

gestive that conformational preference is the factor responsible for the magnetic nonequivalence of the methylene protons of IV.

Further support for this conclusion is provided by the n.m.r. spectrum of the substituted cyclobutenone VI (Fig. 2). The resonance centered on 113 cps is of the methylene protons of the ethyl group of this compound and is the rather complicated AB part of an ABC₃X system. The resonance of the methine proton at the 4-position of the cyclobutene ring is centered on 233 cps and is split into two equally intense doublets, rather than a 1:2:1 triplet. This splitting is most simply explained as the result of unequal coupling between the methine proton and the two adjacent methylene protons, arising from a preference for a conformation for the molecule in which the methine proton is *trans* to one methylene proton and *gauche* to the other.

* Supported in part by the Office of Naval Research and the National Science Foundation.

† Contribution No. 2842.

¹ Pople, J. A., W. G. Schneider, and H. Bernstein, *High-Resolution Nuclear Magnetic Resonance* (New York: McGraw-Hill Book Co., 1959), pp. 88, 119-123.

² Nair, P. M., and J. D. Roberts, *J. Am. Chem. Soc.*, **79**, 4565 (1957).

³ Shafer, P. R., D. R. Davis, M. Vogel, K. Nagarajan, and J. D. Roberts, these PROCEEDINGS, **47**, 49 (1961).

⁴ Kaplan, F., and J. D. Roberts, *J. Am. Chem. Soc.*, **83**, 4666 (1961).

⁵ Pople, J. A., *Mol. Phys.*, **1**, 1 (1958).

⁶ Waugh, J. S., and F. A. Cotton, *J. Phys. Chem.*, **65**, 562 (1961).

⁷ Prepared by reaction of methyl bromide-*d*₁ with sodium 1-cyclopropylethoxide.

A CHANGE FROM NONSENSE TO SENSE IN THE GENETIC CODE

BY SEYMOUR BENZER AND SEWELL P. CHAMPE

DEPARTMENT OF BIOLOGICAL SCIENCES, PURDUE UNIVERSITY

Communicated May 28, 1962

For the genetic information in a cistron to be translated into a polypeptide chain' each coding unit in the nucleotide sequence must correspond to one of the twenty or so amino acids. If not every possible sequence corresponds to an amino acid, mutations that substitute one base for another could, in certain cases, cause the continuity of the information to be interrupted, and such "nonsense" mutations would block completion of the polypeptide chain.

By virtue of a mutant with special properties, it is possible to identify nonsense mutations within the A cistron of the *rII* region of phage T4. In this paper the criterion for nonsense is applied to certain "ambivalent" *rII* mutants,¹ i.e., ones whose phenotypes can be reversed by suppressor mutations in the bacterial host, *Escherichia coli*. The results show that an ambivalent mutation that behaves like nonsense in one bacterial host may nevertheless make sense in a second (suppressor containing) host. This suggests that a suppressor mutation in the bacterium can result in addition to the cell's dictionary of a new sensible coding unit, constituting a change in the genetic code of the bacterial cell.

The r1589 System as a Genetic Test for Nonsense Mutations.—The *rII* genetic region of phage T4 consists of two contiguous regions, A and B, each behaving as a

separate functional unit or cistron.² For the phage to grow in *E. coli* strain KB, both the A and B activities are needed. This requirement may be satisfied by infecting the cell with two mutants, one damaged in the A, the other damaged in the B, in which case the mutants are said to complement one another. Full complementation occurs when one puts together any A mutant and any B mutant having point mutations or deletions of any size within the cistron boundaries. Thus, ordinarily, a defect in one cistron has no effect upon the functioning of the other. As illustrated in Figure 1, each cistron can be thought to produce a specific messenger RNA molecule that is in turn translated into a polypeptide chain.

An exceptional mutant ($r1589$) has been described in which the two cistrons are effectively joined together.³ In $r1589$, a segment is deleted that includes the divide between the cistrons and a portion of each. The A function is thereby lost, but the B function remains, as shown by the fact that $r1589$ will complement with any mutant that provides an intact A cistron. Apparently, the deleted tip of the B cistron is nonessential. However, due to the absence of the cistron-dividing element, the B fragment in $r1589$ no longer functions independently of the A. Crick *et al.*⁴ showed that the B function of $r1589$ can be turned off by crossing certain deletions into the A fragment. The same effect was produced by some mutations induced by proflavine and believed to be single nucleotide deletions or additions. Their explanation of this effect was that the nucleotide sequence is read in successive coding units starting from a fixed point, so that a deletion in the A cistron of length not equal to an integral multiple of the coding ratio would cause a shift of the reading frame, with disastrous effect on the translation of the B fragment. Thus, the properties of $r1589$ can be understood if the A and B fragments are transcribed into a single messenger RNA which is then translated into a single polypeptide chain, as shown in Figure 2A.

As illustrated in Figure 2B, the B function of $r1589$ might also be impaired, without a shift in the reading frame, by a substitution mutation changing a coding unit into one that does not correspond to any amino acid. In such a case, protein synthesis could not continue beyond that point. Substitution mutations of the "transition" type,⁵ i.e., such that one base pair is exchanged for another and the orientation of purine and pyrimidine with respect to the two DNA chains remains unchanged, can be identified by the fact that they are inducible to revert by DNA base analogues such as 2-aminopurine. To test whether an analogue-revertible A cistron mutation is nonsense, it is inserted (by genetic recombination) in series with $r1589$ and the double mutant tested for B cistron activity. If the mutation in question is nonsense, the B activity should be cut off. If, on the other hand, the mutation is "missense," i.e., if the coding unit corresponds to a different amino acid from the original, the B activity should not be cut off, as illustrated in Figure 2C.

The ambivalent phage mutants and bacterial host strains: Certain *rII* mutants of

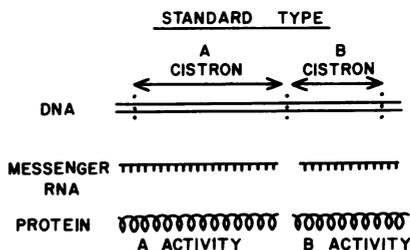


FIG. 1.—Scheme illustrating the independent functioning of the two cistrons of the *rII* region of standard type phage T4.

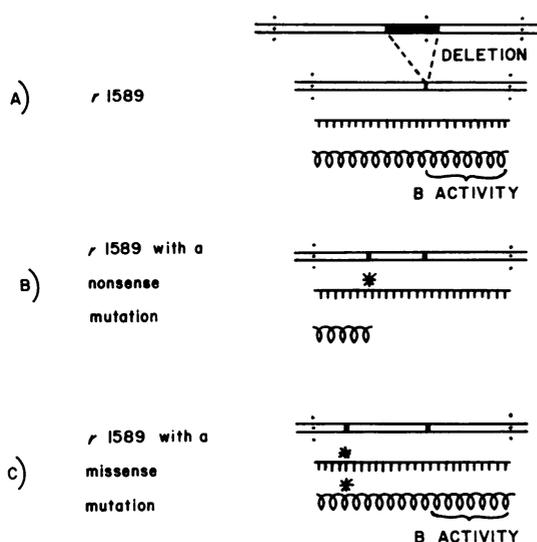


FIG. 2.—Scheme illustrating the properties of the mutant *r1589*. In this mutant, a deletion has occurred so that fragments of the two cistrons are joined, the B fragment retaining its activity. A nonsense mutation in the A cistron cuts off the B activity, but a missense mutation has no effect.

Although the illustration shows protein synthesis proceeding from left to right, this assumption is not necessary. An incomplete protein, blocked by a nonsense mutation, might never be released from the template.

All the subset 1 mutants listed in Table 1 are inducible to revert by 2-aminopurine and thus presumably arose by substitutions of the transition type. The specificity of reversion induction indicates that, in all five cases, the transition involved in

phage T4 are "ambivalent," i.e., inactive in one strain of *E. coli* but active in another.¹ Modification of the bacterial host (by mutation) can lead to activation of an entire group of mutants with defects located at various points in the *rII* region of the phage genome. The altered bacterium is thus said to contain a suppressor mutation that suppresses a specific ambivalent subset of phage mutations. The ambivalent subset relevant to the following discussion is subset 1, and is defined as those mutants which are active on strain KB-3 but not on KB. All of the known members of subset 1 located in the genetic map to the left of *r1589* are shown in Figure 3. The effect of the suppressor in KB-3 on the subset 1 *rII* mutants is shown by the data in Table 1.

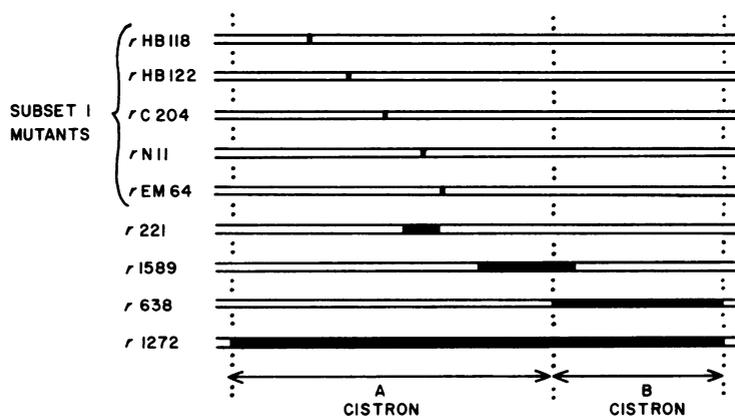


Fig. 3.—Genetic map of the *rII* region showing the locations of the mutations cited in this paper. The first five mutations are revertible by base analogues. The last four are "deletions," indicated by solid black lines. In crosses of the deletion *r1589* with *r638*, or with other mutants located in the tip of the B cistron shown covered by *r1589*, no standard type recombinants have ever been detected in as many as 10^6 progeny. By the method of shrinkage of distance between outside markers,²¹ *r1589* has been shown to be a true deletion.²²

TABLE 1
ACTIVITIES OF VARIOUS rII MUTANTS ON TWO DIFFERENT *E. coli* STRAINS

	r^+	Burst size	
		<i>E. coli</i> KB 229.	<i>E. coli</i> KB-3 190.
Standard type			
Deletions	$r1589$	0.01	0.01
	$r221$	0.03	0.01
	$r1272$	0.04	0.01
Analogue-revertible mutants of ambivalent subset 1	$rHB118$	0.05	157.
	$rHB122$	0.1	150.
	$rC204$	0.08	187.
	$rN11$	0.04	175.
	$rEM64$	0.09	168.
Analogue-revertible mutants not of subset 1	$rAP129$	1.8	2.5
	$rNA27$	0.09	0.04
	$rN74$	0.2	0.05
	$rAP218$	0.1	0.1
	$r1814$	0.3	0.1
	$rH221$	0.1	0.08
	$r607$	0.00	0.01
	$rEM114$	0.3	0.3

The procedure for measurement of burst sizes, as well as the complementation measurements of Table 2, has been previously described.³ KB-3, derived from KB, contains a suppressor mutation that specifically reverses the phenotype of ambivalent mutants of subset 1. In this respect, it is similar to strain KT previously used.¹

the original mutations is GC \rightarrow AT. All except $rHB122$ are phenotypically reversible by 5-fluorouracil.⁶

Characterization of the ambivalent mutations: Each mutant to be tested was first crossed with $r1589$. The progeny were plated on *E. coli* B, on which all produce plaques, and from them the double mutants were isolated. This was done with the help of replica plating, testing each progeny plaque for recombination against the two parent phages. A suspected double mutant was replated on B, a pure stock made from a single plaque, and tested to assure that it gave no recombination (less than 10^{-2} per cent) with either parent type, or with deletions overlapping either end of the $r1589$ deletion. The latter test ruled out the possibility of a false double mutant due to a newly arising point mutation within the $r1589$ segment. All the double mutants were also tested to assure that they gave recombination with mutants located at sites neighboring the original point mutation. This ruled out the possibility that an apparent double mutant might be due to a newly arising deletion overlapping the original site. These checks are necessary, as spurious double mutants sometimes do appear.

In Table 2, the various mutants are analyzed to determine which ones represent nonsense mutations. Note first the properties of mutant $r1589$ by itself. The burst size on strain KB is negligible, but when the cells are simultaneously infected with $r638$ (to supply the A function), a large burst occurs, showing that the B function in $r1589$ is intact. Thus, $r1589$ behaves in the same way as $r221$, a deletion restricted to the A cistron. For the large deletion $r1272$, covering both cistrons, no B activity is present. All three mutants give the same results regardless of whether KB or KB-3 is used as host.

When the deletion $r221$ is crossed into $r1589$, it cuts off the B function of the latter. This is the effect described by Crick *et al.*⁴ due to the interdependence of the A and B fragments in $r1589$, and suggests that the length of $r221$ is a nonintegral number of coding units.

The application of the test to the subset 1 point mutants shows that all cut off

TABLE 2
DETERMINATION OF NONSENSE OR SENSE CHARACTER OF VARIOUS MUTATIONS

	<i>E. coli</i>		<i>E. coli</i>		Conclusion
	KB	Burst Size	KB(+r638)	Burst Size	
Standard type		112.		135.	
Deletions	r ⁺	0.01		77.	
	{ r1589	0.03		109.	
	{ r1272	0.04		0.01	
Double mutant of r221 and r1589	r221-r1589	0.02		0.025	
Double mutants of r1589 with analogue-revertible mutants of ambivalent subset 1	{ rHB118-r1589	0.00		0.00	Nonsense
	{ rHB122-r1589	0.00		0.02	Nonsense
	{ rC204-r1589	0.00		0.00	Nonsense
	{ rN11-r1589	0.00		0.01	Nonsense
	{ rEM64-r1589	0.00		0.00	Nonsense
Double mutants of r1589 with analogue-revertible mutants not of subset 1	{ rAP129-r1589	0.00		58.	Sense
	{ rNA27-r1589	0.08		84.	Sense
	{ rN74-r1589	0.08		73.	Sense
	{ rAP218-r1589	0.07		85.	Sense
	{ r1814-r1589	0.1		28.	Sense
	{ rH221-r1589	0.09		76.	Sense
	{ r607-r1589	0.02		121.	Sense
	{ rEM114-r1589	0.07		80.	Sense

If a mutation in the A cistron cuts off the B activity of r1589, no burst is obtained in the complementation test with r638 (second and fourth columns). For mutants of ambivalent subset 1, the burst size is greatly increased by using KB-3 as the host in the complementation test (fourth column), indicating that the suppressor mutation in KB-3 has changed the reading of these mutations from nonsense to sense. No distinction is made here between "sense" and "missense," since it should be immaterial whether a correct or incorrect amino acid is inserted. Burst size differences less than a factor of about two are not significant.

the B activity of $r1589$ when KB is the host, i.e., they behave as if they were nonsense mutations. However, on switching to the suppressor strain, KB-3, the B function is expressed. In other words, a mutation that behaves as nonsense in strain KB becomes sense by virtue of a suppressor mutation in the host. One of the five mutants ($rHB122$) gives a considerably weaker response. This mutant also differs from the others in not responding to 5-fluorouracil.⁶

For the other eight analogue-reverting mutants listed in Tables 1 and 2, the B cistron function (of the $r1589$ double mutant) is unimpaired in the KB host. Thus, these mutations do not interrupt the reading of the genetic information and are interpreted as missense mutations in which the nucleotide change is such that the normal amino acid is replaced by a different one. Among seven other analogue-revertible mutants that have been tested qualitatively, three were of the missense type while four were nonsense.⁷ The latter were not members of ambivalent subset 1, so that nonsense substitutions are not restricted to that group.

Discussion.—The above analysis is based on the assumption, which also underlies the work of Crick *et al.*,⁴ that the A and B cistrons of the rII region control the amino acid sequences of polypeptide chains. Although there is as yet no direct evidence for this, a property common to certain rII mutations and to certain mutations affecting the enzyme alkaline phosphatase gives support to this assumption. That is, the same suppressor that acts on a subset of mutations in the structural cistron of *E. coli* for the phosphatase enzyme acts concomitantly on subset 1 rII mutations of phage T4.⁸ Furthermore, as shown by Garen and Siddiqi in the accompanying paper,⁹ the phosphatase mutants in question produce no detectable phosphatase protein in the absence of the suppressor, as would be expected if nonsense mutations were involved. Thus, mutations in unrelated cistrons, one in the phage and one in the bacterium, that appear, by quite independent criteria, to be nonsense, are also related by sensitivity to the same suppressor. Recent evidence by Brody and Yanofsky¹⁰ in the tryptophan synthetase system shows that suppressors can act by modifying protein structure. The properties of rII mutants are therefore readily understandable on the assumption that the products of the rII cistrons are proteins.

A second assumption on which our criterion for nonsense is based is that the product of the A cistron fragment of $r1589$ containing the mutation in question does not modify the B activity except in determining whether or not translation into a polypeptide chain is possible. It might be argued that some altered forms of the A (protein) fragment could interact with the B fragment in such a way that the B activity would be affected. It seems most unlikely, however, that the present data could be due to such an effect, since it is implausible that the effects would be the same for the interaction of the A with the B fragment in rII and for the interaction of the phosphatase molecule with itself.

To characterize a mutation as "nonsense," as distinguished from the "gibberish" of Crick *et al.*,⁴ produced by a shift of the reading frame, the mutation must have arisen by a base pair substitution. We have used as a criterion for a substitution of the transition type that the mutation be revertible by the base analogue 2-aminopurine. This is consistent with many of the facts of mutagenic specificity.^{5, 6} However, it cannot be ruled out that base analogues may have effects in addition to causing substitutions.¹¹

Within these limitations, the genetic analysis indicates that certain mutations give rise to a coding unit that does not correspond to an amino acid, so that the genetic code in *E. coli* must not be completely degenerate. Further, the same coding unit, in the phage genome, that is nonsense in strain KB of *E. coli* becomes sense by virtue of a suppressor mutation in strain KB-3. Thus, unless KB-3 can use unusual amino acids, this strain translates at least two coding units into the same amino acid. This implies, in agreement with other evidence,^{4, 12, 13} that the genetic code is partially degenerate.

As previously suggested,^{1, 14-16} the action of external suppressors can be understood in terms of the mechanism of protein synthesis. The translation of genetic information from messenger RNA into amino acid sequences takes place via the mediation of sRNA adaptors having the dual function of accepting a specific amino acid and attaching to a specific nucleotide sequence on the messenger RNA template.^{17, 18} The role of sRNA as an adaptor has recently been demonstrated experimentally.¹⁹ Since the attachment of each amino acid to the correct sRNA depends upon a specific enzyme, it is the set of sRNA adaptors and activating enzymes that determines the genetic code. If the structure of each activating enzyme and each sRNA adaptor is specified by a cistron in the organism, then the genetic code is under the genetic control of the organism itself and could change by mutation.

Thus, the action of the suppressor mutation in KB-3 could be explained by the appearance of a new or modified sRNA adaptor that fits a coding unit absent from the KB dictionary. Alternatively, an adaptor might have always been present but unable to accept an amino acid with any of the activating enzymes, in which case the suppressor mutation might act by creating a new activating enzyme (or modifying the structure of one already present). It has recently been shown that the degeneracy of leucine observed *in vitro*^{12, 13} is due to the existence of two sRNA adaptors having different coding specificities.²³

The changes in the code involved in suppressor mutations may be relatively minor ones having to do with appearance or disappearance of degeneracies. Since the available evidence²⁰ favors a close similarity of the codes in various organisms, strongly selective factors must force the set of adaptors and activating enzymes, in spite of changes in their individual structures,²⁴ to maintain a quasi-universal code.

Summary.—Genetic analysis indicates that certain *rII* mutations of phage T4 give rise to coding units that do not correspond to an amino acid, i.e., they are nonsense mutations. Mutations that behave like nonsense in one bacterial host nevertheless make sense in another host containing a suppressor mutation. The suppressor mutation thus constitutes, in a limited sense, an hereditary alteration of the genetic code.

We wish to thank Mes. Daine Auzins, Judith Berry, and Barbara Williams for assistance in isolation of the double mutants. This research was supported by grants from the National Science Foundation and the National Institutes of Health.

¹ Benzer, S., and S. P. Champe, these PROCEEDINGS, **47**, 1025 (1961).

² Benzer, S., these PROCEEDINGS, **41**, 344 (1955).

³ Champe, S. P., and S. Benzer, *J. Mol. Biol.*, **4**, 288 (1962).

⁴ Crick, F. H. C., L. Barnett, S. Brenner, and R. J. Watts-Tobin, *Nature*, **192**, 1227 (1961).

- ⁵ Freese, E., *J. Mol. Biol.*, **1**, 87 (1959).
- ⁶ Champe, S. P., and S. Benzer, these PROCEEDINGS, **48**, 532 (1962).
- ⁷ Crick, F. H. C., personal communication, for four of the mutants.
- ⁸ Benzer, S., S. P. Champe, A. Garen, and O. H. Siddiqi, manuscript in preparation.
- ⁹ Garen, A., and O. H. Siddiqi, these PROCEEDINGS, **48**, 1121 (1962).
- ¹⁰ Brody, S., and C. Yanofsky, manuscript in preparation.
- ¹¹ Trautner, T. A., M. N. Swartz, and A. Kornberg, these PROCEEDINGS, **48**, 449 (1962).
- ¹² Martin, R. G., J. H. Matthaei, O. W. Jones, and M. W. Nirenberg, *Biochem. Biophys. Res. Comm.*, **6**, 410 (1962).
- ¹³ Speyer, J. F., P. Lengyel, C. Basilio, and S. Ochoa, these PROCEEDINGS, **48**, 441 (1962).
- ¹⁴ Lieb, M., and L. A. Herzenberg, unpublished.
- ¹⁵ Yanofsky, C., and P. St. Lawrence, *Ann. Rev. Microbiol.*, **14**, 311 (1960).
- ¹⁶ Yanofsky, C., D. R. Helinski, and B. Maling, in *Cellular Regulatory Mechanisms*, Cold Spring Harbor Symposia on Quantitative Biology, vol. 26 (1961), p. 11.
- ¹⁷ Hoagland, M. B., *Brookhaven Symposia in Biol.*, **12**, 40 (1959).
- ¹⁸ Crick, F. H. C., "The Biological Replication of Macromolecules," in *Symposium Soc. Expt. Biol.*, **12**, 138 (1958).
- ¹⁹ Chapeville, F., F. Lipmann, G. von Ehrenstein, B. Weisblum, W. Ray, and S. Benzer, these PROCEEDINGS, **48**, 1086 (1962).
- ²⁰ von Ehrenstein, G., and F. Lipmann, these PROCEEDINGS, **47**, 941 (1961).
- ²¹ Nomura, M., and S. Benzer, *J. Mol. Biol.*, **3**, 684 (1961).
- ²² Bode, W., doctoral dissertation, University of Cologne (1962).
- ²³ Weisblum, B., S. Benzer, and R. Holley, manuscript in preparation.
- ²⁴ Benzer, S., and B. Weisblum, these PROCEEDINGS, **47**, 1149 (1961).

SUPPRESSION OF MUTATIONS IN THE ALKALINE PHOSPHATASE STRUCTURAL CISTRON OF *E. COLI**

BY A. GAREN AND O. SIDDIQI

BIOLOGY DIVISION, UNIVERSITY OF PENNSYLVANIA

Communicated by Seymour Benzer, May 28, 1962

A suppressor mutation is defined as one that overcomes the defect caused by another mutation. Of particular interest are the "external" suppressors that are located outside the cistron in which the suppressed mutations occur, and are active only on some of the mutations within the cistron.^{1, 2} The present communication describes the properties of an external suppressor for a class of mutations in the structural cistron for alkaline phosphatase of *E. coli*.³⁻⁵ The results suggest that this class of phosphatase-negative mutations produces a nucleotide configuration that is nonsense (unable to specify any amino acid) in the absence of the suppressor but which functions as a sense configuration when the suppressor is present.

Materials and Methods.—Bacterial strains: In all experiments, the *Hfr* was *E. coli* strain *K10* and the *F*⁻ was *E. coli* strain *W1*. The characteristics of these strains have been described elsewhere.³⁻⁶ Several of the *P*⁻ mutants were previously isolated in collaboration with C. Levinthal and E. Lin. The selective markers employed for the genetic crosses were *T*⁺ and *L*⁺ (ability to grow in a medium lacking threonine and leucine), *S*^r (ability to grow in the presence of 0.1 mg/ml of streptomycin), and *M*⁺ (ability to grow in a medium lacking methionine).

Media: The composition of the low phosphate medium used to prepare cultures for alkaline phosphatase assays was as follows: 1.2×10^{-1} M tris buffer; 0.2% glucose; 6.4×10^{-5} M KH_2PO_4 ; 8×10^{-2} M NaCl; 2×10^{-2} M KCl; 2×10^{-2} M NH_4Cl ; 3×10^{-3} M Na_2SO_4 ;