*IN VITRO SYNTHESIS OF T4 LYSOZYME BY SUPPRESSION OF AMBER MUTATIONS**

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Mutations giving rise to nonsense codons result in termination of protein synthesis at the site of the mutation so that incomplete polypeptide fragments are produced.[1] Suppressors of these mutations bring about insertion of an amino acid at the site specified by the nonsense codon, thus allowing production of complete proteins which may, however, be altered by an amino acid substitution at the mutant site. Bacterial strains carrying amber suppressors Su_I, Su_{II}, or Su_{III} are able to insert serine, glutamine, or tyrosine, respectively, for the amber nonsense triplet UAG.[2]

We recently found that active T4 lysozyme can be made *in vitro* under the direction of RNA extracted from cells infected with bacteriophage T4.[3] In contrast, RNA from cells infected with lysozyme amber mutant eH26 was not able to direct synthesis of the enzyme in a nonpermissive *in vitro* system. These findings raised the possibility of developing an enzymatic assay for suppression *in vitro*.

Here we show that S-30 extracts from strains carrying suppressors can give efficient *in vitro* suppression of lysozyme amber mutants, thereby producing active enzyme. In addition to substantiating the *de novo* nature of the lysozyme synthesis, these results provide an easy and direct assay for suppression, which we have used to investigate the Su_I, Su_{II}, and Su_{III} amber suppressors.

Studies of *in vitro* suppression of RNA bacteriophage amber mutants[4–7] have revealed that the amber Su_I and Su_{III} suppression is due to new serine and tyrosine sRNA's, not present in the nonpermissive cells. Results of genetic experiments and of analyses of ribosomal proteins have suggested that Su_{II} suppression is a result of altered ribosomes which allow translation of UAG as glutamine.[8, 9] However, Wilhelm[10] recently presented evidence from *in vitro* experiments, again using RNA phage, showing that sRNA is responsible for Su_{II} suppression, as in the case of Su_I or Su_{III}.

Our results show that suppression of lysozyme amber mutants is mediated by sRNA in all three amber suppressor strains, in agreement with Wilhelm concerning the Su_{II}.

Materials and Methods.—Bacterial strains: E. coli strains RNase I_{10}^-, CR63 (Su_I), B^E, and C600 (Su_{II}) came from the laboratory collection. CA244 (Hfr H, λ, lac⁻*am*, tryp⁻*am*, Su⁻) and two strains derived from it by spontaneous mutation, CA266 (Hfr H, λ, lac⁻*am*, tryp⁻*am*, Su_I) and CA265 (Hfr H, λ, lac⁻*am*, tryp⁻*am*, Su_{III}), were the gift of Dr. Sydney Brenner. CA180 (Hfr H, λ, lac⁻*am*, B_1^-, Su_{II}), derived by P1 transduction of the suppressor from C600 into CA85,[11] was also obtained from Dr. Brenner.

Bacteriophage: The T4 lysozyme amber mutant eM74 was the gift of Dr. George Streisinger. T4 D and the lysozyme mutant *am* eH26 came from the collection of Dr. Richard Epstein.

Growth of phage: Isolation of the total RNA from infected cells is carried out as

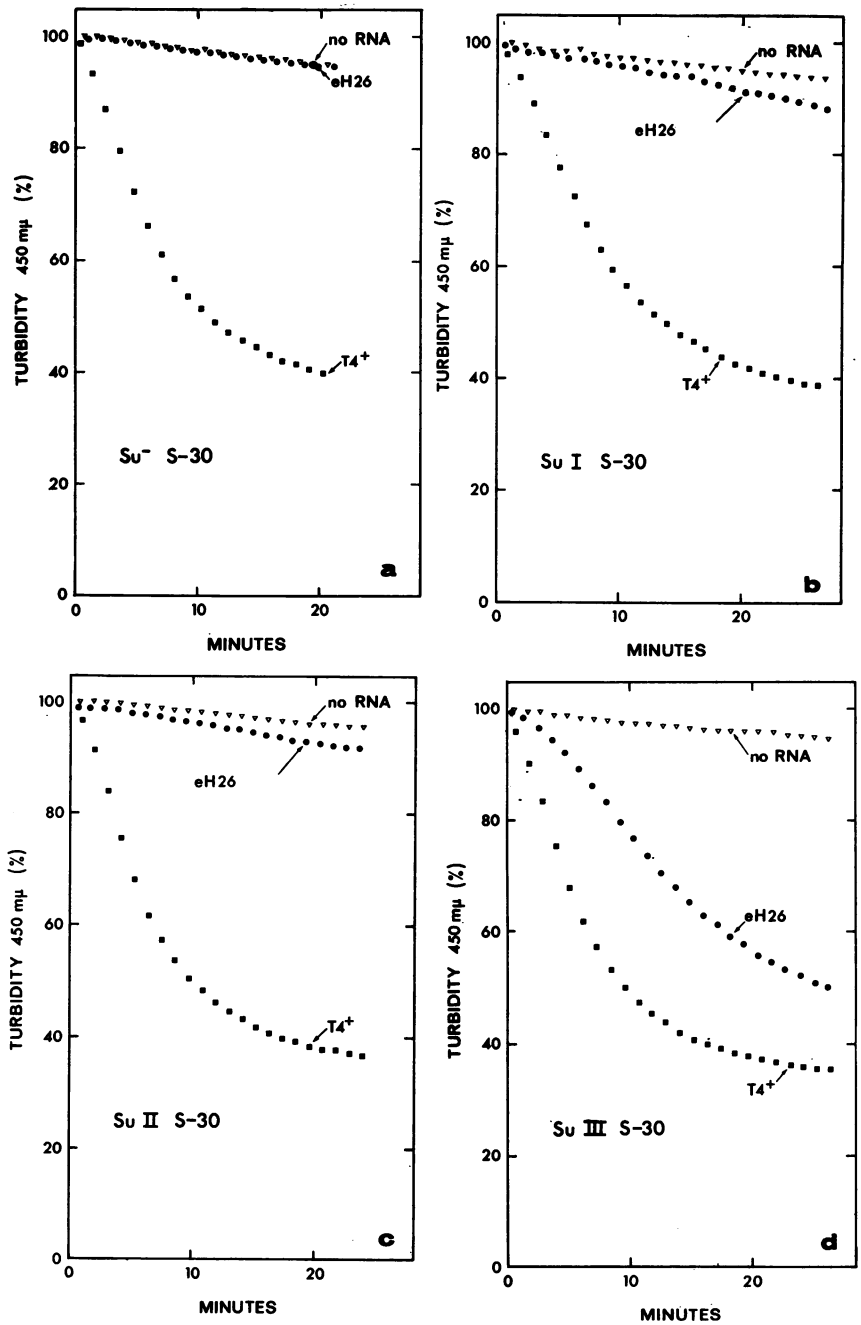

FIG. 1.—One ml of the chloroform-treated *E. coli* lysozyme substrate (initial OD_{450} of 0.4) is mixed with 0.1 ml of the appropriate *in vitro* reaction mixture which has been incubated for 20 min at 36°C in the absence and presence of the indicated RNA samples Details are given in the legend to Table 1. The decrease in OD_{450} is measured on a Gilford recording spectrophotometer at a constant temperature of 25°C.

described previously[3, 12] except that in all cases the RNA is extracted 20 min after infection at 30°C and tryptophane (40 μg/ml) is added just before infection with the mutants of T4 B to improve phage adsorption.

Preparation of sRNA: sRNA from strains CA244 (Su^-), CA266 (Su_I), CA180 (Su_{II}), and CA265 (Su_{III}) is purified and stripped of amino acids by the method of Littauer as described by Capecchi.[13]

In vitro protein synthesis: The S-30 extracts and the conditions of incubation have been described previously.[3, 14] The final magnesium concentration is 8.5 mM and N^5 formyl-tetrahydrofolate has been added to make a final concentration of 0.15 mM. In this work and in the previous work[3] the concentration of Cleland's reagent (dithiothreitol) in the final reaction mixture was 2.25 mM instead of the level reported before.

Lysozyme assay: Enzyme activity is measured by following the decrease in turbidity of chloroform-treated *E. coli* B^E cells as described by Sekiguchi and Cohen.[15] The maximum rate of turbidity decrease is converted to units of enzyme activity by means of a standard curve. Because the components of the *in vitro* system influence the efficiency of the reaction, the standard curve was prepared (as in the previous work[3]) by assaying reaction mixtures (identical to those used in the experiment except for the addition of chloramphenicol to prevent protein synthesis) to which known amounts of a T4 lysozyme standard extract had been added. This lysozyme standard has been described previously.[3] Since the lysozyme substrates vary, a new standard curve was prepared with each series of assays. The relation between the maximum rate of decrease in turbidity and the amount of the standard lysozyme extract is nearly linear.

Results.—T4 lysozyme amber mutant eH26 is suppressed efficiently *in vivo* by amber suppressor Su_{III}, which inserts tyrosine at the amber site, and only poorly by the Su_I (serine) and Su_{II} (glutamine) amber suppressors (Streisinger, personal communication; our unpublished results). If suppression of the eH26 mutation works properly *in vitro*, we would expect a similar pattern of efficiency of suppression in extracts from the three suppressor-carrying strains. To test this hypothesis total RNA extracted from Su^- cells infected by T4 D or T4 D *am* eH26 was used as a source of messenger RNA in S-30 extracts from Su^- and Su^+ cells (Fig. 1). RNA from mutant *am* eH26 is inactive for lysozyme synthesis in the Su^- extract (Fig. 1*a*). In the Su_I and Su_{II} extracts a small but detectable amount of lysozyme is made, while in the Su_{III} extract (Fig. 1*d*) a substantial amount of enzyme is produced. Note that the T4 wild-type RNA stimulates approximately the same synthesis of lysozyme in all four extracts. Thus the eH26 amber mutant can be efficiently suppressed *in vitro* and the pattern of suppression (Su_I and $Su_{II} < Su_{III}$) is in qualitative agreement with that observed *in vivo*.

Another lysozyme amber mutant *am* eM74 has a different pattern of suppression *in vivo*. In contrast to eH26, this mutant is efficiently suppressed by both Su_I and Su_{III} strains and is partially suppressed by Su_{II} (our unpublished results). The results of *in vitro* suppression experiments using RNA from eM74 infected Su^- cells are shown in Table 1, which also includes the data for T4 wild-type and eH26. The incorporation of H^3 leucine into total protein is given in each case and is compared with the lysozyme activity. The eM74 RNA is inactive for lysozyme synthesis in the Su^- extract. However, all three suppressor-carrying extracts

synthesize lysozyme with this RNA. It is clear that Su_I and Su_{III} are more efficient suppressors of this mutation *in vitro* than is Su_{II}, again agreeing qualitatively with the *in vivo* suppression levels.

With this rather simple assay for suppression we can see if the suppression is mediated by sRNA. Since suppression is dominant, addition of purified uncharged sRNA from the suppressing strains, to an otherwise Su^- *in vitro* system, should permit lysozyme synthesis from eH26 RNA or eM74 RNA. That this is so for all three amber suppressors is shown in Figure 2 and Table 2. Enzyme synthesis is observed when Su^+ sRNA is added to the Su^- system; moreover, the amount of enzyme obtained is consistent with the *in vivo* pattern of suppression and with the *in vitro* suppression seen in the Su^+ S-30 extracts (Fig. 1, Table 1). Mutant eM74

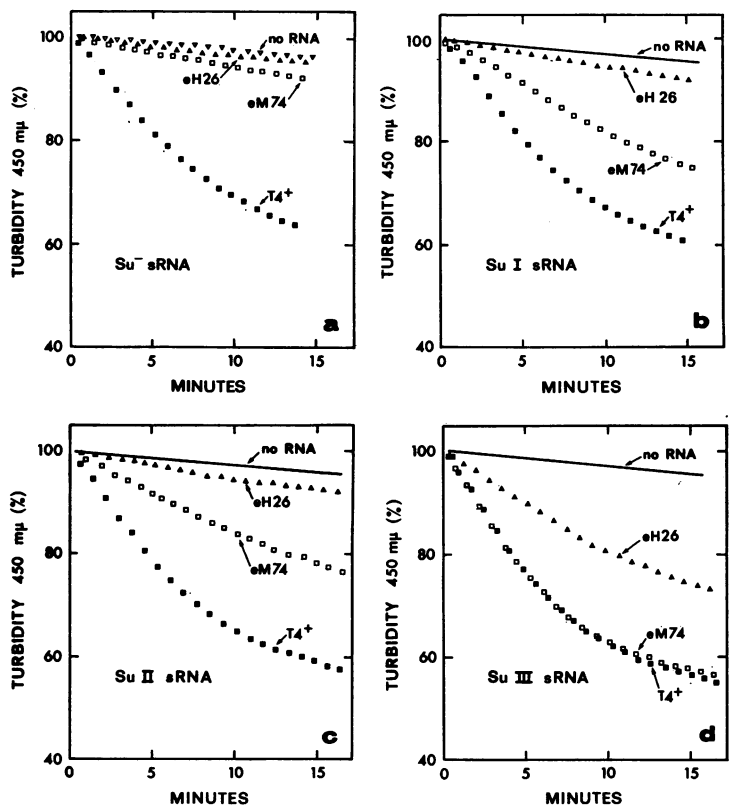

FIG. 2.—The assays are as described in Figure 1. The Su^- *in vitro* system used in all cases is from RNase I_{10}, and is supplemented with the various Su^- and Su^+ sRNA preparations as indicated. See legend to Table 2 for details.

proves to be suppressed by Su_{II} sRNA (Fig. 2*c*), although less efficiently than by Su_I or Su_{III} sRNA (Fig. 2*b* and *d*).

In the experiments illustrated above, RNA extracted from an Su^- host infected with eH26 or eM74 was used so that no suppressing sRNA would be introduced with the messenger preparation (recall that our source of mRNA is the total RNA extracted from the infected cells). We have carried out experiments similar to those

TABLE 1

In vitro SUPPRESSION OF LYSOZYME AMBER MUTANTS

Source of *in vitro* system (S-30)[a]	Incorporation of H^3 leucine ($\mu\mu$moles/ml)[b]	Lysozyme activity[c]	Lysozyme synthesized / Leucine incorporated[d]
	A. Messenger[e] from T4 wild type		
Su^-	1610	54.6	66
Su_I	1100	36.5	65
Su_{II}	1700	38.8	45
Su_{III}	1040	26.4	49
	B. Messenger from T4 *am* eH26		
Su^-	1820	2.0	0
Su_I	1110	4.7	6
Su_{II}	1820	3.4	2
Su_{III}	830	14.5	33
	C. Messenger from *am* eM74		
Su^-	1640	6.2	0.4
Su_I	1080	33.6	61
Su_{II}	1790	16.1	16
Su_{III}	880	29.3	65

[a] The S-30 extracts were prepared from RNase I_{10}^- (Su^-), CR63 (Su_I), C600 (Su_{II}), and CA265 (Su_{III}).

[b] The leucine incorporated into hot acid-insoluble material during the 20-minute incubation is calculated assuming no dilution of the leucine added (0.01 mM leucine of specific activity 22 cpm/$\mu\mu$mole).

[c] The lysozyme activity is calculated from curves such as those in Figure 1. The maximum rate of decrease in OD_{450} is measured and expressed as (ΔOD/min) $\times$ 1000. The backgrounds for the lysozyme assays determined from samples incubated without the addition of messenger RNA were 2.9, 1.3, 1.7, and 1.2 for the Su^-, Su_I, Su_{II}, and Su_{III} S-30 preparations, respectively, and have not been subtracted from these data.

[d] The lysozyme activities in the previous column were converted to μg of protein in a standard lysozyme extract using a standard curve (see *Methods*). This column gives a measure of the specific activity of the lysozyme made *in vitro* and is expressed in μg protein of lysozyme standard $\times$ 1000 divided by the $\mu\mu$moles of leucine incorporated.

[e] The RNA used to program the *in vitro* synthesis was extracted from cells of *E. coli* RNase I_{10}^- (Su^-) infected with T4 wild-type or amber mutant as indicated. For each 0.1-ml reaction mixture, 80 μg of the unfractionated "messenger" RNA was added.

shown in Table 2 using RNA from Su_I-infected cells. The results, while complicated somewhat by a small amount of suppression due to the sRNA in the mRNA preparations, show that the amber mutant RNA from the Su^+ host has roughly the same activity for lysozyme synthesis as that from the Su^- host.

TABLE 2

SUPPRESSION BY PURIFIED sRNA

sRNA added to Su^- extract (RNase I_{10}^-)	Incorporation of H^3 leucine ($\mu\mu$moles/ml)	Lysozyme activity	Lysozyme synthesized / Leucine incorporated
	A. Messenger from T4 wild type		
Su^-	1690	34.6	38
Su_I	1700	38.3	42
Su_{II}	1520	38.6	48
Su_{III}	1950	43.1	42
	B. Messenger from *am* eH26		
Su^-	1730	2.8	0
Su_I	1650	4.9	2
Su_{II}	1600	4.1	2
Su_{III}	1790	17.7	17
	C. Messenger from *am* eM74		
Su^-	1350	4.6	3
Su_I	1820	17.4	16
Su_{II}	1900	11.4	9
Su_{III}	2210	44.2	38

Uncharged sRNA from strains CA 244 (Su^-), CA 266 (Su_I), CA 180 (Su_{II}) or CA 265 (Su_{III}) was added as indicated to make 0.5 mg/ml. The other conditions are as described in Table 1. The lysozyme activities shown have not been corrected for the background of the assay, which was 2.9 (ΔOD $\times$ 1000/min).

Discussion.—The results presented here provide conclusive evidence that the lysozyme activity produced during *in vitro* protein synthesis represents *de novo* synthesis directed by the phage lysozyme messenger. When the RNA used to program the *in vitro* system is obtained by infecting an Su^- host with a lysozyme amber mutant, there can be no question of contamination of the RNA by phage lysozyme (such as denatured lysozyme molecules that might refold during the *in vitro* incubation to produce active enzyme): the amber mutation prevents *in vivo* lysozyme synthesis. This mutant messenger does not program lysozyme synthesis in an Su^- *in vitro* extract. The addition of purified sRNA from Su^+ cells allows efficient lysozyme synthesis to occur by suppression of the amber mutation during the *in vitro* translation. Finally, the *in vitro* suppression of lysozyme amber mutations shows the same specificity as *in vivo* suppression: efficient suppression *in vitro* is found only with those suppressor-mutant pairs which show efficient suppression *in vivo*.

The suppression of lysozyme amber mutations by sRNA from the three amber suppressors in an otherwise Su^- system, is in accord with the results of similar experiments using amber mutants in the coat protein of RNA bacteriophages.[4–6, 10] The specific sRNA species involved in suppression by Su_I and Su_{III} have been identified.[4, 7] In the case of Su_{II}, the preliminary data of Wilhelm[10] show that the sRNA fraction from Su_{II} can suppress RNA bacteriophage amber mutations *in vitro*. However, ribosome alterations have been found in some Su_{II} strains, and genetic data suggest that the suppression and the altered ribosomes are related, as if the suppression were mediated by ribosomes. Our data support the conclusion of Wilhelm[10] that Su_{II} suppression is due to altered sRNA, presumably a new glutamine sRNA species not present in the Su^- cells. Thus the altered ribosomes do not seem to be responsible for suppression.

Amber mutations occurring early in a polycistronic messenger are often polar with respect to synthesis of proteins beyond the mutation. That this polarity also extends to the mRNA has recently been shown for the tryptophane[16] and lactose[17, 18] operons, where a deficiency of mRNA beyond the amber mutation is found. Our preliminary results comparing the lysozyme amber messenger from Su^- and Su^+ cells suggest that a substantial amount of complete messenger is found in the nonpermissive cells. This implies that the untranslated portion of the lysozyme messenger (that beyond the amber mutation) is not missing or obligatorily degraded. However, since mapping of the lysozyme mutations is not complete and since polarity may not exist in the lysozyme gene in the same sense that it does with the polycistronic messenger, these results do not necessarily contradict those from the lactose and tryptophane operons.

It has been suggested that transcription may be coupled with and contingent on translation.[19, 20] However, in the case of lysozyme messenger, the fact that the amber mRNA from Su^- cells is active shows that translation of the *whole* lysozyme messenger is not necessary for its complete transcription.

Summary.—RNA extracted from cells infected with phage T4 carrying an amber mutation in the structural gene for phage lysozyme programs the *in vitro* synthesis of lysozyme activity in extracts from Su^+ cells but not in extracts from Su^- cells. This provides proof of the *de novo* nature of the *in vitro* synthesis of this enzyme and also provides a sensitive assay for *in vitro* suppression. This assay has been used

to show that sRNA is responsible for the suppression in the case of amber suppressors Su_I, Su_{II}, and Su_{III}. Translation (*in vivo*) of the entire lysozyme messenger is apparently not necessary for its transcription since RNA from an Su^- host infected with lysozyme amber mutants programs the *in vitro* synthesis of active enzyme in the appropriate Su^+ extracts.

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[12] One of the steps in the extraction of the RNA[3] involves heating the cells briefly with SDS in a 64°C bath until lysis occurs. It may be difficult to recognize lysis in the very concentrated cell suspensions used. Consequently we have measured the temperature reached by the cell suspensions during this step and have found that lysis occurs at about 42°C under our conditions.

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[14] The *in vitro* synthesis of lysozyme does not require the use of polyethyleneglycol or tetrahydrofolate (although they were used in all of the experiments reported here). Details will be reported in a subsequent communication.

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