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Yeast Super-Suppressors Are Altered tRNAs Capable of Translating a Nonsense Codon in Vitro

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Summary

tRNA isolated from two different yeast super-suppressor strains translates a known nonsense mutation in vitro, whereas tRNA from a closely related nonsuppressing strain does not. Suppression was assayed by translation of RNA isolated from an amber coat mutant of bacteriophage Qβ (GB11) in a protein-synthesizing system derived from mouse tissue culture cells (L cells). Suppressed forms of Qβ coat protein synthesized in vitro were quantitatively detected by a specific immunoprecipitation assay. The L-cell protein-synthesizing system also responds to E. coli suppressor tRNA. This indicates that the biochemical mechanism for nonsense suppression is very similar in yeast and E. coli. These findings also provide additional evidence that the amber codon (UAG) functions as one of the mammalian chain-terminating codons. Since the suppression assay utilizes protein-synthesizing components isolated from mammalian cells, it should prove useful in the search for mammalian nonsense suppressors.

Introduction

The codons UAA (ocher), UAG (amber), and UGA (opal) serve as signals for polypeptide chain termination in bacteria (Brenner, Stretton, and Kaplan, 1965; Weigert and Garen, 1965; Sambrook, Fan, and Brenner, 1967; Zipser, 1967). Recent studies suggest that these codons act as polypeptide chain termination signals in mammalian cells and yeast as well (Beaudet and Caskey, 1971; Martin, 1974; Stewart et al., 1972; Stewart and Sherman, 1972, 1973). A class of mutations can be isolated in bacteria, called nonsense mutations, in which a chain termination codon arises in the interior of a structural gene. Since polypeptide chain termination occurs at the site of the genetic alteration in such mutants, an amino terminal polypeptide fragment is produced by the affected gene.

In bacteria, nonsense mutations may be reverted by extragenic suppressors. These suppressors have been characterized and are known to be altered tRNA molecules (Capecchi and Gussin, 1965; Engelhardt et al., 1965; Goodman et al., 1968). The suppressor tRNAs operate by inserting a specific amino acid at the nonsense codon, thereby allowing completion of the polypeptide chain.

There are also mutations in yeast which are suppressible by a second unlinked mutation called a super-suppressor (Hawthorne and Mortimer, 1963, 1968; Gilmore, Stewart, and Sherman, 1971; Sherman et al., 1973). Although the mode of action of the super-suppressors has not yet been directly examined, evidence suggests that some of the suppressible alleles are nonsense mutations. Analysis of the amino acid replacements in intragenic revertants of suppressible mutants of iso-1-cytochrome c have indicated that the mutants arose by alteration of a wild-type codon to a UAA or UAG codon (Gilmore et al., 1971; Stewart and Sherman, 1972, 1973; Stewart et al., 1972). Biochemical and genetic studies of suppressible mutations indicate that they can be polar (Manney, 1964; Fink, 1966) and, in some cases, that they may lead to the production of incomplete gene products (Manney, 1968; Shaffer, Rytka, and Fink, 1969). Furthermore, tyrosine and serine have been shown to be inserted in vivo at UAA or UAG codons by some super-suppressor strains (Gilmore et al., 1971; Sherman et al., 1973; Liebman, Stewart, and Sherman, 1975; F. Sherman, personal communication). These are two of the amino acids inserted at nonsense codons by known bacterial nonsense suppressors (Weigert and Garen, 1965; Stretton and Brenner, 1965; Notani et al., 1965; Weigert, Lanka, and Garen, 1965; Kaplan, Stretton, and Brenner, 1965). Thus the suppressible alleles in yeast appear to be nonsense mutations. These data are consistent with, but do not prove, the hypothesis that yeast super-suppressors operate by a mechanism similar to that of bacterial nonsense suppressors.

In this paper we demonstrate that tRNA isolated from two strains of yeast carrying different super-suppressor mutations exhibits suppressor tRNA activity in vitro. tRNA isolated from a nonsuppressing yeast strain shows no suppressor activity in vitro. The assay for detecting suppressor tRNA in yeast used a protein-synthesizing system derived from mouse L cells. It was programmed with RNA isolated from the bacteriophage Qβ or a derivative of Qβ (GB11) which contains an amber mutation in the coat protein gene. The L-cell system recognizes the amber codon in GB11 RNA as a chain-terminating codon in vitro. Addition of E. coli suppressor tRNA results in the synthesis of intact coat protein molecules. The system was therefore optimized for suppression using E. coli suppressor tRNA. Gesteland and co-workers, using a different in vitro protein-synthesizing system, have reached similar conclusions about the mechanism of nonsense suppression in yeast (R. Gesteland, personal communication).

There are no known examples of either nonsense mutations or nonsense suppressors in mammalian

cells. The cell-free protein-synthesizing system described in this paper should be useful in the search for mammalian suppressor tRNAs, since it uses only mammalian cell protein synthesis components and responds to known suppressor tRNAs.

Results

Synthesis of Qβ Coat Protein in the Mammalian Cell-Free Extracts

To show that super-suppression in yeast is caused by altered tRNA molecules capable of translating nonsense codons, we have developed a mammalian cell-free protein-synthesizing system sensitive to suppressor tRNAs. The cell-free system is programmed with RNA isolated from the bacteriophage Qβ or a derivative of Qβ (GB11) which contains an amber mutation (UAG) in the viral coat protein gene. Aviv et al. (1972), Schreier et al. (1973), and Morrison and Lodish (1973, 1974) have shown that Qβ coat protein is one of the polypeptide products synthesized in mammalian cell-free extracts programmed with Qβ RNA.

We have increased the sensitivity of the assay for Qβ coat protein synthesized in such mammalian cell-free systems by preparing an antibody directed against purified viral coat protein. Qβ coat protein synthesized in vitro was selectively and quantitatively immunoprecipitated by this antibody (see Experimental Procedures). The immunoprecipitates were then analyzed by sodium dodecyl sulfate–urea polyacrylamide gel (SDS-urea PAG) electrophoresis. The gels were internally calibrated with fluorescenated protein molecular weight markers (Hughes, Wahl, and Capecchi, 1975). The specific antibody reaction provides further evidence for the synthesis of Qβ coat protein in such cell-free systems. Figure 1B shows that mouse L-cell extracts programmed with Qβ RNA synthesize a protein product which reacts with anti-Qβ coat protein serum and has the same molecular weight as Qβ coat protein. No such material is synthesized in the extracts without added Qβ RNA (A), nor is any such material synthesized in response to Qβ RNA containing an amber mutation in the viral coat protein cistron (C). The specificity of the antibody reaction is illustrated in the control experiment shown in Figure 1D. This panel demonstrates that Qβ coat protein synthesized in vitro is not precipitated by a nonimmune serum. We can conclude from these experiments that Qβ coat protein is synthesized in these mammalian cell-free extracts. The low background in the controls permits detection of very small amounts of Qβ coat protein (~10^{-15} moles).

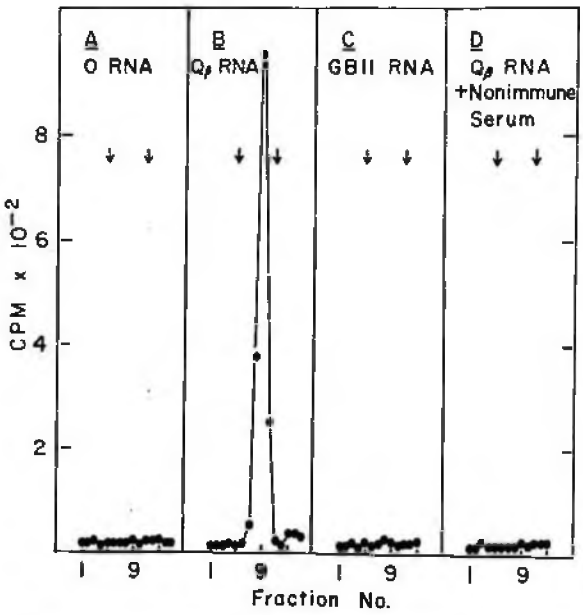

Figure 1. Synthesis of Qβ Coat Protein by a Cell-Free Protein-Synthesizing System Derived from Mouse L Cells

In vitro protein synthesis was performed and the reaction mixtures immunoprecipitated, washed, electrophoresed, and processed as described in Experimental Procedures. The arrows indicate the position of migration of the fluoresceinated molecular weight markers cytochrome (left arrow, molecular weight 12,500 daltons) and myoglobin (right arrow, molecular weight 17,000 daltons). The fluoresceinated molecular weight markers migrate with their unlabeled counterparts on SDS-urea gels (Hughes et al., 1975). Qβ coat protein has a monomer molecular weight of 14,000 daltons (Konigsberg et al., 1970). Each point on the graphs represents the radioactivity present in a 1 mM slice of the gel.

(A) shows the pattern of radioactivity on an SDS-urea PAG of a reaction (immunoprecipitated with anti-Qβ coat serum) in which protein synthesis was done without adding Qβ RNA. The endogenous level of amino acid incorporation was 5 × 10^4 cpm. (B) shows the pattern of immunoprecipitable radioactivity seen after adding Qβ RNA to the L-cell protein-synthesizing system. (C) shows the pattern of radioactivity obtained when the in vitro system is programmed with GB11 RNA (a mutant of Qβ containing an amber mutation in the coat protein gene). (D) is the same experiment as shown in (B), except that the immunoprecipitation reaction was done with serum from a rabbit which had not been immunized with Qβ coat protein.

The System Responds to E. coli Suppressor tRNA

Figure 2C shows that addition of partially purified E. coli suppressor tRNA (isolated from a strain containing an *Su 1* suppressor gene, that is, Su+) to the mammalian cell-free system programmed with GB11 RNA allows synthesis of complete coat protein molecules. Figure 2B demonstrates that identically purified tRNA from the isologous E. coli strain lacking the *Su 1* suppressor gene (Su−) fails to permit the synthesis of coat protein. This mammalian cell-free system therefore responds to suppressor tRNA. These experiments imply that the amber codon (UAG) functions in mammalian cells in an analogous fashion to its role in bacterial systems

(that is, as a polypeptide chain termination signal). Figure 3 shows that the addition of increasing amounts of purified E. coli suppressor tRNA results in increasing levels of suppression. This indicates that there is a competition between the suppressor tRNA and the mammalian polypeptide chain termination machinery for the UAG codon. These experiments also provide an assay for detecting suppressor tRNAs isolated from other cell sources, such as yeast or mammalian cells.

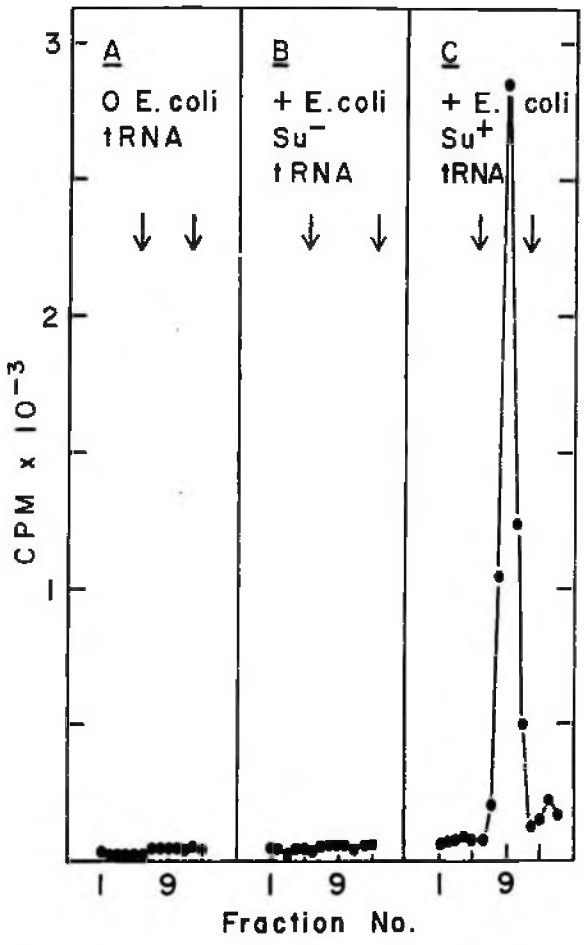

Figure 2. Suppression of the Amber Coat Mutant GB11 in a Mammalian Cell-Free Extract by E. coli Suppressor tRNA

L-cell extracts were programmed with GB11 RNA. The reactions were immunoprecipitated, electrophoresed, and processed as described in Experimental Procedures. The arrows represent the position of migration of the fluoresceinated molecular weight markers cytochrome c (left arrow, molecular weight 12,500 daltons) and myoglobin (right arrow, molecular weight 17,000 daltons).

(A) shows the pattern of immunoprecipitable radioactivity from an in vitro protein synthesis reaction programmed with GB11 RNA. (B) shows the pattern of immunoprecipitable radioactivity from an identical reaction to that in (A), to which 20 μg of fractionated S26 tRNA (see Experimental Procedures) were added. S26 is a suppressor negative strain. (C) is as (B), except that the 20 μg of fractionated tRNA were isolated from S26RIE, a strain which contains *Su 1* suppressor tRNA. S26 and S26RIE are isologous strains except for the suppressor gene. The tRNA used in the experiments shown in (B) and (C) was prepared identically, and the corresponding fractions were used.

Yeast Super-Suppressor Strains Contain Suppressor tRNAs

Experiments analogous to those described above were performed to test the hypothesis that yeast super-suppressors contain altered tRNA molecules capable of translating nonsense codons. tRNA was isolated from a yeast super-suppressing strain (D599-4B) which contains the SUP7-2 suppressor gene. This strain inserts tyrosine at UAG codons in vivo (Sherman et al., 1973). The tRNA was fractionated by benzoylated DEAE cellulose (BD cellulose) chromatography, and the tyrosine-accepting fractions were pooled. The experiment illustrated in Figure 4 shows that this tRNA fraction is capable of suppressing the UAG codon in GB11 RNA. Figure 4B demonstrates that the same tRNA fraction from the closely related yeast strain (SL110) which does not contain a super-suppressor gene fails to permit synthesis of intact coat protein molecules. These experiments demonstrate that the super-suppressor strain D599-4B contains altered tRNA molecules capable of translating the amber codon in the GB11 coat protein cistron.

Figure 5 shows the optical density profile of the D599-4B tRNA eluted from the BD cellulose column.

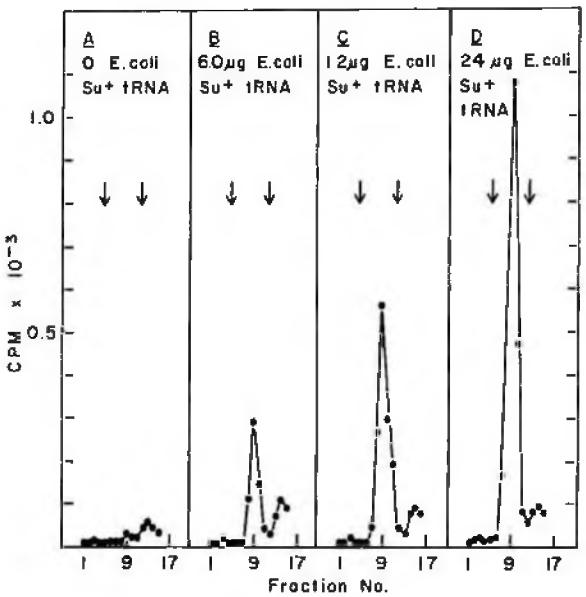

Figure 3. Suppression of the Amber Coat Protein Mutant GB11 with Increasing Amounts of *Su 1* (E. coli) tRNA

L-cell extracts were programmed with GB11 RNA, and the reactions immunoprecipitated, electrophoresed, and processed as described in Experimental Procedures. For the experiments shown in panels A–D, 0 μg, 6 μg, 12 μg, and 24 μg of fractionated S26RIE tRNA were added.

The tRNA was pooled into three fractions as indicated on the graph. The tyrosine-accepting specific activity of each fraction was determined and is given in Table 1. Figure 6 indicates that fraction III contains the highest tyrosine-accepting specific activity and also the highest suppressor specific activity. Fraction 1 of the D599-4B tRNA, which had a very low tyrosine-accepting specific activity, contained no detectable suppressor tRNA activity. Each fraction of the D599-4B tRNA has been measured for suppressor tRNA activity over a range of tRNA concentrations (data not shown). For the experiments shown in Figure 6, an equal amount of tRNA (OD_{260}) from each fraction was added to the reaction mixtures. Since the suppressor tRNA activity chromatographs with the tyrosine acceptor activity, these experiments are consistent with the hypothesis that the suppressor tRNA arose from an altered tyrosyl tRNA. In vitro suppression was not

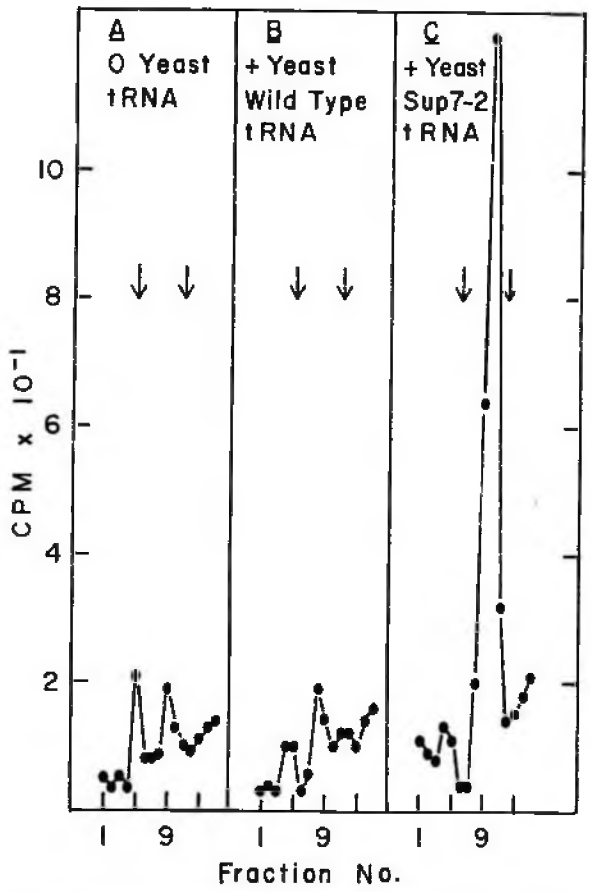

Figure 4. Suppression of the Amber Mutation in GB11 RNA by Yeast SUP7-2 tRNA

L-cell extracts were programmed with 60 μg of GB11 RNA. Reactions were immunoprecipitated with anti-Qβ coat sera, electrophoresed, and processed as described in Experimental Procedures.

(A) shows the immunoprecipitable radioactivity from a control experiment with no added tRNA. (B) shows the same reaction with the addition of 20 μg of BD cellulose-fractionated tRNA (see Experimental Procedures) isolated from the nonsuppressing strain SL-110. (C) shows the immunoprecipitable radioactivity synthesized when 20 μg of fractionated tRNA isolated from the super-suppressing yeast strain D599-4B (SUP7-2-containing) were added to the reaction mixture. The tRNA from SL-110 and D599-4B was prepared identically, with analogous fractions used for the experiments presented in (B) and (C).

Table 1. Amino Acid Acceptor Activities of Fractionated Yeast tRNA

BD Fraction	D599-4B (SUP7-2) Relative Tyrosyl tRNA Specific Activity	L-374 (SUQ5-2) Relative Seryl tRNA Specific Activity
Fraction I	3	29
Fraction II	33	62
Fraction III	100	100
Fraction IV		87

Relative amino acid acceptor activity in the benzoylated DEAE cellulose fractions of tRNA isolated from super-suppressing yeast strains.

The tRNAs were fractionated on benzoylated DEAE cellulose as shown in Figures 5 and 7. tRNA charging assays were done as described in Experimental Procedures. Specific activity of tRNA charging was normalized to the tRNA fraction containing the highest in vitro suppressor activity.

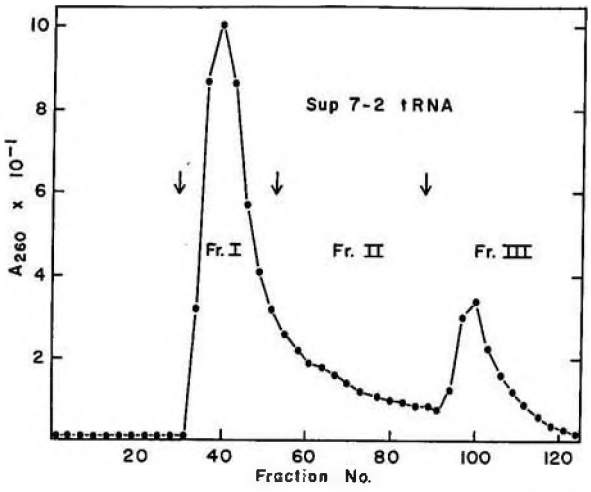

Figure 5. Fractionation of D599-4B tRNA by Benzoylated DEAE Cellulose chromatography

285 mg of purified tRNA from D599-4B (a SUP7-2-containing strain) were loaded onto a 1.4 cm × 50 cm column of BD cellulose in 25 mM Tris–Cl (pH 7.4), 10 mM $MgCl_2$, 1 mM DTT. The column was washed with 200 ml of this buffer, followed by 200 ml of 25 mM Tris–Cl (pH 7.4), 10 mM $MgCl_2$, 200 mM NaCl, and 1 mM DTT. The tRNA was eluted in two steps. The first wash, which removed ~80% of the tRNA bound to the column, was 25 mM Tris–Cl (pH 7.4), 10 mM $MgCl_2$, 200 mM NaCl, 1 mM DTT (pH 7.4). The remaining ~20% of the tRNA, containing most the suppressor activity (see Table 1), was eluted with 150 ml of 25 mM Tris–Cl, 10 mM $MgCl_2$, 1.1 m NaCl, 10% methoxyethanol, 1 mM DTT (pH 7.4). tRNA was pooled into fractions as shown by the arrows.

observed with tRNA prepared from the nonsuppressing strain SL110, either before fractionation on BD cellulose or with the fractions obtained after BD cellulose chromatography (data not shown).

Figures 7 and 8 show analogous experiments using a different super-suppressing yeast strain (L374). This strain contains the SUQ5-2 suppressor gene which inserts serine at UAG codons in vivo (F. Sherman, personal communication). The L374 tRNA was fractionated on a BD cellulose column and pooled into four fractions, as illustrated in Figure 7. Table 1 shows the relative serine-accepting specific activity for each of these fractions. Figure 8 demonstrates that the SUQ5-2 strain does contain tRNA capable of translating the UAG codon in GB11 RNA. In addition, the suppressor-specific activity correlates well with the serine-accepting specific activity. These results suggest that the SUQ5-2 suppressor tRNA was derived from an alteration in a seryl tRNA species.

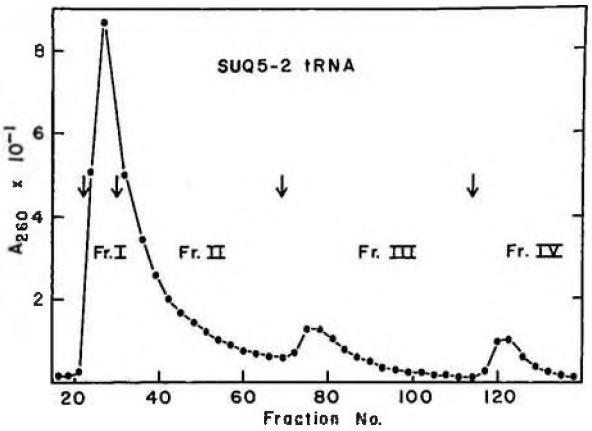

Figure 7. Fractionation of L-374 (SUQ5-2) tRNA by BD Cellulose Column Chromatography

249 mg of purified L-374 were prepared and loaded onto the benzoylated DEAE cellulose column as described for the D599-4B tRNA (see Figure 5). The tRNA was eluted in three steps. The first wash was with 200 ml of 25 mM Tris–Cl (pH 7.4), 10 mM $MgCl_2$, 650 mM NaCl, 1 mM DTT. This was followed by 200 ml of 25 mM Tris–Cl, 10 m $MgCl_2$, 1.1 M NaCl, 1 mM DTT (pH 7.4). The final elution was with 200 ml of 25 mM Tris–Cl (pH 7.4), 10 mM $MgCl_2$, 1.1 M NaCl, 10% methoxyethanol, 1 mM DDT (pH 7.4). The tRNA fractions were pooled as indicated by the arrows.

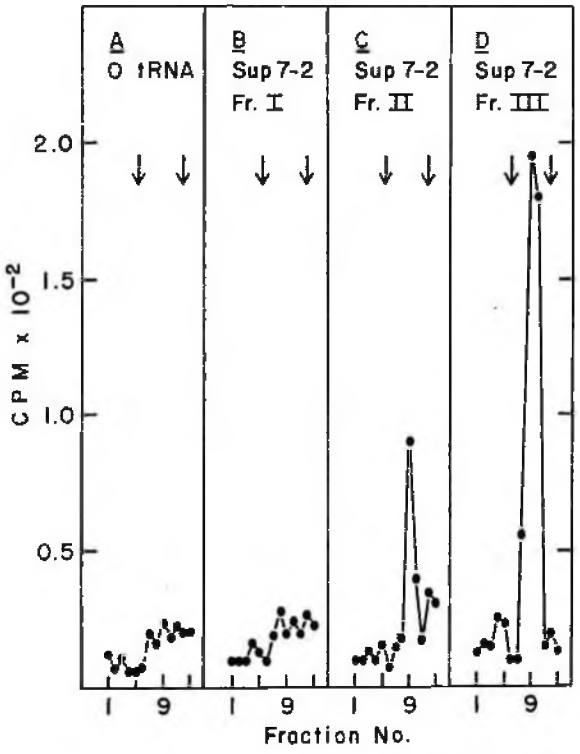

Figure 6. In vitro Suppression with Benzoylated DEAE Fractions of tRNA from the SUP7-2-Containing Yeast Strain D599-4B

Fractions obtained in Figure 5 were assayed for suppressor activity as described in Experimental Procedures. (A) shows the pattern of counts from a control experiment in which no tRNA was added to the mammalian in vitro protein-synthesizing system programmed with GB11 RNA. The experiments shown in (B), (C), and (D) used 20 μg of tRNA from D599-4B fractions I, II, and III.

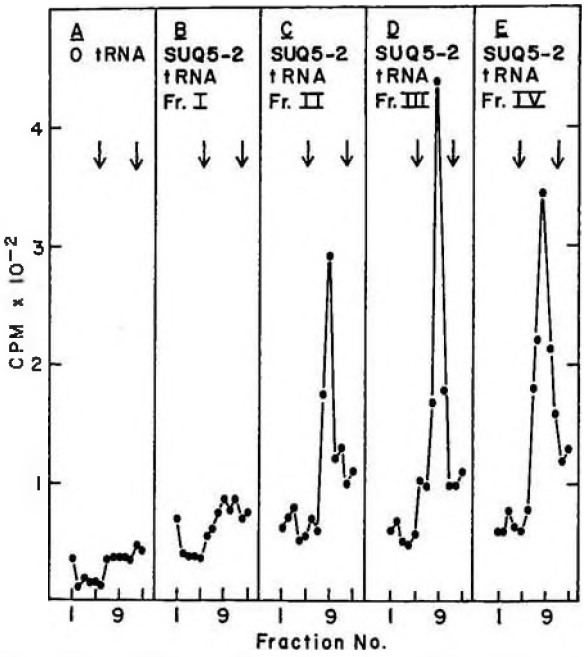

Figure 8. In Vitro Suppression with Benzoylated DEAE Fractions of tRNA Isolated from the SUQ5-2-Containing Yeast Strain L-374

The tRNA fractions are those shown in Figure 7. The assay for suppressor activity is as described in Experimental Procedures and Figure 1. (A) is the pattern of immunoprecipitable radioactivity on an SDS-urea polyacrylamide gel of the control experiment without added tRNA. (B) is the experiment with 20 μg of fraction I tRNA. (C) shows 20 μg of fraction II tRNA, (D) 20 μg of fraction III tRNA, and (E) 20 μg of fraction IV tRNA.

Efficiency of Suppression

The data presented in this paper cannot be used to calculate the in vivo efficiency of suppression for two reasons. First, these experiments were performed over an extended time period and used different mouse L-cell extracts. The different extracts varied in their ability to translate Qβ RNA. Since this ability was measured for each extract, the data could be normalized for this parameter. A more serious difficulty is determining the degree of purity of the suppressor tRNA species. In theory, using pure suppressor tRNA, we should be able to approach 100% suppression in the cell-free system simply by adding increasing amounts of suppressor tRNA. 100% suppression is defined to mean the synthesis of as much Qβ coat protein by the L-cell system programmed with GB11 RNA and suppressor tRNA as is synthesized with Qβ RNA. (The efficiency of suppression could not be calculated by measuring the amount of amino terminal fragment produced relative to suppressed coat protein because the fragment is degraded in this system.) Very high levels of suppressor tRNA should compete out the polypeptide chain termination machinery at the appropriate nonsense codons. In practice, the saturating levels of suppression observed in the heterologous system are less than 100%. Using the E. coli suppressor tRNA purified on BD cellulose (Gilham et al., 1967) and RPC-5 (Pearson, Weiss, and Kelmers, 1971), we observe saturation at approximately 60% suppression. Addition of more tRNA results in inhibition of amino acid incorporation and reduced levels of detectable Qβ coat protein (data not shown). With the yeast suppressor tRNAs fractionated by BD cellulose chromatography, we observe saturation at approximately 15% suppression. Again, the addition of more tRNA results in inhibition of translation and decreased levels of Qβ coat protein synthesis. These experiments indicate that the tRNA fractions contain an inhibitor. It is reasonable to suspect that the inhibitors are tRNA molecules, since we are working with a heterologous system (that is, E. coli or yeast tRNA in a mammalian cell-free protein-synthesizing system).

Discussion

Our data demonstrate that the super-suppressors in yeast (Saccharomyces cerevisiae) and the nonsense suppressors in bacteria act by a similar biochemical mechanism. Both of the super-suppressor strains D599-4B (SUP7-2) and L374 (SUQ5-2) were shown to contain tRNA molecules capable of translating in vitro the amber (UAG) codon in the coat protein ciston of the mutant Qβ phage GB11. tRNA from the closely related nonsuppressing strain SL-110 does not suppress GB11 in vitro. Previous work has shown that the strains D599-4B (SUP7-2) and L374 (SUQ5-2) insert tyrosine and serine, respectively, at UAG codons in vivo (Sherman et al., 1973; F. Sherman, personal communication). The tRNA isolated from D599-4B was fractionated by chromatography on a benzoylated DEAE cellulose column. The suppressing activity from D599-4B is coincident with the tyrosine-accepting tRNA fraction (Table 1). L374 (SUQ5-2) tRNA was also fractionated on benzoylated DEAE cellulose. The suppressor activity chromatographs with the serine-accepting tRNA fractions. This suggests that SUP7-2 was derived from a tyrosyl tRNA and that SUQ5-2 was derived from a seryl tRNA.

The cell-free protein-synthesizing system used in these experiments was prepared from mouse tissue culture cells (L cells). This system, when programmed with GB11 RNA, responds to E. coli *Su 1* suppressor tRNA. This directly relates the suppression observed in vitro with yeast tRNA to the well characterized nonsense suppressors of E. coli.

The conditional lethal system provided by nonsense mutations and their suppressors has been extremely valuable for genetic and biochemical analysis of bacteria and yeast. Development of a nonsense mutant suppressor system in cultured mammalian cells and their viruses should also be very useful. First, it would provide a nonleaky conditional lethal system for genetic analyses. Because of the narrow temperature range over which mammalian cell lines can be propagated, temperature-sensitive mutations often exhibit partial activity at the nonpermissive temperature. Second, the nonsense mutant system can provide a direct means of identifying the mutant gene product. In a nonsuppressing cell line, the nonsense mutation would cause an amino terminal fragment to be produced from the affected gene.

Since the suppression assay employed in these experiments used a protein-synthesizing system derived from mouse L cells, it should be compatible with mammalian suppressor tRNAs. The demonstration that yeasts can tolerate nonsense suppressors indicates that it is reasonable to seek such suppressors in mammalian tissue culture cells. In addition, it is now clear that, at least in vitro, a mammalian protein-synthesizing system is capable of nonsense suppression if provided with an appropriate tRNA and message.

Experimental Procedures

Materials

RPC-5 was obtained from Astro Enterprises. Benzoylated DEAE cellulose, ^{14}C–tyrosine (460 mCi/mmole), ^{3}H–leucine (50 Ci/mmole), and ^{3}H–lysine (47 Ci/mmole) were purchased from Schwarz-Mann. ^{14}C–serine (153 mCi/mmole) was obtained from

New England Nuclear. ATP, GTP, CTP, pyruvate kinase (PK), and creatine phosphokinase (CPK) were purchased from Calbiochem. Triton X-100 was the product of Rohm and Haas Co. (Philadelphia, Pennsylvania). Acrylamide and N,N' methylene bisacrylamide were obtained from Eastman Kodak. Ultra-pure SDS was purchased from the Pierce Chemical Co. (Rockford, Illinois). Freund's complete adjuvant was obtained from Miles. The purified Qβ coat protein used to prepare antiserum was a gift from Dr. A. Wiener. The coat protein was prepared from purified virus by chromatography on a DEAE-urea column. Phenol was redistilled before use and stored in the dark at −20°C.

Cells and Strains

The mouse tissue culture cells (L cells) used to prepare the cell-free extracts have been previously described (Sharp, Capecchi, and Capecchi, 1973). The yeast strains D599-4B, L374, and SL110 were supplied by Dr. F. Sherman. D599-4B contains the amber super-suppressor SUP7-2. This strain was cloned on minimal agar (Manney, 1964), supplemented with histidine, leucine, and lysine. D599-4B also contains the Tyr7-1 and Trp1-1 alleles which are suppressed by SUP7-2. The cells were grown at 30°C in a broth culture containing 1% yeast extract, 2% peptone, and 3% dextrose (YEPD). For preparation of yeast tRNA, the cells were grown in 10 l cultures and harvested at 10 g/l. In YEPD medium, the SUP7-2-containing cells grow more slowly than cells which have lost the suppressor activity. We therefore plated out the cells just before harvesting to measure the ratio of suppressor-containing to suppressor negative cells. More than 80% of the cells used for isolation of SUP7-2 tRNA exhibited suppressor activity, as demonstrated by their ability to grow on the appropriate agar plates.

L-374, which contains the amber super-suppressor SUQ5-2 and is ψ+ (Cox, 1965), was also cloned on histidine-, leucine-, and lysine-containing minimal agar plates. This strain contains the SUQ5-2 suppressible alleles met8–1, trp1–1, and tyr7–1. Cells were grown as described for D599-4B. More than 95% of the L-374 cells used to prepare the tRNA still retained the SUQ5-2 suppressor gene when the culture was harvested. SL-110, which does not contain a super-suppressor, was grown from a single colony picked from YEPD agar plates. E. coli strains containing suppressors were routinely tested with bacteriophage λ and T4 tester strains to ensure that they retained the appropriate suppressing activity. E. coli strains were grown in broth containing 0.2 g $MgCl_2 6H_2O$, 1.98 g $(NH_4)_2SO_4$, 2.72 g KH_2PO_4, 14 g K_2HPO_4, 4 g vitamin-free casamino acids (Difco), and 10 g of glucose per liter. Cells were harvested at 5 g/l. All tRNAs isolated from suppressor-containing E. coli strains were shown to contain suppressor activity in an E. coli in vitro protein-synthesizing system (Capecchi, 1966). Qβ and GB11 (a derivative of Qβ containing an amber mutation in the coat protein gene) were provided by Dr. P. Kaesberg. Qβ and GB11 were grown on S26 and the isogenic *Su 1*-containing strain S26RIE.

Preparation of Qβ and GB11 RNA

Qβ and GB11 were grown and purified according to the procedure of A. Wiener (1973). RNA was extracted from purified phage with distilled phenol as described by Capecchi (1966).

tRNA Preparation

tRNA was prepared from yeast and E. coli according to the methods of Capecchi (1966) with the following modifications. tRNA was extracted from freshly harvested cells at 4°C using 1 vol (per weight of cells) of phenol and 1 vol of 50 mM NaH_2PO_4 (pH 7.0), 1 mM dithiothreitol (DTT). For yeast tRNA, SDS was added to the extraction buffer to give a final concentration of 1%. The tRNA samples were purified by extraction with 1 M NaCl containing 1 mM DTT, and DEAE cellulose chromatography (Capecchi, 1966). The purified tRNA samples were fractionated on benzoylated DEAE cellulose according to Gilham et al., 1967. The *Su 1*-containing tRNA fraction purified by benzoylated DEAE cellulose chromatography of S26RIE tRNA was further purified by chromatography on RPC-5 (Pearson et al., 1971). S26 tRNA was prepared and purified in an identical manner so that analogous fractions of tRNA could be obtained from the nonsuppressing cell line.

Assays of tRNA Acceptor Activity

Each 50 μl assay for serine or tyrosine tRNA acceptor activity contained 5 μl of an appropriate dilution of tRNA, Tris–HCl (pH 7.4) (67.5 mM), KCl (108 mM), $MgCl_2$ (4.5 mM), EDTA (0.45 mM), BSA (1 mg/ml), ATP (1.6 mg/ml), yeast S-300 (supernate from a 300,000 × g centrifugation, 4 mg protein/ml), PEP (5 mM), PK (2.7 I.U/ml), DTT (1.8 mM), ^{14}C–serine (1.8 μCi/ml), or ^{14}C–tyrosine (1.26 μCi/ml). For each assay a range of tRNA concentrations (10–50 μg/50 μl reaction) was tested to ensure that the measured acceptor activity was within the linear range.

Preparation of Antisera

Two 10 lb male New Zealand white rabbits were injected suprascapularly at weekly intervals with 1.5 mg of purified Qβ coat protein. The coat protein was suspended in 1 ml of 50 mM Tris–HCl (pH 7.4), 7 mM $MgCl_2$, and then an emulsion was made with 1 ml of Freund's complete adjuvant. After 6 weeks of injections, the animals were bled and titered by precipitation of radioactive Qβ coat protein synthesized in vitro. One rabbit showed a response. After a 6 week rest, injections were resumed on a weekly basis. Blood (approximately 50 ml) was collected from the ear veins every 2 weeks. When the titer reached a plateau level, the serum was used for the detection of Qβ coat protein synthesized in vitro. Antisera were processed and purified by $(NH_4)_2SO_4$ fractionation as previously described (Wahl, Hughes, and Capecchi, 1975).

Immunoprecipitation and Gel Electrophoresis

The in vitro protein-synthesizing reactions were analyzed for Qβ coat protein by quantitative immunoprecipitation with anti-Qβ coat serum followed by electrophoresis of the precipitate on calibrated SDS-urea polyacrylamide gels (Hughes et al., 1975). 5 mg of antibody directed against purified Qβ coat protein were added to each 100 μl L-cell reaction mixture. After reacting overnight at 4°C, 10 mg of goat anti-rabbit gammaglobulin were added and allowed to react for 4 hr at 4°C. The resulting precipitate was collected by centrifugation for 30 min at 1000 × g. The pellets were washed twice at 4°C with 400 μl of buffer containing 25 mM Tris–HCl (pH 7.4), 3.5 mM $MgCl_2$, 300 mM KCl, 2% Triton X-100, and once with buffer containing 50 mM Tris–HCl (pH 7.4), 7 mM $MgCl_2$. The pellet was recovered after each wash by centrifugation at 1000 × g for 30 min. Resuspension of the pellet in SDS-urea buffer, electrophoresis of the sample on SDS-urea polyacrylamide gels, and processing of the gels for analysis of radioactivity have been previously described (Hughes et al., 1975). The only modification in these studies is the use of 15% instead of 11.1% polyacrylamide gels. Each gel was internally calibrated with fluoresceinated myoglobin and cytochrome c (Hughes et al., 1975).

Quantitative Immunoprecipitation of Qβ Coat Protein

In developing the immunoprecipitation assay for the detection of in vitro synthesized Qβ coat in the L-cell extracts, two criteria were required: the immunoprecipitation had to be quantitative, and the serum should recognize in vitro Qβ coat protein containing a number of different amino acid substitutions at the site of the amber mutation in GB11 (position 17). Both of these parameters were tested using an E. coli protein-synthesizing system [illegible] free extracts programmed with Qβ RNA. Qβ coat [illegible] predominant protein synthesized. In Figure 9 we [illegible] rylamide gel electrophoretic analyses of the [illegible] product and immunoprecipitable product [illegible] tions: the reaction programmed with Qβ RNA; [illegible] reaction programmed with GB11 RNA; [illegible] tyrosyl suppressor tRNA (*Su 3* tRNA) [illegible] in Figure 9 that the immunoprecipitation

wild-type coat protein (which contains glutamine at position 17) and GB11 suppressed coat (which contains tyrosine at position 17). We have also demonstrated that the GB11 coat with serine inserted at the amber site is quantitatively immunoprecipitated (data not shown). These experiments demonstrate that the antisera can be used to quantitatively precipitate suppressed forms of Qβ coat.

It is interesting that GB11 will not form plaques on an *Su 3* (tyrosine-inserting) strain (data not shown). This is not a problem of efficiency of suppression, since an *Su 1* strain (serine-inserting) which has a lower level of suppression than *Su 3* (Garen, 1968) nevertheless is a permissive host for GB11. This leads to the conclusion that active phage cannot be formed with tyrosine at position 17 of the coat protein, but that at least some of the antigenic determinants are still present.

L-Cell Extracts

Mouse L cells were grown in spinner culture (8 l) to a density of 5×10^5 cells per ml and harvested by centrifugation at 4°C for 10 min at 160 × g. The cells were then washed 2 times with 150 ml of buffer containing 35 mM Tris–HCl (pH 7.4), 146 mM NaCl, and 11 mM glucose. The washed cells were resuspended in 20 ml of buffer containing 10 mM Tris–HCl (pH 7.4), 1.5 mM $MgCl_2$, 15 mM KCl, and 2 mM DTT. The cells were allowed to swell for 10 min at 4°C, and then were broken by 30 strokes in a tightly fitting Dounce homogenizer. 2.5 ml of buffer containing 200 mM Hepes (pH 7.2), 30 mM $MgCl_2$, 1 M KCl, and 20 mM DTT were added to the crude homogenate. The cell homogenate was clarified by centrifugation at 20,000 × g for 30 min. To reduce the endogenous level of protein synthesis, the supernatant was incubated at 32°C for 30 min in the presence of 2 mM ATP, 0.2 mM GTP, 7.5 mM creatine phosphate (CP), and 100 μg of creatine phosphokinase. After the incubation, the reaction mixture was centrifuged at 1000 × g for 20 min and chromatographed on a 300 ml G25 Sephadex column. The macromolecular fractions were pooled and concentrated by dialysis against 2L of 20 mM Hepes (pH 7.2), 3 mM $MgCl_2$, 100 mM KCl, 2 mM DTT, and 15% polyethylene glycol (Union Carbide).

Mammalian Cell-Free Protein-Synthesizing System

A standard 100 μl reaction mixture contained 50 mM Hepes (pH 7.2), 3.5 mM $MgCl_2$, 90 mM KCl, 2 mM ATP, 0.6 mM GTP, 12.5 mM CP, 6 μg CPK, 40 μM of each amino acid except lysine and leucine, 2 mM DTT, 2.5 μCi of ^{3}H–leucine, 2.5 μCi of ^{3}H–lysine, 60 μl of L-cell extract (prepared as described above), and, unless otherwise stated, 60 μg of either Qβ RNA or GB11 RNA. The amount and type of tRNA added to the reaction mixtures are indicated in the figure legends. The reaction mixtures were incubated at 33°C for 45 min. At the end of the incubation, the reaction was terminated by dilution with an equal volume of ice cold buffer (0°C) containing 50 mM Hepes (pH 7.2) and 3 mM $MgCl_2$. The reactions were then reacted with anti-Qβ coat serum as described in Immunoprecipitation and Gel Electrophoresis.

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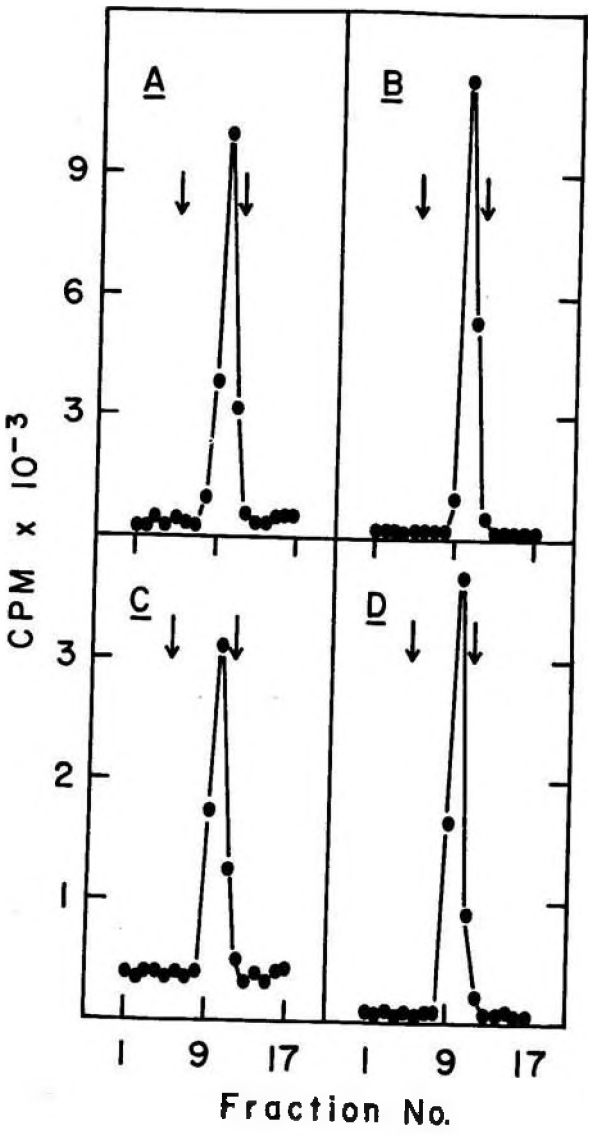

Figure 9. Immunoprecipitation and TCA Precipitation of Qβ Coat Protein Synthesized in Vitro

E. coli extracts were programmed with Qβ or GB11 RNA, and protein synthesis carried out as described by Capecchi (1966). After incubation at 36°C for 30 min, the reactions were divided into two equal aliquots, one of which was immunoprecipitated as described in Experimental Procedures. 150 μl of 1 M NaOH were added to the other, followed by 2 ml of 7% TCA. TCA precipitates were collected by centrifugation, the pellets resolubilized in 1 M NaOH, and then reprecipitated with 2 ml of 7% TCA. The precipitates were collected by centrifugation, the pellets washed twice with ice cold ether-ethanol (2 vol:1 vol), and dried with a stream of nitrogen. The pellets were then dissolved in loading buffer, electrophoresed, and the gels analyzed as described in Experimental Procedures.

(A) shows the electrophoretic pattern obtained from an E. coli reaction mix programmed with Qβ RNA and TCA precipitated as described. The arrows indicate the position of migration of the fluorescein-labeled molecular weight standards cytochrome c (left arrow, molecular weight 12,500 daltons) and myoglobin (right arrow, molecular weight 17,000 daltons). (B) is the electrophoretic pattern of an equal aliquot of the reaction mix used in (A), but precipitated with antibody directed against purified Qβ coat protein. The peak of radioactivity seen in (A) and (B) migrates at the position of Qβ coat protein. The amount of radioactivity in the peak at the Qβ coat position in (A) and (B) is identical. (C) and (D) are analogous to (A) and (B), except that the reaction mixture was programmed with GB11 RNA and a saturating level (10 μg per 50 μl reaction) of purified E. coli tyrosine suppressor tRNA. The peaks in (C) and (D) have identical amounts of radioactivity.

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