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Mechanism Studies of Suppressor-Gene Action[1]

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Abstract

Brody, Stuart (Stanford University, Stanford, Calif.), and Charles Yanofsky. Mechanism studies of suppressor-gene action. J. Bacteriol. **90**:687–695. 1965.—Mutations which change the primary structure of the A protein of the tryptophan synthetase of *Escherichia coli* can be reversed by allele-specific suppressor mutations. Normally, the suppressors of a particular A mutant lead to the appearance of small amounts of a wild-type-like A protein (su-A protein), in addition to the cross-reacting material antigenically similar to the normal A protein (CRM-A protein). In some cases, the particular ratio of su-A protein to CRM-A protein, indicative of a given suppressor gene, was increased when that suppressor gene was transduced into a different strain, such as a K-12 Hfr stock of *E. coli*. In these cases, there was a general correlation between an increased ratio and a marked instability of the suppressor gene. However, stable suppressed stocks were isolated in the Hfr strain, which also produced a high proportion of su-A protein. The ratios of su-A protein to CRM-A protein remained relatively constant under conditions of tryptophan repression in three different suppressor stocks, suggesting that the formation of each of the su-A proteins does not involve the interaction of a CRM-A protein with any other cellular constituent. It would appear, then, that the changes in the primary structure of the A protein which lead to the formation of the su-A proteins are determined before or during, but not after, the synthesis of the polypeptide chain. The specificity of amino acid activation was investigated in strains bearing one of the suppressor genes. These studies failed to reveal any significant alteration in the amino acyl ribonucleic acid (RNA) synthetases or the transfer RNA molecules for arginine, glycine, histidine, and tyrosine.

In recent years, it has become clear that many cellular components are involved in the translation of genetic information. It might be expected, therefore, that mutational alteration of any one of these components could ultimately affect the expression of genetic material, or of individual genes. In particular, changes in the specificity of a component involved in translation could directly affect the structure of gene products, and subsequently their biological activity. Such alterations in the proper translation of genetic information, or "mistakes" in protein synthesis, have been proposed as an explanation for the allele-specific suppressor mutations of the tryptophan synthetase A mutants of *Escherichia coli* (Yanofsky, Helinski, and Maling, 1961; Brody and Yanofsky, 1963), as well as for suppressors of many other types of mutants (Benzer and Champe, 1961; Garen and Siddiqi, 1962).

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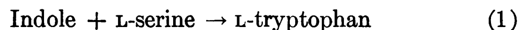
As reported previously (Crawford and Yanofsky, 1959; Brody and Yanofsky, 1963), allele-specific suppressor mutations reverse the effects of only certain mutations in the tryptophan synthetase A gene. Although many different mechanisms of suppression may exist, it has been shown that suppressors of the A mutants lead to an alteration in the primary structure of the A protein (Brody and Yanofsky, 1963). The most reasonable explanation for such changes is an alteration in the specificity of some component involved in protein synthesis, so that during synthesis a particular amino acid is erroneously incorporated into a protein at a low frequency in place of another amino acid. This type of "mistake" in protein synthesis might affect the structure of many other proteins as well as the A protein, if the suppressor mutation altered a component which was common to the synthesis of many proteins.

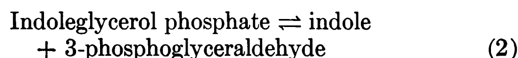
The specificity of particular components common to the synthesis of many proteins was investigated in an effort to uncover the mechanism of action of one particular suppressor. It is the purpose of this paper to present additional evi-

dence on the "mistake level" found in certain suppressed strains, to describe the characteristic instability of some suppressor genes, and to report the lack of observable effects of a suppressor mutation on the specificity of activation of certain amino acids.

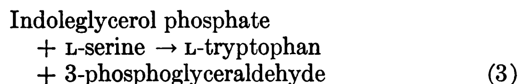
Pertinent facts about the tryptophan synthetase enzyme complex. The tryptophan synthetase enzyme complex is composed of two separable protein subunits (Crawford and Yanofsky, 1958), designated A and B. When complexed, these two proteins will catalyze the following three reactions (Crawford and Yanofsky, 1958; Crawford, 1960):



Indole + L-serine → L-tryptophan (1)



Indoleglycerol phosphate ⇌ indole + 3-phosphoglyceraldehyde (2)



Indoleglycerol phosphate + L-serine → L-tryptophan + 3-phosphoglyceraldehyde (3)

Many mutant strains (A mutants) have been isolated which produce an altered A protein, designated CRM-A protein (cross-reacting material antigenically similar to the normal A protein) or A-CRM, which reacts with antibodies to the normal A protein (Yanofsky and Crawford, 1959). All of the CRM-A proteins studied to date can combine with the normal B component, and this complex can catalyze reaction 1, but not the other two reactions. The effect of allele-specific suppressor mutations on different A mutants was shown to involve the restoration of small amounts of reaction 2 activity to mutant strains lacking this activity (Crawford and Yanofsky, 1958; Yanofsky et al., 1961; Brody and Yanofsky, 1963). This restored activity was found to be associated with a small amount of a second type of A protein, which resembled the wild-type A protein. In the one strain which was thoroughly examined (strain A-36 su_{36}), the wild-type-like A protein was shown to differ from the corresponding CRM-A protein at one position in its primary structure (Brody and Yanofsky, 1963).

Materials and Methods

Culture procedures. The A mutants and suppressed mutants mentioned in this paper were described previously (Yanofsky and Crawford, 1959; Yanofsky et al., 1961). The *E. coli* strain designated as K-12 Hfr $B_1^-R_t^-$ is resistant to inhibition by 5-methyltryptophan (5-MT) and was kindly supplied by Francois Jacob. (B_1^- and R_t^- designate a thiamine requirement and an insensitivity to tryptophan repression, respectively.) *Try*$^-$ markers were introduced into this strain by transduction techniques employing phage P1kc. These techniques, as well as the procedures for the growth of the various strains, were described previously (Yanofsky and Lennox, 1959).

The procedures for enzymatic assays were also described previously (Yanofsky and Crawford, 1959).

Determination of su_{36} stability was performed by inoculating liquid cultures, in minimal broth (Vogel and Bonner, *unpublished data*) or L broth (Lennox, 1955), with cells from an appropriate single-colony isolate, and incubating the cultures with shaking at 37 C overnight. After suitable dilution, approximately 100 to 150 cells were plated on minimal-tryptophan agar. The colonies were then tested for tryptophan dependence by the replica plating technique, or by appropriate test streaking.

All large-scale cultures used for enzyme analysis or transfer ribonucleic acid (t-RNA) preparation were similarly examined for suppressor-gene stability by appropriate dilution and plating on minimal-tryptophan agar. The values for su_{36} gene stability in large-scale cultures are similar to those given in Table 1.

Possible reversion of the original mutation in the A gene to the wild-type form can be detected by plating, after suitable dilution, on minimal agar and on a minimal agar containing 0.01 μg/ml of 5-MT. Suppressor strains will form much smaller colonies on the 5-MT agar than on the minimal agar, whereas wild-type strains form colonies of the same size on either medium.

Assay method for amino acid activation. A procedure similar to that of Berg et al. (1961) was employed for the determination of the amount of C^{14}-labeled amino acid bound to t-RNA. A reaction mixture of 1 ml contained the following: 0.5 μmole of adenosine triphosphate (ATP); 5.0 μmoles of $MgCl_2$; 2.0 μmoles of reduced glutathione; 50 μmoles of tris(hydroxymethyl)aminomethane (Tris)-maleate buffer, *p*H 7.2; 0.01 to 0.05 μmole of a t-RNA preparation; a dialyzed *E. coli* enzyme extract containing 100 μg of protein or more; and 5 to 30 mμmoles of a C^{14}-labeled amino acid. After 20 min of incubation at 37 C, the tubes were chilled for 5 min, and 3 volumes of a cold salt-ethyl alcohol solution were added. This salt-ethyl alcohol solution was 70% ethyl alcohol, 0.5 M with respect to NaCl. After vigorous stirring, and an additional 5 min of incubation at 0 C, the tubes were centrifuged at 8,000 × *g* for 10 min. The precipitates were washed twice with the salt-ethyl alcohol solution and were finally dissolved in water. Insoluble material was removed by centrifugation at 8,000 × *g*, and the radioactivity and optical density of the supernatant solution were measured. Radioactivity was determined by the addition of 0.2 ml of the supernatant solution to 8 ml of a dioxane scintillator solution (Werbin, Chaikoff, and Imada, 1959), and this solution was then counted in a Packard Tri-Carb scintillation spectrometer. The optical density (OD) at 260

TABLE 1. *Suppressor-gene activity and stability*

Stock	Origin of suppressed strain	Mistake level (ratio of su-A protein to CRM-A protein)*	Stability (percentage of observed colonies that are *try*⁻)†
A-36 su_{36}-6‡	Ultraviolet-induced	1:12	0.5–2
A-36 su_{36}-2‡	Ultraviolet-induced	1:11	<0.5
A-36 su_{36}‡	Ultraviolet-induced	1:12	0.2–0.5
Hfr R_t^- A-36 su_{36} #1	Transduction	1:12	<1.0
Hfr R_t^- A-36 su_{36} #15	Transduction	1:5	40–50
Hfr R_t^- A-36 su_{36} #7	Transduction	1:2	40–50
A-36 su_{36} #12	Transduction	1:12	0.4–0.5
Hfr $R_t^-B_1^-$ A-36 su_{36R}	Ultraviolet-induced	2:5	<0.2
Hfr $R_t^-B_1^+$ A-36 $su_{36R}su_{36}$	Transduction	1:1	<0.1

* Calculated directly from the ratio of indoleglycerol phosphate → tryptophan activity to indole → tryptophan activity in crude extracts, assuming that the su-A protein is similar to the wild-type A protein in its enzymatic properties.

† Testing procedure described in Materials and Methods.

‡ A-36 su_{36}-6, A-36 su_{36}-2, and A-36 su_{36} are separately isolated suppressed mutants derived from mutant A-36. The derivation of other strains is given in the Results section.

mμ of a 1:300 dilution of the supernatant solution was determined with a spectrophotometer, and a conversion factor of 24 OD units = 1 mg of t-RNA was used for calculations.

Enzyme preparation. Cells from freshly grown overnight 1-liter minimal cultures were harvested and ground with alumina, and the pastes were suspended in 10 ml of cold 0.1 M Tris buffer (*p*H 7.4) containing 0.01 M $MgCl_2$ + 0.001 M mercaptoethanol. All manipulations were then carried out at 4 C. After centrifugation at 8,000 × *g* for 20 min and 100,000 × *g* for 90 min, the supernatant fluids were dialyzed overnight against 2 liters of 0.01 M Tris buffer at *p*H 7.4, containing 0.001 M mercaptoethanol. This preparation was then used immediately or after 1 to 3 days of storage at −15 C.

Preparation of t-RNA. t-RNA was prepared by a modification of a procedure of U. Littauer (*personal communication*). A 58-g amount of frozen *E. coli* cell paste was thawed in 58 ml of 0.001 M Tris buffer (*p*H 7.4) containing 0.01 M $MgCl_2$; 92 ml of 90% phenol were added with stirring, and the layers were allowed to separate (room temperature, 60 min), with occasional swirling. The mixture was chilled for 10 min and was then centrifuged at 16,000 × *g* for 20 min; the aqueous layer was collected. The phenol layer was extracted with 40 ml of the Tris-$MgCl_2$ buffer, and the procedure was repeated. A 200-ml amount of cold 95% ethyl alcohol (with 2% potassium acetate) was added to the combined aqueous extracts, and the mixture was stored at −15 C for 1 hr. After centrifugation at 8,000 × *g* for 20 min, the precipitate was washed twice with 5 ml of cold 1 M NaCl, and the supernatant solutions were combined. The t-RNA was then precipitated by the addition of 3 volumes of 95% ethyl alcohol and was centrifuged; the precipitate was suspended in 30 ml of 0.1 M Tris buffer (*p*H 7.4). This solution was applied to a column of diethylaminoethyl Selectacel (5 by 16 cm) which had been previously equilibrated with 0.1 M Tris buffer (*p*H 7.4). The sample was washed into the column with this Tris buffer, and an additional 300 ml of this buffer were run through the column. After 200 ml of 0.1 M Tris buffer, containing 0.2 M NaCl, had passed through the column, the t-RNA was eluted with 0.1 M Tris buffer (*p*H 7.4) containing 0.7 M NaCl (200 ml, total volume). Additional elution with Tris buffer, containing higher NaCl concentrations, yielded very little t-RNA. The eluate was chilled and mixed with 3 volumes of cold 95% ethyl alcohol, and was kept at −15 C for 1 hr; the precipitate was collected by centrifugation at 10,000 × *g* for 20 min. The precipitates were dissolved in water, insoluble material was removed by centrifugation, and the solutions were stored at −15 C.

Radioactive chemicals. C^{14}-DL-arginine (13 μc/μmole), C^{14}-glycine (6 μc/μmole), C^{14}-DL-histidine, (9.8 μc/μmole), and C^{14}-DL-tyrosine (12 μc/μmole), purchased from Calbiochem, were purified separately by column chromatography by use of a Spinco amino acid analyzer. The resulting solutions were found to be free from detectable radioactive impurities (<0.01%), as determined with a Packard liquid scintillation-flow cell detector.

Periodate-inactivation procedure. A small amount of a t-RNA preparation (4 to 20 mg) was charged with an excess of a nonradioactive amino acid under the conditions employed for the assay procedure (scaled up 10-fold); the t-RNA was precipitated, washed once with the NaCl-ethyl alcohol mixture, and finally suspended in 1.0 ml of 0.1 M sodium acetate buffer (*p*H 5.0). The denatured protein and any other insoluble material were removed by centrifugation at 12,000 × *g* for 10 min, and the supernatant solution was treated with 2.0 ml of 0.1 M $NaIO_4$. After 20 min at 37 C, the t-RNA was precipitated with 3 volumes of the NaCl-ethyl alcohol solution, washed once with

3.0 ml of this solution, and suspended in 1.0 ml of water. A 2-ml amount of 0.33 M Tris buffer (*p*H 9.3) was then added, and the solution was incubated at 37 C for 90 min. The t-RNA was again precipitated with the NaCl-ethyl alcohol solution, centrifuged, allowed to drain well, and finally suspended in a volume of distilled water equivalent to the original volume of the t-RNA solution. This material was then assayed for its acceptor ability according to the assay method described above. Control experiments differed only in that amino acid solutions were not employed in the initial charging procedures.

RESULTS

Activity and stability of the suppressor gene. From previous studies (Brody and Yanofsky, 1963), it appeared that the activity of individual suppressor genes, judged in terms of the ratio of su-A protein (the A protein found in suppressed mutants which is active in reaction 3) to CRM-A protein, was a fairly stable characteristic of each suppressed mutant. Therefore, it was somewhat surprising when the introduction of the su_{36} gene by transduction into the Hfr R_t^- A-36 strain gave try^+ stocks with different colony sizes on minimal medium. [The designation su_{36} is used when the suppressor gene is present in an active form. When the suppressor gene is present in an inactive (nonsuppressing) form, an allele designation is not given. For the suppressor genes studied, the original inactive form is the wild-type form.] Three distinguishable types were identified, and many representatives of each try^+ type were analyzed for their enzymatic activities. It was found that each type of try^+ stock had a different ratio of su-A protein to CRM-A protein. One type was very similar to the parental A-36 su_{36} strain (it had a ratio of 1:12), another had an average ratio of 1:5, and the third exhibited an average ratio of 1:2. The control transduction of su_{36} into the A-36 strain gave only one type of try^+ colony, which had an su-A protein to CRM-A protein ratio of 1:12.

Genetic stability tests were performed to determine whether the apparent increased effectiveness of the su_{36} gene in the Hfr R_t^- strains might be related to an instability of this gene. These tests indicated that the su_{36} gene in the parental A-36 su_{36} strain as well as the su_{36} gene in those Hfr R_t^- strains which had a low ratio of su-A protein to CRM-A protein, exhibited slight instability (Table 1). However, in most of the strains which had a high su-A protein to CRM-A protein ratio, the su_{36} gene was extremely unstable (see Table 1). In those strains in which the su_{36} gene was very unstable, the observed ratio of su-A protein to CRM-A protein is probably lower than the true value for the suppressed mutants, since a considerable proportion of the cells did not have an active su_{36} gene. It was also found that the B_1^+ marker, which is very closely linked to the su_{36} gene, exhibited great instability (40 to 50%), but only when introduced with the su_{36} gene by cotransduction. Therefore, it appeared that a fragment of genetic material introduced by P1 transduction was unstable in the Hfr strain and that this instability generally led to an increased effectiveness of the su_{36} gene.

Two suppressed strains were also isolated in the Hfr R_t^- A-36 strain after irradiation with ultraviolet light. These suppressor strains obtained in the Hfr R_t^- stock differed in two respects from the suppressor strains isolated in the original A-36 stock. The su_{36R} gene of one of these suppressed mutants (designated su_{36R} since it was isolated in the Hfr R_t^- strain) was not linked to the B_1^+ marker by transduction, and the su_{36R} gene appeared to be quite stable (Table 1). The appearance of this type of suppressor strain (stable, high ratio of su-A protein to CRM-A protein) indicated that there need not be any correlation between the A protein ratios and the instability of a su_{36} gene.

Strains bearing two su_{36} genes were prepared by the transduction A-36 su_{36} → Hfr $R_t^-B_1^-$ A-36 su_{36R}, selecting for those cells which were B_1^+. Since B_1^+ and su_{36} show 90% joint transduction, most of the B_1^+ colonies should carry both su_{36} and su_{36R}. A strain bearing both suppressor genes was isolated. The results of analyses of one doubly suppressed strain are shown in Table 1, where it can be seen that extremely high su-A protein to CRM-A protein ratios were found. The stability of the second su_{36} gene (introduced by transduction) could not be readily determined, since those strains which might lose this su_{36} gene would still be try^+.

Although studies were not performed on the nature of the su-A protein formed in all of these different strains, examination of physical, chemical, and enzymatic properties of the su-A protein and the CRM-A protein in strain Hfr R_t^- A-36 su_{36} #15 indicated that these two A proteins closely resembled the corresponding two A proteins found in the A-36 su_{36} strain (Brody, Ph.D. Thesis, Stanford Univ., Stanford, Calif., 1964). Primary structure studies indicated that the su-A protein had a glycine residue instead of an arginine residue at a particular position in the A protein (Brody, Ph.D. Thesis, Stanford Univ., 1964). Therefore, it appeared likely that the action of the su_{36} gene was the same in the

TABLE 2. *Repression of su-A and CRM-A protein formation in suppressed mutants**

Strain	Growth medium (μg L-tryptophan/ml)	A protein activity of extracts†				InGP → Try to In → Try activity ratio
		In → Try reaction		InGP → Try reaction		
		Amt	Specific activity‡	Amt	Specific activity	
		units/ml		*units/ml*		%
A-36 su_{36}	0	130	6.0	5.50	.250	4.2
	5	30	1.25	1.20	.050	4.1
	10	24	1.08	1.08	.049	4.5
	50	17	0.83	0.97	.048	5.8
A-11 su_{11}	0	1,380	55	12.00	.480	0.87
	5	1,180	47	8.80	.352	0.75
	10	760	33	5.50	.239	0.73
	20	33	1.5	0.20	.009	0.59
K-12$_p$ R_t^- A-3 su_3	0	1,490	62	6.30	.261	0.42
	5	1,560	65	6.80	.282	0.43
	10	380	19	1.20	.061	0.32

* In = indole, Try = tryptophan, InGP = indoleglycerol phosphate. A 1-ml amount of a minimal overnight culture was used as inoculum for 1 liter of minimal medium. Samples of a sterile tryptophan solution (2 mg/ml) were added to give the desired concentration. Each 2-liter flask was incubated at 37 C on a rotary shaker until maximal growth was obtained, and then standard harvesting and extract preparation procedures were employed.

† Assayed in the presence of excess B protein.

‡ Units per milligram of protein.

Hfr R_t^- A-36 su_{36} strains as in the A-36 su_{36} strain, but with a greatly increased effectiveness.

Tryptophan repression. Three different suppressor strains were cultured in minimal medium supplemented with different amounts of tryptophan, and the A protein activities of their extracts were determined according to standard procedures (Yanofsky and Crawford, 1959). The rationale for this experiment was as follows: if the formation of the su-A protein involved some type of bimolecular interaction between the CRM-A protein and another cellular component, then lowering the amount of CRM-A protein (by tryptophan repression) should increase the relative proportion of su-A protein to CRM-A protein. The results of the experiments summarized in Table 2 indicated that the respective su-A protein to CRM-A protein ratios remained relatively constant, even under conditions in which the synthesis of the A protein was severely repressed. This result would tend to rule out a bimolecular type of reaction as being responsible for the formation of the su-A protein.

Studies on amino acid activation. As mentioned previously, the change in the primary structure of the A protein could be explained as an alteration of the specificity of some component involved in protein synthesis. Although any one of many components could have been altered, it was decided to examine the amino acid activation components, since they appeared to be the most amenable to experimental analysis. A reconstituted amino acid-activating system was employed, consisting of a purified t-RNA fraction, believed to contain all of the t-RNA(s), plus a crude enzyme preparation. Homologous systems were generally tested, i.e., t-RNA and activating enzymes from the same strain. If an altered specificity was found with the components from the su_{36} strain, then a similar experiment involving heterologous mixtures should permit the localization of any alteration in specificity to the t-RNA or to the activating enzyme fraction.

Additivity of amino acid activation. Experiments were performed to determine whether there was an independent activation of certain amino acids by the components of a reconstituted activating system, or whether there was some competition between these amino acids for a given t-RNA or amino acyl RNA synthetase. One method of detecting this type of competition would be to separately measure the acceptor ability of a given t-RNA preparation for two different amino acids, and to compare the sum of these two values with the total acceptor ability found with a mixture of the two amino acids. The finding of 100% additivity of the two in-

TABLE 3. *Additivity of amino acid activation*

C^{14}-labeled amino acids present	Source of t-RNA and activating enzymes	
	Hfr R_t^- A-36 su_{36}*	Hfr R_t^- A-36
Histidine	2,120†	5,120
Tyrosine	1,750	2,440
Histidine + tyrosine	3,780	7,240
Per cent of calculated sum	97	96
Arginine	4,440	8,120
Glycine	2,480	6,480
Arginine + glycine	6,880	14,680
Per cent of calculated sum	99	100

* Ratio of su-A protein to CRM-A protein of 1:5.

† Attachment of C^{14}-amino acids to t-RNA, expressed as counts per minute per milligram of RNA. The assay method described in Materials and Methods was employed. The amount of t-RNA was 0.02 μmoles, and the amounts of C^{14}-labeled amino acids were: glycine, 10 mμmoles; arginine, 10 mμmoles; tyrosine, 6 mμmoles; histidine, 6 mμmoles.

dividual values would be indicative of separate activation of the amino acids tested, whereas nonadditivity would presumably represent some type of competition between these amino acids for some component of the reconstituted activation system.

The optimal conditions employed in these experiments involved the following: an excess of amino acyl RNA synthetase for the amino acids tested, a 50-fold excess of C^{14}-labeled amino acid, and a limiting amount of t-RNA. Assays were performed in duplicate, at two different limiting concentrations of t-RNA, and the optical density of every final t-RNA sample was determined. Approximately equal concentrations of the isotopic amino acids were used, but in some experiments the ratios were varied from 3:1 to 1:3 without any apparent effect on the results.

The additivity of the activation of arginine and glycine was tested because of the known replacement of arginine by glycine in the su-A protein (Brody and Yanofsky, 1963); the histidine and tyrosine activation was determined for other reasons. Strains bearing the su_{36} gene contain 8% more tyrosine and 8% less histidine in their total soluble protein than strains lacking the su_{36} gene. However, the same analyses do not indicate any statistically significant deviations in arginine and glycine content. Hfr R_t^- A-36 strains with and without the su_{36} gene were employed as the sources of the components used in these studies, since the su_{36} gene appeared to be more active in the Hfr R_t^- strain and since primary structure studies had been performed on the su-A and CRM-A proteins from this strain (Brody, Ph.D. Thesis, Stanford Univ., 1964).

The data obtained (Table 3) indicated that there was no consistent or significant departure from additivity for the two amino acid pairs tested with the components from either strain. These experiments were also performed with heterologous mixtures, with the same result. The lower activation values for the t-RNA preparation from the su_{36} strain were due to the presence of ultraviolet-absorbing material, probably RNA, which did not accept amino acids (Brody, Ph.D. Thesis, Stanford Univ., 1964). The variability of these assays was 5% or less; thus, if competition between amino acids results in a 10% reduction in activation, it should have been detectable. It can be concluded from these experiments that 95% of the t-RNA molecules that will accept these four amino acids (histidine, tyrosine, glycine, and arginine) will not accept more than one of these amino acids under the experimental conditions employed.

Experiments were also performed to determine the effect of relatively high concentrations of one amino acid (unlabeled) on the activation of another amino acid (labeled). The same pairs of amino acids were employed in these studies. Although the data obtained indicated that high concentrations of certain amino acids decreased the activation of other amino acids somewhat, these effects appeared to be nonspecific and could not be correlated with the source of the t-RNA or amino acyl RNA synthetase used in the experiment.

Inactivation of t-RNA by periodate treatment. It has been shown that periodate oxidation can be employed to obtain information on the specificity of t-RNA acceptor ability (Preiss et al., 1959). Periodate oxidation will only inactivate uncharged t-RNA, and, therefore, only those t-RNA molecules charged with an amino acid prior to the periodate treatment will be protected against inactivation. For example, if a species of t-RNA existed which could accept either glycine or arginine, the initial charging of the t-RNA with glycine or arginine would protect the acceptor ability of this t-RNA species from periodate inactivation. After removal of the protecting amino acid, this t-RNA should be able to accept either of these amino acids.

Some representative results of periodate inactivation experiments are presented in Table 4. It is clear that there was significant self-protec-

TABLE 4. *Amino acid protection of t-RNA against periodate inactivation*†

t-RNA source	Protecting amino acid	Per cent of original acceptor ability remaining for			
		Histidine	Tyrosine	Arginine	Glycine
su_{36}*	L-Histidine	83	10		
	L-Tyrosine	4	40		
	None	1	8		
Non-su_{36}	L-Histidine	67	12		
	L-Tyrosine	2	40		
	None	1	8		
su_{36}*	L-Arginine			48	52
	Glycine			6	50
	None			7	56
Non-su_{36}	L-Arginine			50	50
	Glycine			5	65
	None			5	57

* Ratio of su-A protein to CRM-A protein of 1:5.
† See Materials and Methods for a description of the test procedures employed.

tion of acceptor ability for three of the four amino acids tested. It is also clear that there was little if any cross-protection for these four amino acids in any of the t-RNA preparations. In most of the experiments, only 5 to 10% of the initial acceptor ability remained, and, therefore, a cross-protection of 10% of these molecules would have been easily detected against this background. The glycine acceptor ability appeared to be protected in all cases, presumably owing to the production of small amounts of glycine by the crude enzyme extract. It was, therefore, not possible to determine whether there was any protection of glycine acceptor ability by arginine with the crude enzyme system. However, glycine does not appear to protect any t-RNA capable of accepting arginine under the conditions employed. Thus, in these experiments it also was not possible to detect a t-RNA species in preparations from the su_{36} strains (or any other strains examined) which had the ability to accept more than one of the amino acids tested.

DISCUSSION

The finding that the su_{36} gene appeared to be more active in restoring A protein activity in the indoleglycerol phosphate → tryptophan reaction in the Hfr R_t^- strains than in the original K-12 strains suggests that there is an increased amount of the su_{36} gene product in the Hfr R_t^- strains. It seems likely that the su_{36} gene contains the information for the structure of this active suppressor product, and that the mutation in the su_{36} gene results in an alteration in the structure and function or specificity of this product. However, the possibility has not been ruled out that this suppressor mutation is a regulatory mutation, allowing the production of a product whose formation was previously repressed. In either case, it is probable that the increase in the relative amount of su-A protein due to the presence of an unstable su_{36} gene or two su_{36} genes can be correlated with an increased amount or activity of the suppressor-gene product.

The suppressor-gene product does not appear to be a cellular component which interacts with the CRM-A protein to form the su-A protein. This conclusion is based on the results of the tryptophan repression experiment, assuming that the postulated component which would react with the CRM-A protein would be limiting, and that the level of this postulated component would not be influenced by tryptophan repression. In some of the strains examined, it would appear that the mechanism leading to the production of the su-A protein is indeed limiting, since other suppressed strains were obtained which exhibited much higher levels of su-A protein. If one accepts the latter conclusion, then the findings obtained would indicate that the suppressor mechanism probably operates during the synthesis of the A protein rather than after the formation of the A protein.

The nature of the suppressor-gene product remains to be determined. The experiments performed in an attempt to detect changes in amino acid acceptor specificity for the pertinent amino acids were unsuccessful. Furthermore, no sig-

nificant differences were found between su_{36} and non-su_{36} strains as to the amount of glycine t-RNA acceptor activity relative to the total amino acid acceptor ability of the t-RNA preparations. Comparisons of chromatographic elution profiles (on methylated albumin kieselguhr columns) of su_{36} and non-su_{36} t-RNA preparations individually labeled with C^{14}-arginine or C^{14}-glycine also failed to reveal differences. In view of these negative results, it would appear that the su_{36} mutation either does not affect the specificity of amino acid activation or brings about a quantitative minor change.

Since changes in the specificity of activation may be unlikely in this case, it is of value to consider other possible mechanisms of suppression within the framework of the "mistake in synthesis" hypothesis. The su_{36} mutation could alter a particular type of t-RNA molecule so that it occasionally pairs incorrectly with the messenger RNA-ribosome complex, incorporating glycine instead of arginine. Similarly, an alteration of some ribosomal component (Davies, Gilbert, and Gorini, 1964) could lead to a change in the specificity of t-RNA attachment and amino acid incorporation. It is also conceivable that suppression results in an alteration of the specificity of an enzyme which modifies the structure of nucleotides, e.g., a methylating enzyme, so that a particular nucleotide would be altered in the A gene, the A protein messenger, or any other cellular component involved specifically in the synthesis of the A protein. If a deoxynucleotide in the A gene were altered and were transcribed incorrectly, or if an altered ribonucleotide in the A gene messenger paired incorrectly with ribonucleotides in t-RNA or ribosomal RNA, then this change in structure could lead to the incorporation of a different amino acid at a particular position in the A protein. Along the same lines, it is conceivable that some type of base analogue is produced by suppressed mutants which could have any of the above-mentioned effects if it were incorporated only at particular positions in nucleic acid.

At present, it seems unnecessary to consider these postulated mechanisms in great detail, since so little is known about the possibilities mentioned. However, since "mistakes" in amino acid incorporation do occur, it is likely that suppressor mechanisms could involve alterations in any one of the components that participate in the transcription and ultimate translation of the nucleotide sequence in DNA.

The instability of the su_{36} gene is noteworthy, although the relationship between instability and the mechanism of suppression is not clear. Genetic instability appears to be frequently associated with suppressor genes (Dawson and Smith-Keary, 1963; Gunderson, 1963; Hill, 1963; Schwartz, 1963), and it may well be that some suppressor mutations involve genetic changes quite different from the single base pair substitutions believed to be typical of many mutations.

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LITERATURE CITED

BENZER, S., AND S. CHAMPE. 1962. A change from nonsense to sense in the genetic code. Proc. Natl. Acad. Sci. U.S. **48**:1114–1121.

BERG, P., F. BERGMANN, E. OFENGAND, AND M. DIECKMANN. 1961. The enzymic synthesis of amino acyl derivatives of ribonucleic acid. J. Biol. Chem. **236**:1726–1734.

BRODY, S., AND C. YANOFSKY. 1963. Suppressor gene alteration of protein primary structure. Proc. Natl. Acad. Sci. U.S. **50**:9–16.

CRAWFORD, I. 1960. Identification of the triose phosphate formed in the tryptophan synthetase reaction. Biochim. Biophys. Acta **45**:405–406.

CRAWFORD, I., AND C. YANOFSKY. 1958. On the separation of the tryptophan synthetase of *Escherichia coli* into two protein components. Proc. Natl. Acad. Sci. U.S. **44**:1161–1170.

CRAWFORD, I., AND C. YANOFSKY. 1959. The formation of a new enzymatically active protein as a result of suppression. Proc. Natl. Acad. Sci. U.S. **45**:1280–1287.

DAVIES, P., W. GILBERT, AND L. GORINI. 1964. Streptomycin, suppression and the code. Proc. Natl. Acad. Sci. U.S. **51**:883–890.

DAWSON, G., AND P. SMITH-KEARY. 1963. Episomic control of mutation in *Salmonella typhimurium*. Heredity **18**:1–20.

GAREN, A., AND O. SIDDIQI. 1962. Suppression of mutations in the alkaline phosphatase structural cistron of *E. coli*. Proc. Natl. Acad. Sci. U.S. **48**:1121–1127.

GUNDERSEN, W. 1963. New type of streptomycin resistance resulting from action of the episome-like mutator factor in *Escherichia coli*. J. Bacteriol. **86**:510–516.

HILL, R. F. 1963. The stability of spontaneous and ultraviolet-induced reversions from auxotrophs in *Escherichia coli*. J. Gen. Microbiol. **30**:289–297.

LENNOX, E. 1955. Transduction of linked genetic characters of the host by bacteriophage P1. Virology **1**:190–206.

PREISS, J., P. BERG, E. OFENGAND, F. BERGMANN, AND M. DIECKMANN. 1959. The chemical nature of the RNA-amino acid compound formed by amino acid-activating enzymes. Proc. Natl. Acad. Sci. U.S. **45**:319–328.

SCHWARTZ, N. 1963. The nature of ethylmethanesulfonate induced reversions of *lac*⁻ mutants of *E. coli*. Genetics **48**:1357–1375.

WERBIN, H., L. CHAIKOFF, AND M. IMADA. 1959. A rapid sensitive method for determining H^3-water in body fluids by liquid scintillation spectrometry. Proc. Soc. Exptl. Biol. Med. **102**:8–12.

YANOFSKY, C., AND I. CRAWFORD. 1959. The effects of deletions, point mutations, reversions and suppressor mutations on the two components of the tryptophan synthetase of *E. coli*. Proc. Natl. Acad. Sci. U.S. **45**:1016–1026.

YANOFSKY, C., D. HELINSKI, AND B. MALING. 1961. The effects of mutation on the composition and properties of the A protein of *Escherichia coli* tryptophan synthetase. Cold Spring Harbor Symp. Quant. Biol. **26**:11–24.

YANOFSKY, C., AND E. LENNOX. 1959. Transduction and recombination study of linkage relationships among the genes controlling tryptophan synthesis in *Escherichia coli*. Virology **8**:425–447.