded that they arose from glutamine codons (CAG → UAG).

The opal mutations (UGA) can arise from tryptophan (UGG) or arginine (CGA) codons.

The tryptophan origin signifies a mutagenic event in the sense strand of the DNA, and the arginine origin signifies a mutagenic event in the antisense strand. By the previous arguments, the three paired opals probably arose from tryptophan codons. The three unpaired opal sites may be presumed to have come from arginine codons.

Ochre mutations (UAA) may be induced in the tryptophan codon (UGG) or the glutamine codon (CAA). The former is unlikely, since (i) it would require two simultaneous mutations occurring in adjacent nucleotides and (ii) one would expect to find the products of a single mutagenic event (amber or opal) paired with the ochre mutations. The latter was not found. Therefore, the ochre mutations have probably been induced in the glutamine codon (CAA).

In summary, the paired amber-opal sites indicate the presence of tryptophan codons (UGG); the unpaired amber and opal mutations may tentatively be assigned to glutamine (CAG) and arginine (CGA) codons, respectively, and the ochre mutants suggest the presence of glutamine (CAA) codons in the wild-type phage. Thus, there are at least 3 tryptophan residues, 6 and possibly 12 glutamine residues, and, tentatively, 3 arginine residues in the wild-type phage protein of the A cistron.

Four amber-opal combinations have previously been described in the rII region. In the A cistron, amber rN97 is found with opal rx665. In the B cistron, amber rHB74 and opal rx655 are paired, amber rx237 and opal rN65 are paired (8), and amber rHB232 and opal rHB243 are paired

(W. H. McClain, Ph.D. Thesis, Purdue University, 1968).

Amber rN97 is located in the A3a segment (Champe, *personal communication*), suggesting that the amber-opal pair isolated in this investigation and found in segment A3a is identical to the pair described by Brenner et al. (8). The other two pairs (segments A5c2c2 and A6c1) represent newly identified combinations.

McClain and Champe (11) used ^{14}C -labeled tryptophan as an aid in identifying a peptide coded by the rII B cistron. Genetic arguments had suggested the presence of this amino acid in the rII protein (7). Since tryptophan occurs relatively infrequently in proteins (6, 14), it can be present or absent in polypeptide fragments of considerable size, thereby facilitating identification. In the present investigation, genetic evidence suggests the existence of at least three tryptophan residues in the rII A cistron protein, the presence of which suggests that the McClain and Champe technique could be used for further analytical work on the rII products.

Since the amino acid codon assignments for the origins of the unpaired amber and opal mutants (glutamine and arginine) require verification, it would be premature to conclude that the distribution of these amino acids is atypical. However, it is reasonable to assume that the ochre mutations have arisen from CAA glutamine codons (13). Six ochre sites have been identified, and it is interesting to note that four of the sites are found within the first five segments (A1a to A2a). A total of 40 sites, of an entire sum of about 240 known within the A cistron, have been found within these five segments (5). Thus, this region represents a relatively short piece of DNA. Glutamine may be locally enriched in the corresponding peptide, a possibility that may aid in identifying an rII A cistron peptide by methods based on the McClain and Champe procedure and utilizing ^{14}C -glutamine.

Finally, it is of interest to know the degree of saturation of the map achieved with HA-inducible nonsense mutations. Twenty-one sites have been found. The number of undiscovered sites may be estimated by applying the Poisson distribution to the number of single-occurrence (seven) and double-occurrence (five) sites (Table 2). Assum-

ing that the mutations at these sites follow the Poisson distribution, this calculation predicts about five sites yet to be found, or a saturation of approximately 80%.

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