JOURNAL OF BACTERIOLOGY, July 1973, p. 129-138

Vol. 115, No. 1
Printed in U.S.A.

Ambiguity in a Polypeptide-Synthesizing Extract from *Saccharomyces cerevisiae*

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Received for publication 19 December 1972

Environmental factors known to induce ambiguity in bacterial extracts were tested in an in vitro cytoplasmic polypeptide-synthesizing system derived from *Saccharomyces cerevisiae*. Increasing concentrations of magnesium, spermine, and spermidine resulted in extensive leucine-phenylalanine ambiguity in polyuridylic acid-directed polypeptide synthesis. Kinetic studies showed that spermine-mediated stimulation of leucine incorporation occurred when phenylalanine was being actively incorporated. In addition to leucine, the amino acids isoleucine and serine were incorporated in the presence of added magnesium and spermine. Ambiguity in the presence of a high Mg^{2+} concentration was decreased when the pH of the reaction mixture was lowered. Ethanol and neomycin enhanced ambiguity to a small, but significant, extent. Streptomycin and temperature had no effect on ambiguity. Leucine, isoleucine, and serine were not attached to phenylalanine transfer ribonucleic acid (tRNA) when the aminoacylation reaction was performed at increasing Mg^{2+} and spermine concentrations. On the other hand, increasing levels of Mg^{2+} and spermine stimulated the incorporation of leucine from tRNA into polypeptide during the transfer reaction. The formal similarity between the findings in the yeast and *Escherichia coli* systems implies the existence of a tRNA-screening site on the yeast ribosome comparable to that suggested for bacteria. A proposal is made as to the manner in which this site may function to produce the ambiguous codon translation observed.

Details of the molecular mechanism for accurate translation of the genetic code are not known. The first indication of the involvement of a factor other than codon-anticodon pairing came from Davies et al. (10) who showed that the ribosome contributes to the accuracy of translation. Ribosomes from wild-type *Escherichia coli*, but not from a streptomycin-resistant strain, gave ambiguous translation of messenger ribonucleic acid (RNA) in the presence of streptomycin.

Further studies revealed that, in addition to aminoglycoside antibiotics (10, 11), other environmental factors, such as elevated magnesium concentrations (18, 38), decreased temperature (18, 38), polyamines (18), and ethanol (35), induced a high level of ambiguity in translation. By examination of the amino acid response to various synthetic messengers (10, 11, 12, 18), ambiguity was found to be nonrandom; a specific group of amino acids, those having connecting codons, was incorporated in response to each messenger employed. Parameters of the protein-synthesizing machinery, namely, transfer RNA (tRNA) concentration (11, 22, 36), availability of amino acids (36, 38, 41), and the chain length of messenger RNA (30), influenced the level of ambiguity observed.

The study of ambiguous translation is a powerful tool for characterizing the parameters of the protein-synthesizing machinery which determine faithful translation of the code. This approach, used in bacterial systems at the subcellular and cellular levels, has yielded substantive information and insight into the solution of the coding problem (9, 20, 24, 43).

In contrast to the marked sensitivity of bacterial systems to environmentally induced ambiguity, little or none has been reported in crude amino acid-incorporating systems obtained from eukaryotic cells (5, 13, 37, 42). Therefore, there is no system available from higher organisms to compare with bacterial extracts in reference to the mechanisms of fidelity. In this communication, we report the induction of reproducibly high levels of ambiguity in a crude

cell-free system derived from *Saccharomyces cerevisiae*.

(This paper is part of a thesis submitted by the senior author in partial fulfillment of the requirements for the Ph.D. degree from the City University of New York, N.Y., 1972. A preliminary account of part of this work was presented at the 70th Annual Meeting of the American Society for Microbiology, Boston, Mass., 26 April to 1 May, 1970 [Bacteriol. Prac., p. 150, 1970].)

MATERIALS AND METHODS

Growth of cells. A starter culture of *S. cerevisiae* ATCC 9767 was grown for 20 to 22 h in broth containing (in grams per liter): tryptone (Difco), 10.0; yeast extract (Difco), 5.0; NaCl, 5.0; glucose, 2.0; at PH 7.0. Incubation was at 30 C and 300 rpm on a Gyrotary Shaker (New Brunswick Scientific Co., Inc.). A 900-ml amount of the broth described was seeded with 20 ml of the starter culture and grown until mid-log phase (A_{550} = 0.78, Spectronic 20, Bausch and Lomb). Flasks were plunged into an ice-water mixture, and the cells were harvested by centrifugation, washed with buffer [5 mM magnesium acetate, 20 mM potassium chloride, 10 mM tris(hydroxymethyl)aminomethane (Tris)-hydrochloride (pH 7.8), 6 mM β-mercaptoethanol], and stored at −20 C.

Preparation of subcellular fractions. All procedures were carried out at 2 to 4 C. Frozen pellets were ground by hand to a liquid consistency in a mortar and pestle at 4 C with twice their wet weight of acid-washed glass beads (0.11 mm diameter), assuming 1 ml of beads equals 1 g. It was important to use a rapid motion in grinding in order to obtain a highly active extract. The broken cells were extracted with 1 vol of buffer A (5 mM magnesium acetate, 20 mM potassium chloride, 10 mM Tris-hydrochloride [pH 7.8], 0.33 mM dithiothreitol [DTT]) equal to two times the wet cell weight. After centrifugation at 30,000 × *g* for 30 min, 3 μg of deoxyribonuclease (EC 3.1.4.5) per ml was added, and after 5 min the extract was again centrifuged at 30,000 × *g* for 30 min. The supernatant fluid was passed through a G-25 Sephadex column, and the peak macromolecular fractions, identified by their yellow opalescence, were pooled and designated the S-30 cell fraction. For S-30 fractions at pH 7.0 and 6.5, the column was equilibrated with buffer A at the respective pHs. The pooled fractions at the different pH levels were adjusted to the same A_{260} before use.

S-122 fractions were prepared by centrifuging the S-30 extract at 122,000 × *g* for 165 min and reserving the top two-thirds of the supernatant fluid. The ribosome pellet was suspended in buffer A with a Teflon pestle and centrifuged at 10,000 × *g* for 10 min, and the soluble portion was adjusted to 500 A_{260} units per ml.

All cell fractions were shell-frozen in dry ice-acetone and stored at −72 C. Activity of ribosome suspensions was maintained through at least two cycles of thawing and refreezing and for at least 3 months of storage.

Preparation of aminoacyl tRNA synthetases. All procedures were carried out at 2 to 4 C. For use in experiments involving the aminoacylation of purified phenylalanine tRNA ($tRNA^{phe}$), endogenous tRNAs were removed from the S-122 fraction (3). This fraction (average A_{280}/A_{260} = 0.7) was applied to diethylaminoethyl (DEAE)-cellulose equilibrated with buffer I (20 mM K_2HPO_4-KH_2PO_4 [pH 6.9],1 mM magnesium acetate, 20 mM β-mercaptoethanol, 10% glycerol [vol/vol]); the column was washed with the same buffer; and the material was eluted with buffer II (buffer I in 250 mM K_2HPO_4-KH_2PO_4 [pH 6.9]). The A_{280} peak fractions were pooled and concentrated by bringing the mixture to 70% saturation with ammonium sulfate. The precipitate was solubilized in buffer A and passed through G-25 Sephadex. The peak A_{280} fractions were pooled (A_{280}/A_{260} = 1.6) and stored as above.

When synthetases were prepared for the aminoacylation of tRNAs used in the transfer reaction, the portion of the S-122 fraction which precipitated between 35 and 80% saturation with ammonium sulfate was solubilized in buffer A made 10% (vol/vol) in glycerol, and passed through a G-25 Sephadex column, and the A_{280} peak fractions were pooled.

Preparation of aminoacyl tRNAs. The reaction mixture contained the following components in a volume of 1.0 ml: 6 mM magnesium acetate, 20 mM potassium chloride, 10 mM tris-hydrochloride (pH 7.8), 0.33 mM DTT, 1.5 mM adenosine 5'-triphosphate (ATP), 10 mM phosphoenolpyruvate, 100 μg of phosphoenolpyruvate kinase (EC 2.7.1.40), 0.2 mM cytidine 5'-triphosphate (CTP), 1.5 nM *l*-[^{14}C]-amino acid (specific activities of phenylalanine and leucine were 455 Ci/mol and 312 Ci/mol, respectively), 15 nM each of *l*-[^{12}C]amino acids excluding the radioactive amino acid, 1.6 mg of aminoacyl synthetase protein, and 1 mg of yeast tRNA. Incubation was at 30 C for 40 min, and the aminoacyl tRNAs were extracted (43) and stored in 10 mM potassium acetate buffer (pH 5.6). The tRNA content was estimated by assuming that 24 A_{260} units equal 1 mg of tRNA.

Amino acid incorporation in the standard S-30 system. Components of the standard incubation mixture (see Fig. 1) were incubated at 30 C for 35 min. The concentration of *l*-[^{14}C]amino acids used in different experiments varied from 244 to 1,115 pmol, and specific activities ranged from 112 to 513 Ci/mol. S-30 fractions contained 0.51 to 1.4 mg of protein and 0.08 to 0.27 mg of RNA per 0.1 ml. Reactions were stopped by the addition of 0.7 ml of 8% trichloroacetic acid and 3% vitamin-free Casamino Acids (Difco) and were heated at 85 C for 30 min. Precipitates were collected on Whatman no. 1 paper or Whatman glass-fiber paper (GF/A) and washed with trichloroacetic acid and 70% ethanol. Radioactivity was determined on a Nuclear-Chicago low-background gas-flow counter at an assumed efficiency of 25%. Net polyuridylic acid (poly U) stimulation of a given amino acid refers to the incorporation in the presence of poly U minus that in its absence.

Aminoacylation of $tRNA^{phe}$. The basic reaction mixture contained the following components in a volume of 0.1 ml: 7 mM magnesium acetate, 20 mM potassium chloride, 10 mM Tris-hydrochloride (pH 7.8),

89 μM DDT, 1 mM ATP, 10 mM phosphoenolpyruvate, 10 μg of phosphoenolpyruvate kinase (EC 2.7.1.40), 0.2 mM CTP, 500 pmol of the indicated *l*-[^{14}C]amino acid (specific activities ranged from 112 to 455 Ci/mol), 0.026 mg of aminoacyl tRNA synthetases, and 2.1 μg (approximately 84 pmol) of tRNA^{phe}. After incubation at 30 C for 35 min, the tubes were chilled, and 100 μg of carrier yeast RNA and 1 ml of cold 10% trichloroacetic acid were added. Precipitates were collected on membrane filters, washed with 2 ml of cold 10% trichloroacetic acid and 1 ml of cold 70% ethanol, and counted on a Nuclear-Chicago low-background gas-flow counter.

Transfer of amino acids from aminoacyl tRNAs into polypeptide. The reaction mixture contained the following in a volume of 0.4 ml: magnesium acetate and spermine as indicated, 20 mM potassium chloride, 10 mM Tris-hydrochloride (pH 7.8), 83 μM DTT, 0.33 mM guanosine triphosphate (GTP), ribosome fraction containing 0.5 mg of protein and 0.4 mg of RNA, and yeast tRNA charged with either *l*-[^{14}C]-phenylalanine or *l*-[^{14}C]leucine, and a mixture of [^{12}C]amino acids exclusive of the radioactive amino acid, as indicated. Reactions were carried out in the presence and absence of 100 μg of poly U, and the net poly U stimulation of a given amino acid was calculated. Incubation was at 30 C for 50 min, and reactions were terminated with 1 ml of 10% trichloroacetic acid and heated at 85 C for 30 min. Precipitates were collected on membrane filters and washed with 5% trichloroacetic acid and 70% ethanol. Filters were dried and counted in toluene-based Omnifluor on a Nuclear-Chicago liquid scintillation counter at 60% efficiency.

Biochemical assays. Protein was determined by the method of Zamenhof (44), and RNA was determined as described by Schneider (31).

Materials. Deoxyribonuclease (EC 3.1.4.5) (ribonuclease-free) was obtained from Worthington Biochemical Corp., Freehold, N.J.; G-25 Sephadex was from Pharmacia Fine Chemicals, Inc., Piscataway, N.J.; DEAE-cellulose was from Bio-Rad Laboratories, Richmond, Calif.; yeast tRNA and [^{14}C]- and [^{12}C]amino acids were from Schwarz/Mann, Orangeburg, N.Y.; poly U was from Miles Laboratories, Inc., Kankakee, Ill.; GTP, ATP, and CTP were from P-L Biochemicals, Milwaukee, Wis.; phosphoenolpyruvate, DTT, spermidine trihydrochloride, and spermine tetrahydrochloride were from Caliochem, Los Angeles, Calif.; phosphoenolpyruvate kinase (EC 2.7.1.40) and tRNA^{phe} were from Boehringer Mannheim Corp., New York, N.Y.; Omnifluor was from New England Nuclear Corp., Boston, Mass.; membrane filters (type B-6) were from Schleicher and Schuell, Keene, N.H.; streptomycin sulfate, neomycin sulfate, and cycloheximide were from Sigma Chemical Co., St. Louis, Mo.; and chloramphenicol was from Mann Research Laboratories, New York, N.Y.

RESULTS

Effect of environmental conditions on fidelity in the S-30 cell-free amino acid-incorporating system. An S-30 system based on those previously developed for *E. coli* (26) and yeast (6, 14, 34) was the standard control in these experiments. A suboptimal Mg^{2+} concentration was employed since it yielded high phenylalanine-incorporating activity but low ambiguity (see Fig. 2E). The kinetics of poly U-directed phenylalanine incorporation in this system (Fig. 1) show that active polypeptide synthesis continued for about 35 min. Based on the assumption that all the S-30 RNA (0.14 mg) was ribosomal, approximately 700 pmol of polyphenylalanine was synthesized per mg of ribosomal RNA, and twice this activity was obtained with some extracts. Response to endogenous messenger was never more than 3 pmol per reaction mixture.

Mitochondria were excluded from the S-30 fraction by centrifugation, and experiments showed that this system was insensitive to chloramphenicol but inhibited 97% by cycloheximide. These results demonstrate that only 80*S* ribosomes of cytoplasmic origin were active in this system (32).

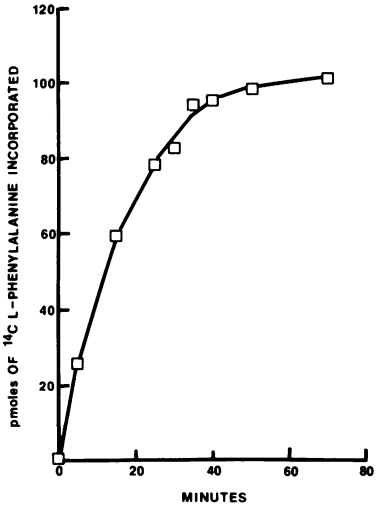

FIG. 1. *Kinetics of phenylalanine incorporation in the standard S-30 amino acid-incorporating system. The reaction mixture contained the following components in a volume of 0.4 ml: 6 mM magnesium acetate; 20 mM potassium chloride; 10 mM Tris-hydrochloride (pH 7.8); 83 μM DTT; 0.033 mM GTP; 0.635 mM ATP; 3.1 mM phosphoenolpyruvate; 12.5 μg of phosphoenolpyruvate kinase (EC 2.7.1.40); 244 pmol of [1-^{14}C]phenylalanine (sp act, 513 Ci/mol); 12,500 pmol of each amino acid in a mixture of [1-^{12}C]amino acids, excluding the radioactive amino acid; 100 μg of poly U; and 0.1 ml of an S-30 subcellular fraction containing 0.87 mg of protein and 0.14 mg of RNA. Incubation was at 30 C for the time indicated. Reactions were stopped and counted as described in Materials and Methods.*

The variables examined were those previously shown to induce ambiguity in bacterial in vitro polypeptide synthesis, namely, Mg^{2+} concentration, temperature, pH, and the addition of polyamines, ethanol and the aminoglycoside antibiotics, streptomycin and neomycin. The degree of fidelity obtained was determined by comparison of the calculated Leu/Phe ratios observed under varied conditions with the ratio found in the control.

The results of an experiment in which the temperature of incubation was varied from 12 to 45 C are shown in Fig. 2A. Optimal poly U-directed phenylalanine incorporation (101.5 pmol) was obtained at 37 C, whereas the highest level of poly U-stimulated leucine response (2.6 pmol) was seen at 30 C. The Leu/Phe ambiguity ratio at the standard temperature, 30 C, was 0.03, and no significant change in this ratio was observed throughout the range of temperatures tested.

In contrast with these results, elevated Mg^{2+} concentrations induced high levels of ambiguity (Fig. 2E). The Leu/Phe ratio obtained at the standard Mg^{2+} concentration (0.006 M) was 0.04. As the Mg^{2+} concentration was increased, progressively higher ratios were obtained. At 0.029 M Mg^{2+}, leucine incorporation exceeded

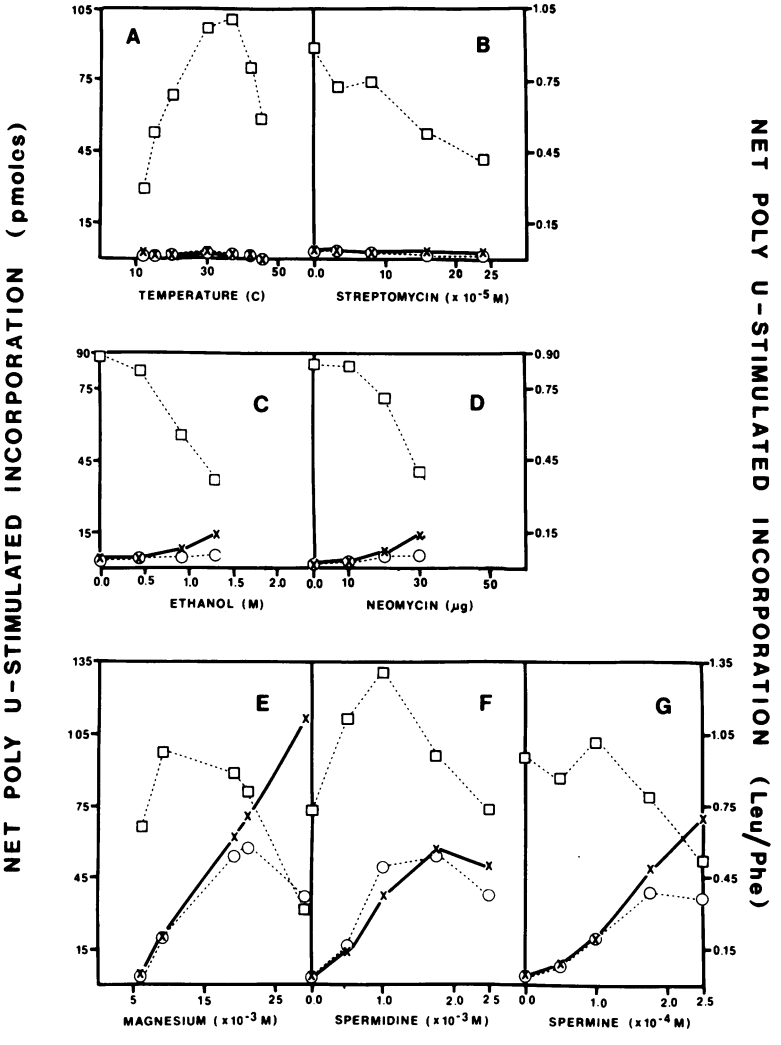

FIG. 2. *Effect of environmental conditions on net poly U stimulation of [1-^{14}C]leucine and [1-^{14}C]phenylalanine incorporation in the standard S-30 system (Fig. 1 and Materials and Methods). Net poly U-stimulated incorporation is the incorporation of [^{14}C]amino acid in the presence of poly U minus incorporation in its absence. Symbols: (□) phenylalanine and (○) leucine (pmoles of incorporation); (×) Leu/Phe.*

that of phenylalanine to give an ambiguity ratio of 1.12. Even when the Mg^{2+} concentration was optimum for phenylalanine incorporation, both leucine incorporation and the level of ambiguity were higher than in the control. In another experiment, the ambiguity observed at an elevated Mg^{2+} concentration (0.016 M) was diminished when the reaction was run at lower pHs (Table 1). This result is similar to the pH effect reported for bacterial extracts (22).

The addition of the polyamines, spermidine and spermine, also gave markedly increased ambiguity in the yeast system. In the presence of 1.8×10^{-3} M spermidine, the level of leucine-phenylalanine ambiguity increased 14-fold, to 0.57 (Fig. 2F). Similar to the effect with Mg^{2+} (Fig. 2E), increasing the spermidine concentration from 0.5×10^{-3} to 1.8×10^{-3} M resulted in increasing leucine incorporation, and leucine-phenylalanine ambiguity was expressed even under conditions optimum for phenylalanine incorporation. It is most likely that spermidine stimulated phenylalanine incorporation because the control was run at a suboptimal Mg^{2+} concentration (6, 7, 39).

When 2.5×10^{-4} M spermine was added to the system, the level of leucine-phenylalanine ambiguity was 0.70, or 18 times that seen in the control (Fig, 2G). Leucine incorporation was increased approximately 10-fold, to 39.1 pmol, by the addition of 1.8×10^{-4} M spermine, and ambiguity was observed even under conditions optimal for the phenylalanine response to poly U.

A study of the kinetics of amino acid incorporation in the absence and presence of 3.0×10^{-4} M spermine showed that the rate of spermine-induced leucine incorporation was highest during the first 35 min, when phenylalanine incorporation was most active (Fig. 3). After 15 min of incubation, well within the linear portion of the curves, the Leu/Phe ratio in the presence of spermine was 0.22, compared with 0.01 in its absence. Thus, the observed spermine-induced error cannot be explained by leucine incorporation occurring after a preferential inhibition of polyphenylalanine synthesis.

TABLE 1. *Effect of pH on net poly U-stimulated leucine and phenylalanine incorporation in the standard S-30 system*[a]

pH	Net poly U stimulation[b] (pmol)		
	Leu	Phe	Leu/Phe
7.8	25.6	95.3	0.27
7.0	7.4	55.9	0.13
6.5	3.7	33.2	0.11

[a] See Fig. 1 and Materials and Methods. Reactions were carried out at 0.016 M magnesium, in the presence and absence of poly U.

[b] Calculated as in Fig. 2.

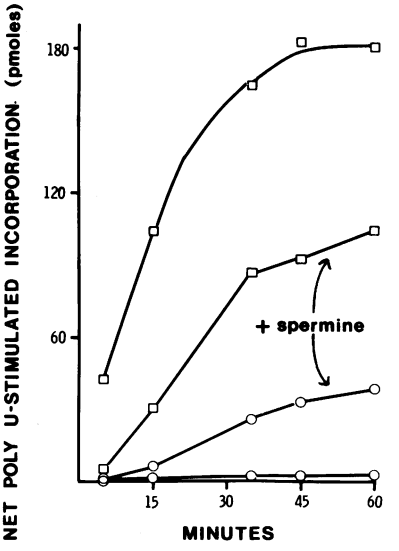

FIG. 3. *Kinetics of net poly U-stimulated leucine and phenylalanine incorporation in the S-30 system in the absence and presence of* 3.0×10^{-4} *M spermine (Fig. 1 and 2, and Materials and Methods). Symbols: □, phenylalanine; ○, leucine.*

As in bacterial systems (35), the addition of ethanol induced ambiguity in the yeast extract (Fig. 2C), although lower values for the ambiguity ratio were obtained than with Mg^{2+} and added polyamines. Also in contrast with these agents, ethanol stimulated the incorporation of leucine only while decreasing that of phenylalanine.

Streptomycin did not alter the fidelity of translation, although moderately low levels inhibited protein synthesis (Fig. 2B). (Resistance to the antibiotic was demonstrated at the cellular level.) On the other hand, neomycin increased ambiguity sevenfold, to 0.14 (Fig. 2D). The effect of neomycin on the system followed the same pattern as that of ethanol (Fig. 2C, D).

In bacteria, resistance to streptomycin and neomycin appears to be determined by separate genes (1). Mutants with lesions in the *strA* locus are no longer susceptible to streptomycin-induced ambiguity in vitro, whereas they retain their response to neomycin (10). The different effects of streptomycin and neomycin seen in this yeast system may also result from the expression of different genes.

In summary, similar to bacterial systems, markedly high levels of the ambiguous leucine-phenylalanine response to poly U were induced

in the yeast system by Mg^{2+} and added spermine and spermidine; ethanol and neomycin induced ambiguity, but to a decidedly lower degree; and lowering the pH of the reaction mixture reduced the level of ambiguity obtained at an elevated Mg^{2+} concentration.

Amino acids responding to poly U at elevated Mg^{2+} and spermine concentrations. The possibility that increased levels of Mg^{2+} and spermine induce the incorporation of amino acids other than leucine was examined. Ambiguity ratios were obtained for a series of [^{14}C]amino acids in poly U-stimulated incubations run at high Mg^{2+} and spermine concentrations and were compared with those observed under standard conditions.

Of the 10 amino acids tested at 0.016 M Mg^{2+}, only leucine, isoleucine, and serine showed significantly increased incorporation and amino acid/Phe ratios (Table 2). Leucine, isoleucine, and serine incorporation and their respective ambiguity ratios were also increased in the presence of spermine (Table 3). With the possible exception of tyrosine, spermine did not stimulate the response of any other amino acid tested.

Thus, there is specificity in ambiguity in the yeast system, and Mg^{2+} and added spermine induce the incorporation of the same amino acids, namely, leucine, isoleucine, and serine. The effectiveness of both agents in stimulating ambiguity was in the order: leucine > isoleucine ≫ serine.

Effect of elevated Mg^{2+} and spermine concentrations on the fidelity of aminoacylation of tRNA. Fidelity of translation in the S-30 amino acid-incorporating system requires specific recognition in (i) the charging of tRNAs with their specific amino acids, and (ii) in the transfer of amino acids from tRNAs into peptide linkage on a messenger-ribosome complex. The fidelity of these operational steps was examined in isolated systems.

TABLE 2. *Amino acid specificity of magnesium-induced ambiguity in the standard poly U-stimulated S-30 system*[a]

Amino acid	0.006 M Mg		0.016 M Mg	
	Net poly U stimulation[a] (pmol)	Amino acid/Phe ratio	Net poly U stimulation[a] (pmol)	Amino acid/Phe ratio
Phenylalanine	62.3		62.0	
Leucine	2.0	0.03	18.0	0.29
Isoleucine	0.4	0.01	12.6	0.20
Serine	0.7	0.01	1.9	0.03
Tyrosine	0.2	0.00	0.0	0.00
Valine	0.3	0.01	0.4	0.01
Proline	0.0	0.00	0.0	0.00
Methionine	0.1	0.00	0.0	0.00
Lysine	0.2	0.00	0.0	0.00
Arginine	0.7	0.01	1.0	0.02
Histidine	0.1	0.00	0.0	0.00

[a] See Fig. 1 and 2, and Materials and Methods.

TABLE 3. *Amino acid specificity of spermine-induced ambiguity in the standard poly U-stimulated S-30 system*[a]

Amino acid	No spermine		1.8×10^{-4} M spermine	
	Net poly U stimulation[a] (pmol)	Amino acid/Phe ratio	Net poly U stimulation[a] (pmol)	Amino acid/Phe
Phenylalanine	100.8		74.2	
Leucine	6.3	0.06	50.7	0.68
Isoleucine	2.0	0.02	27.5	0.37
Serine	2.1	0.02	3.3	0.04
Tyrosine	0.2	0.00	0.7	0.01
Valine	1.2	0.01	0.5	0.01
Proline	0.8	0.01	0.3	0.00
Methionine	0.1	0.00	0.0	0.00
Lysine	0.7	0.01	0.1	0.00

[a] See Fig. 1 and 2, and Materials and Methods.

Purified $tRNA^{phe}$ was charged in reaction mixtures containing increasing Mg^{2+} and spermine concentrations and one [^{14}C]amino acid at a time (phenylalanine, leucine, isoleucine, or serine). No other amino acids were included.

In reaction mixtures containing from 7 to 100 mM Mg^{2+}, $tRNA^{phe}$ accepted 46.8 to 49.1 pmol of phenylalanine and only 0.8 pmol or less of leucine, isoleucine, and serine (Table 4). Additional experiments demonstrated the presence of high activities for leucyl, isoleucyl, and seryl tRNA synthetases. Levels of each of the nonspecific amino acids bound remained constant at increasing Mg^{2+} concentrations, and these low values were probably due to the presence of contaminating tRNAs. When increasing levels of spermine were tested in aminoacylation, similar results were obtained (Table 4).

Effect of Mg^{2+} and spermine on fidelity in the transfer reaction. Yeast tRNA was charged with either [^{14}C]leucine or [^{14}C]-phenylalanine and a mixture of cold amino acids, excluding the radioactive amino acid, at a low magnesium concentration and in the absence of added spermine. After purification, this charged tRNA was incubated with ribosomes and poly U at increasingly high Mg^{2+} and spermine levels in transfer reaction mixtures. The ribosome preparations were unpurified and postribosomal

TABLE 4. *Effect of magnesium and spermine on aminoacylation of tRNA^{phe} with phenylalanine, leucine, isoleucine, and serine*[a]

Conditions	Amino acid (pmol) bound to tRNA[b]			
	Phenyl-alanine	Leucine	Isoleucine	Serine
Mg (M)				
0.007	46.8	0.8	0.3	0.8
0.026	46.9	0.6	0.2	0.7
0.05	46.9	0.8	0.3	0.8
0.1	49.1	0.6	0.1	0.8
Spermine (M)				
2.4×10^{-4}	45.5	0.7	0.2	0.8
0.001	44.9	0.5	0.7	0.8
0.01	44.5	0.5	0.3	0.8
0.06	35.0	0.3	0.0	0.4

[a] See Materials and Methods.
[b] Blank values were subtracted.

supernatant fluid was not required for polypeptide formation.

In the presence of increasing Mg^{2+} levels, the transfer of leucine increased, while that of phenylalanine decreased (Table 5). The Leu/Phe ratio was 0.07 at 0.004 M Mg^{2+} and was elevated to 0.83 at 0.019 M Mg^{2+}. Similar results were obtained when the effect of spermine was tested. The ambiguity ratio increased from 0.08 in the control to 0.70 in the presence of 3.9×10^{-4} M spermine (Table 5). Thus, ambiguity at the transfer level can account for the ambiguity observed in the S-30 protein-synthesizing system.

DISCUSSION

Environmentally induced ambiguity has been observed and partially characterized in a cell-free polypeptide-synthesizing system from yeast. Added magnesium and polyamines, ethanol, and neomycin stimulate ambiguous translation and, in addition to phenylalanine, leucine, isoleucine, and serine, are incorporated in response to poly U. Furthermore, ambiguity was shown to occur at the transfer reaction level, suggesting that the ribosome is the site of action of environmental agents.

The same agents that induce ambiguity in yeast are also active in *E. coli* (10, 11, 35, 38) and *B. stearothermophilus* (18), and the same amino acids that are involved in the ambiguous response to poly U in yeast have been reported in the bacterial systems (10, 11, 18, 35, 38). Furthermore, the order of stimulation in both yeast and *B. stearothermophilus* is Leu > Ile ≫ Ser (18). In *E. coli*, the ribosome has been unequivocally identified as the target of environmental factors (10, 17, 27, 28).

Two characteristics of ambiguity have emerged from these studies: (i) a low level of ambiguity occurs with the wild-type ribosome, and thus nonsense mutations are leaky, and (ii) ambiguity is not random, in that only amino acids with connected codons are incorporated in response to a given message. In the *E. coli* system, Gorini and co-workers (4, 20) have developed evidence for a ribosomal tRNA-screening site which appears to function in regulating the level of ambiguity observed in translation. The *ram* mutation increases the low level of ambiguity seen with wild-type ribosomes, whereas the *strA* mutation reduces it, and the two genes interact to produce intermediate phenotypes (19, 20). The *strA* (28) and *ram* (45) gene products have been identified as proteins of the 30S ribosomal subunit and, thus, are probably components of this site or influence it by allosteric interaction. The addition of streptomycin appears to affect this site in the same manner as *ram* does intrinsically.

We propose that a site on the cytoplasmic ribosomes of yeast corresponding to the putative tRNA-screening site on *E. coli* ribosomes is the basis for the striking similarity in ambiguity observed in the two systems. How does this site function in matching an aminoacyl tRNA molecule with a given codon-ribosome complex? Gorini (4, 20) proposed a basic model in which the primary recognition site is that between codon and anticodon (in ambiguity only con-

TABLE 5. *Effect of magnesium and spermine on net poly U-stimulated transfer of leucine and phenylalanine from tRNA into protein*[a]

Conditions	Net poly U stimulation (pmol)		
	Leu	Phe	Leu/Phe
Mg (M)			
0.004	0.3	4.4	0.07
0.006	0.7	3.8	0.18
0.009	1.3	3.2	0.41
0.014	1.6	2.1	0.76
0.019	1.5	1.8	0.83
Spermine ($M \times 10^{-4}$)[b]			
	0.3	4.0	0.08
0.9	0.6	3.6	0.17
1.8	1.0	2.9	0.34
2.5	1.0	2.0	0.50
3.9	0.7	1.0	0.70

[a] See Materials and Methods. All assays contained approximately 100 μg of yeast tRNA charged with either *l*-[^{14}C]leucine or *l*-[^{14}C]phenylalanine. The specific activities of the [^{14}C]leucyl tRNA and the [^{14}C]phenylalanyl tRNA were 64 and 24 pmol of [^{14}C]amino acid per 100 μg of tRNA, respectively.
[b] In the presence of 0.004 M Mg^{2+}.

nected codons are read) and the secondary interaction is that between the ribosomal tRNA-screening site and a portion of the tRNA outside of the anticodon.

It has been shown that the *strA* ribosome restricts and the *ram* ribosome and the addition of streptomycin stimulate the entry of two classes of tRNAs (4, 20): (i) those involved in the ambiguous response in which mispairing of codon-anticodon bases occurs, and (ii) suppressor tRNAs mutated in the anticodon in which conventional pairing (8) takes place. A feature of the tertiary conformation of the two classes of tRNA may be the same, but different from that of a wild-type tRNA involved in conventional codon pairing. Pairing of a wild-type tRNA with an incorrect codon, such as occurs in ambiguity, and the occurrence of a mutated base in a correctly paired suppressor tRNA molecule may produce similar conformational changes in a distal portion of the tRNA molecule which interacts with the ribosomal tRNA-screening site. Evidence for interplay between the anticodon and a distant portion of the tRNA has been suggested by Hirsh and Gold (23) and Eisinger et al. (16).

Thus, in the context of Gorini's proposal (20), acceptance or rejection of a tRNA by a messenger-ribosome complex would depend primarily on matching the codon and anticodon and also on features of tRNA and ribosome conformation that result in lesser or greater binding affinity between the two components. It is possible that streptomycin and other agents induce a change in the structure of the wild-type ribosomal tRNA-screening site, thereby increasing the affinity of this site for tRNAs involved in unconventional codon pairing. The *ram* mutation may confer a similar conformational change on this site intrinsically. Sherman and Simpson (33) provided evidence supporting the hypothesis that streptomycin stimulates ambiguity by inducing a change in the conformation of the ribosome (10, 11, 29).

In the yeast system, environmental conditions induced poly U-directed incorporation of leucine, isoleucine, and serine, but not of valine and tyrosine. All of these amino acids have connected codons containing two uridylic acid (U) residues. In fact, one valine codon has U's in positions two and three, as does the isoleucine codon containing two U's. The known base sequences of yeast tRNAs were compared with a view to finding bases outside the anticodon which were the same in leucine, isoleucine, and serine, but different in tyrosine and valine tRNAs. A base pair, uracil-adenine, the first in the amino acid stem proximal to the dihydrouracil and thymine-pseudouracil-cytosine loops, is common to the former group (and to $tRNA^{phe}$), whereas a guanine-cytosine pair occurs in tyrosine and valine tRNAs (15). This difference may be responsible for a slightly different conformation of tyrosine and valine tRNAs, resulting in particularly low affinity of these tRNAs for the ribosomal screening site. Further studies are required to establish whether this tRNA site is responsible for the pattern of misreading observed. Preliminary to this, it would be productive to establish which of the various tRNAs for a given amino acid are involved in the ambiguous response to poly U.

Bayliss and Vinopal recently reported streptomycin suppression of auxotrophy in *S. cerevisiae* (2), but the coding properties of the ribosome were not examined. Our demonstration of ambiguous reading of the code in a yeast subcellular system supports the notion (2) that streptomycin suppression of auxotrophy in the now classical sense (10, 21) may be found in yeast.

Crude amino acid-incorporating systems derived from mammalian cells fail to give the high incidence of ambiguous response seen in bacterial and yeast systems. In view of the higher level of ambiguity obtained when reticulocyte ribosomes are incubated at a high concentration of KCl (25), it is possible that a ribosomal site similar to that discussed for bacteria and yeast is present in mammalian ribosomes, but is protected in some way from environmental change.

Mammalian and yeast cytoplasmic ribosomes are of the same physical class and both are sensitive to cycloheximide but resistant to antibiotics affecting 70*S* ribosome function. However, the difference observed at the level of ambiguity indicates that there may be sufficient differences at other sites so that antibiotics which react with ribosomes of eukaryotic pathogens, but not with those of the mammalian host, may yet be found. In this connection, phylogenetic differences between yeast and rat liver mitochondrial protein-synthesizing systems in antibiotic sensitivity have been reported recently (41).

In summary, we describe a yeast system in which high levels of ambiguity are induced, and show that the component affected by magnesium and spermine is probably the ribosome. These findings suggest the feasibility of employing the yeast system as a tool in solving the problem of how coding fidelity is achieved on cytoplasmic ribosomes of eukaryotic cells.

ACKNOWLEDGMENTS

This investigation was supported by Public Health Service grant AI-08231 from the National Institute of Allergy and Infectious Diseases and grant no. 1228 from the City University of New York Faculty Research Program.

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