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Yeast Suppressors of UAA and UAG Nonsense Codons Work Efficiently in Vitro via tRNA

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Summary

A cell-free protein-synthesizing system, containing an S-100 fraction from yeast, ribosomal subunits from Krebs ascites cells, and ribosome initiation factors from rabbit reticulocytes, translates yeast, adenovirus, and rabbit globin messenger RNAs and the RNA from bacteriophage Qβ. An amber mutation in the Qβ synthetase gene is suppressed in vitro if the S-100 fraction is from yeast strains carrying amber suppressor mutations. Suppressor *SUP*6-2 gives 16% suppression, and the recessive lethal suppressor RL-1 gives 50% suppression. Extracts from strain FM6, which has the ochre suppressor *SUP*4-1, give a longer protein product from the normal synthetase gene of Qβ with an efficiency of 63%. This implies that UAA is the terminator for the synthetase gene, and that synthesis of this read through protein can be used as an assay for ochre suppression. Suppression in each of these cases is mediated by tRNA, since purified tRNA is the only fraction from suppressing strains that is required in an otherwise nonsuppressing cell-free system.

Introduction

The nature of many biochemical processes can be most effectively studied by an intimate combination of genetic and biochemical approaches. This is evident from numerous examples in bacteria. With eucaryotic organisms, however, this multiple approach has not been so productive, because of the difficulty of doing extensive genetic manipulations. The fungi, yeast in particular, are an exception. For instance, in yeast, mutations that cause premature termination of protein synthesis within a gene (for example, nonsense or frameshift mutations) have been isolated and characterized. Suppressors of nonsense mutations have been isolated as well (Hawthorne and Mortimer, 1963), and it has been possible to show that these suppressor mutations cause insertion of a particular amino acid (usually tyrosine) at the position specified by the nonsense codon (Stewart and Sherman, 1972; Stewart et al., 1972). In bacteria, the genetic system using suppressor and nonsuppressor cells to study mutations in cell genes or in viral genes (for review see Hartman and Roth, 1973) is especially powerful, since it provides a convenient and general method for identifying a protein with the gene that specifies its primary structure. The yeast suppressors are the only known examples of this phenomenon among eucaryotes.

We have studied the yeast suppressors with the hope of extending a suppression system to mammalian cells. It has not yet been possible to establish a cell-free protein-synthesizing system from yeast with which to study the biochemistry of the suppressor mutants. Apparently, the problem is at initiation of protein synthesis, since polysomes in yeast extracts can complete synthesis of their nascent chains, but cannot initiate synthesis on the endogenous yeast mRNA or on any exogenous mRNA except synthetic polynucleotides such as poly(U) (Gallis and Young, 1975; C. McLaughlin, personal communication).

In this paper we report that yeast extracts, when supplemented with mammalian ribosomal subunits and initiation factors, will synthesize proteins using a variety of natural messenger RNAs to produce a pattern of proteins similar to that found with more standard cell-free protein-synthesizing systems. Using this hybrid cell-free system, we have been able to assay directly the activity of several nonsense suppressors of yeast on translation of the synthetase gene of the RNA coliphage Qβ. We have been able to show that the suppression activity of representative amber- and ochre-specific suppressors of yeast can be recovered in the tRNA fraction. Using similar techniques, Capecchi, Hughes, and Wahl (1975) have reached the same conclusion about yeast amber suppressors.

Properties of Yeast Nonsense Suppressors in Vivo

Mutants of yeast that suppress the nonsense codons UAA (ochre) and UAG (amber) were first isolated through their ability to reverse simultaneously the phenotype of as many as five mutations (Hawthorne and Mortimer, 1963; Gilmore, 1967). They have since been shown to suppress defined UAA and UAG mutations in the gene specifying iso-1-cytochrome c by causing the insertion of tyrosine at the position of the nonsense codon (Stewart et al., 1972; Stewart and Sherman, 1972). Unlike their bacterial analogues, however, the ochre suppressors of yeast are active only on UAA mutations and do not suppress UAG mutations (Gilmore, Stewart, and Sherman, 1971). In extensive searches for sup-

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pressors in ordinary haploid yeast strains, only eight independent loci on six different chromosomes have been identified which can mutate to give efficient nonsense suppressors (Hawthorne and Mortimer, 1963; Gilmore and Mortimer, 1966). Amber and ochre suppressors have been found at each of these eight loci, and in all cases, the amino acid inserted has been found to be tyrosine. All the ochre suppressors are dominant and UAA-specific; their efficiency of suppression is increased in the presence of the cytoplasmically inherited genetic element *psi*+ (Cox, 1965, 1971), to the point that haploid strains carrying both an ochre suppressor and *psi*+ are often inviable. All the amber suppressors at these eight loci are dominant and UAG-specific; they are not affected by the *psi*+ element.

The properties of these nonsense suppressors have led to the belief that the eight loci mutable to yield suppressors are genes specifying a family of eight iso-accepting tyrosine tRNA molecules. The failure of the ochre suppressors at these loci to suppress an amber mutation is usually postulated to be due to a modified base in the first position of the anticodon which prevents "wobble" pairing (Gilmore et al., 1971). Although preliminary evidence for a change in the chromatographic properties of $tRNA^{Tyr}$ in suppressing strains of yeast has been published (Bruenn and Jacobson, 1972), until now no direct evidence has shown the involvement of tRNA in nonsense suppression in yeast.

By beginning in a *psi*+ haploid strain of yeast, Cox (1965) isolated an ochre suppressor which has been found, by Liebman, Stewart, and Sherman (1975), to insert serine into protein at UAA (ochre) codons. This suppressor is so weak as to be undetectable in *psi*− cells. No new amber suppressors were found by this technique, presumably because *psi*+ has no effect on amber suppressors.

Reasoning that the tyrosine iso-accepting tRNA genes might be the only ones in yeast sufficiently redundant to give rise to suppressors in *psi*− haploid strains, Brandriss, Soll, and Botstein (1975) and Hawthorne and Leupold (1974) isolated amber suppressors in diploid strains and found that many of these suppressor mutations were recessive lethal. One of these suppressors was characterized further and found to insert serine into protein at a UAG codon (M. C. Brandriss and J. W. Stewart, personal communication).

In this paper, we examine representatives of the tyrosine-inserting suppressors in both the amber and the ochre form using the yeast-mammalian cell-free protein-synthesizing system. These suppressors are active in extending polypeptide chains beyond amber or ochre codons in vitro; in all the cases examined, we could recover the suppressing activity in the tRNA fraction. Recessive lethal suppressors were also found to function in vitro, and their activity also seems to reside in the tRNA fractions.

Results

Cell-Free System

Crude "S-23" extracts prepared by homogenization of yeast cells support a large endogenous synthesis due to runoff of existing polysomes, but do not respond to addition of exogenous messenger RNA despite systematic alteration of reaction conditions. We found that if the extract is supplemented with ribosomal subunits from mouse ascites cells and rabbit reticulocyte initiation factors (Schreier and Staehelin, 1973; Anderson et al., 1974), then addi-

Table 1. Requirements for Protein Synthesis

	Minus Spermidine	Plus Spermidine
S-23 Mixed System		
Complete	146,321	483,146
Minus Yeast RNA	54,689	219,753
Minus Initiation Factors	14,128	51,527
Minus Ribosomes	14,188	38,446
Minus Initiation Factors and Ribosomes	15,084	45,904
S-100 Mixed System		
Complete		247,528
Minus Yeast RNA		93,538
Minus 30–40 Initiation Factor Fraction		52,115
Minus 40–60 Initiation Factor Fraction		21,211

The reaction mixtures for the S-23 mixed system contained 50% by volume of the S-23 extract prepared from yeast strain S288C and had a final magnesium concentration of 4 mM, which was optimal for this system. When spermidine was added (0.8 mM), the magnesium concentration was reduced to 2.4 mM for optimal activity. The rest of the components were as described in Experimental Procedures. The S-100 mixed system was as described. In all cases the radioactivity represents that found in a 5 μl sample after base treatment and precipitation with trichloroacetic acid.

tion of yeast cytoplasmic RNA stimulates incorporation of ^{35}S-methionine into protein about 3 fold, as seen in Table 1. If either of these components from mammalian cells is omitted, synthesis is decreased over 10 fold. Addition of the polyamine spermidine has a 3–4 fold stimulatory effect on the synthesis, as has been reported for mammalian and wheat germ cell-free protein synthesis (Atkins et al., 1975; R. C. Mulligan and B. E. Roberts, personal communication); consequently, it has been included in all subsequent experiments.

If the yeast ribosomes are removed from the crude extract by preparation of an S-100 fraction, protein synthesis is still demonstrable in the mixed system, but the overall extent is decreased about 2 fold. Table 1 shows in addition that both the initiation factor fractions prepared by ammonium sulfate precipitation are required for maximal protein synthesis. Although the mammalian components are likely to be free of most of the tRNAs, tRNA synthetases, and elongation factors, we have as yet no knowledge of the components that are in fact contributed by the yeast S-100 fraction.

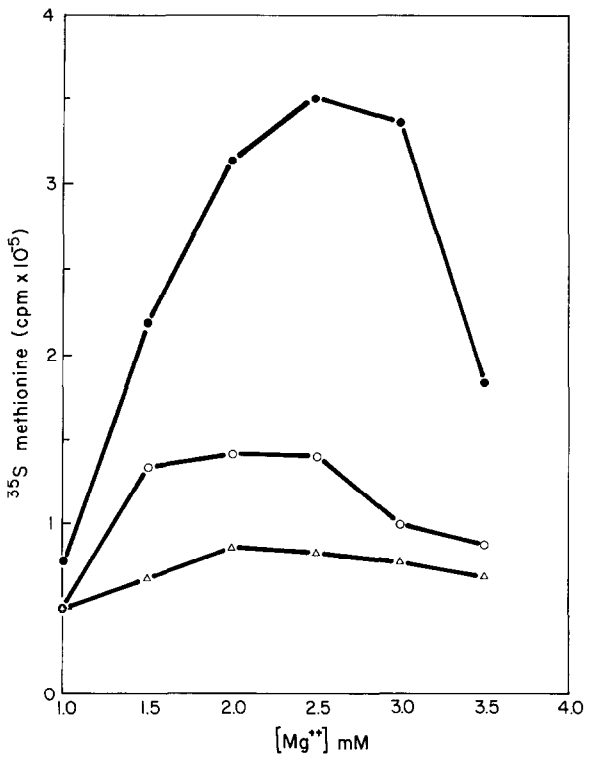

Figure 1. Dependence of Protein Synthesis upon Magnesium^{++} Concentration

The ordinate represents the amount of radioactive methionine incorporated into alkali-resistant, trichloroacetic acid-insoluble material in a 5 μl aliquot from reaction mixtures containing the magnesium acetate concentrations indicated on the abscissa. The other components are as described in Experimental Procedures. (●—●) yeast cytoplasmic RNA; (○—○) Qβ RNA; (△—△) no added RNA.

RNA from bacteriophage Qβ also stimulates protein synthesis in the mixed system, but, as seen in Figure 1, the maximal level is considerably lower than that observed with yeast cytoplasmic RNA. The stimulation ranges from 1.5–2 fold over the background without added RNA, but a large part of the background (usually at least 50%) is not due to protein synthesis, since the unincubated reaction mixture alone shows acid-precipitable ^{35}S-methionine.

The products of cell-free protein synthesis with various messenger RNAs are shown in the autoradiogram of an SDS-polyacrylamide gel in Figure 2.

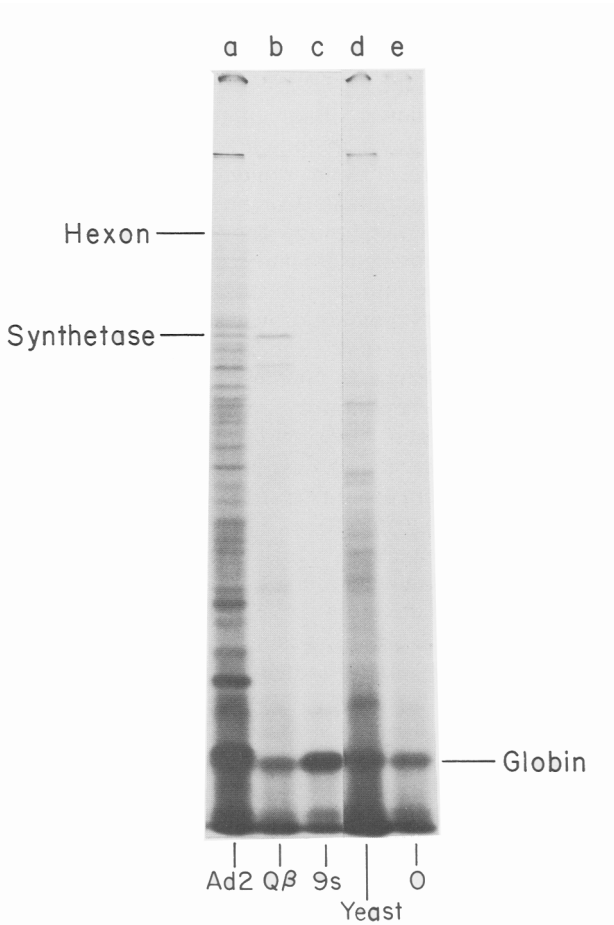

Figure 2. SDS-Polyacrylamide Gel Electrophoresis of Products of Cell-Free Synthesis with Different mRNAs

The autoradiogram shown is a 7 day exposure of a dried 17.5% polyacrylamide gel in which the equivalent of 5 μl of reaction mixtures containing different messenger RNAs was analyzed in the tracks as follows: (a) cytoplasmic RNA from Ad2-infected KB cells; (b) wild-type Qβ RNA; (c) 9S, globin mRNA from rabbit reticulocytes; (d) yeast RNA. Each 50 μl reaction mixture contained 1.25 μl of an S-100 fraction from S288C. "Hexon" denotes the adenovirus protein of molecular weight 120,000 daltons identified by comparison with known hexon synthesized in a fully mammalian cell-free system. "Synthetase" denotes the Qβ phage-coded synthetase protein identified by comparison with proteins made from Qβ RNA in an E. coli cell-free system and by the use of mutants described below.

Cytoplasmic RNAs extracted from yeast cells or from KB cells infected with adenovirus stimulate the synthesis of many polypeptides. Many of these migrate with proteins synthesized in a mammalian cell-free system using these same RNAs as messengers. For instance, the largest protein seen when RNA from Ad2 virus-infected cells is translated is the major virion structural protein—the hexon monomer. Rabbit globin mRNA stimulates synthesis of a major product which presumably is a mixture of the α and β chains. It should be noted that all the samples analyzed in Figure 2 have a band that migrates with the globin mRNA product, because of a small amount of globin mRNA that contaminates the preparations of rabbit reticulocyte ribosome initiation factors.

RNA from bacteriophage Qβ stimulates synthesis of a polypeptide of molecular weight equal to approximately 65,000 daltons, which is the size expected of a Qβ synthetase (Kamen, 1970). This is the product of the synthetase gene, since, as seen in Figure 3, translation of RNA from a mutant phage with an amber mutation in this gene (K. Horiuchi, personal communication) results in loss of the 65,000 dalton protein and the appearance of a fragment of about 55,000 daltons, which presumably results from termination of protein synthesis at the amber site. A 55,000 dalton fragment is also observed when the Qβ *am* RNA is translated in a nonsuppressing E. coli cell-free system (Atkins and Gesteland, 1975).

Amber Suppression

We can now ask whether suppression can be detected if the S-100 extracts are made from strains of yeast known to contain amber suppressors. Extracts of the suppressor strain *SUP*6-2, which inserts tyrosine in response to UAG, do suppress the Qβ amber mutation (Figure 3, c), as shown by synthesis of the 65,000 dalton synthetase polypeptide. The control experiment using an extract from a nonsuppressing strain (Figure 3, a and b) shows that translation of the amber RNA is not suppressed. Figure 4 shows a similar experiment using the recessive lethal amber suppressor RL-1 (Brandriss et al., 1975) either as a diploid or as an aneuploid heterozygote carrying two copies of chromosome 3, compared to using their nonsuppressing related strains—the direct parent in the case of the diploid (Brandriss et al., 1975) and a nonsuppressing haploid segregant in the case of the aneuploid. Again with the extracts from the two suppressing cells, some full length synthetase protein is observed when the amber mutant RNA is translated; however, the level of suppression seen is clearly greater with the extract from the aneuploid strain than with the diploid strain.

The level of suppression can be estimated by scanning autoradiographs with a densitometer. For this purpose, we have used autoradiograms exposed for shorter times than those shown in photographs here, to insure that the film exposure is in a range of linear response. Figure 5 shows an example of suppression by the RL-1 aneuploid compared to a haploid nonsuppressing segregant strain. In the latter case, the largest polypeptide seen in appreciable amounts is the amber fragment.

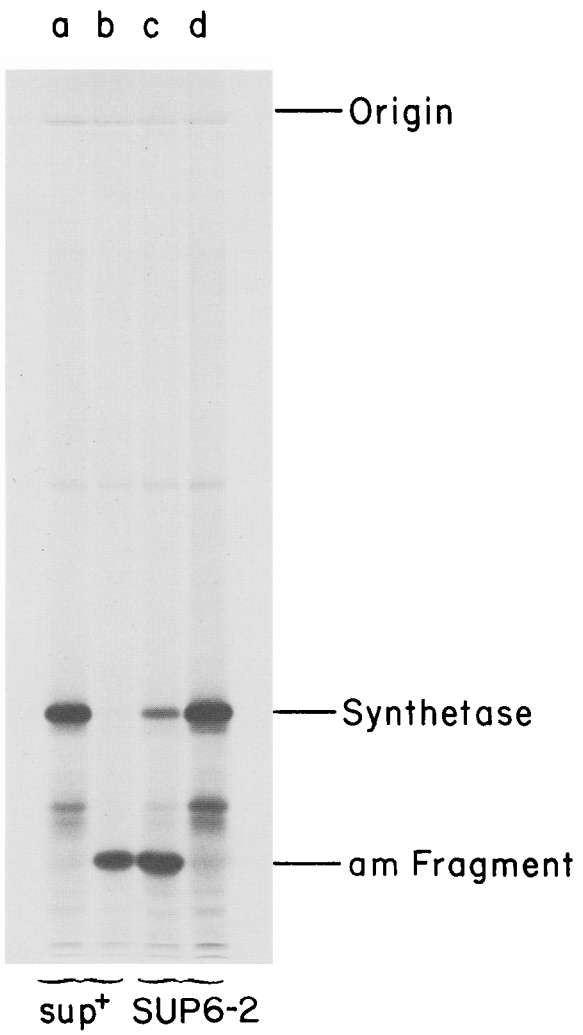

Figure 3. Suppression by *SUP*6-2 Amber Suppressor

The autoradiogram is a 5 day exposure of a 10% SDS-polyacrylamide gel run for 7 hr at 100 v, so that the bromophenol blue sample dye has run the equivalent of 1.5 times the length of the gel. Samples of 10 μl from the following cell-free protein synthesis mixtures were analyzed as follows:

S-100 Source	mRNA
(a) S288C, wild-type yeast	Qβ+
(b) S288C, wild-type yeast	Qβ *am*
(c) CY3, *SUP*6-2 suppressor	Qβ *am*
(d) CY3, *SUP*6-2 suppressor	Qβ+

A small amount of wild-type protein might be indicated and in fact is obvious on autoradiograms exposed for much longer periods of time. This is to be expected, since the preparation of Qβ amber mutant phage unavoidably contained 1–2% wild-type revertants. To a first approximation, we can take the ratio of radioactivity in the wild-type polypeptide to the sum of the radioactivity in the amber fragment plus wild type as an estimate of the efficiency of suppression. We conclude that the RL-1 suppressor in the aneuploid strain promotes about 50% read through beyond the amber terminator. Similar analyses of the RL-1 in the diploid strain indicate a suppression level of about 16%, which is similar to the levels found with the *SUP*4-2 amber suppressor seen in Figure 3.

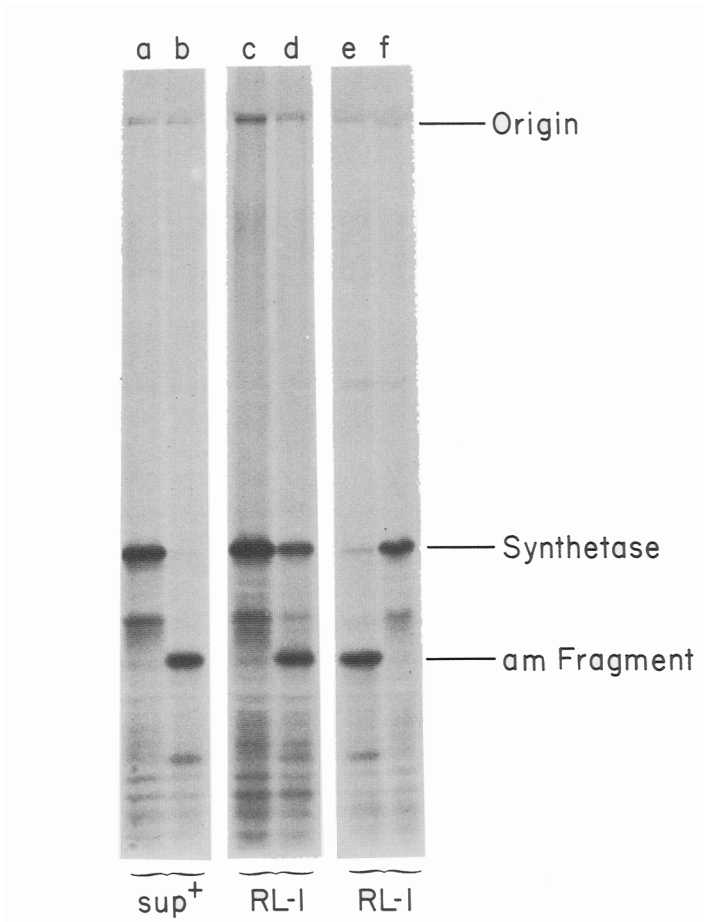

Figure 4. Suppression by Recessive Lethal Amber Suppressor

The autoradiogram is a 5 day exposure of a 10% polyacrylamide gel showing the products of cell-free synthesis as follows:

S-100 Source	mRNA
(a) wild-type parental strain	Qβ+
(b) wild-type parental strain	Qβ *am*I
(c) RL-1 aneuploid suppressor	Qβ+
(d) RL-1 aneuploid suppressor	Qβ *am*I
(e) RL-1 diploid suppressor	Qβ *am*I
(f) RL-1 diploid suppressor	Qβ+

Ochre Suppression

No ochre (UAA) mutants are available in the synthetase of Qβ to test for ochre suppression in the same manner. When wild-type Qβ RNA is translated using an ochre-suppressing strain, however, a new polypeptide is seen that migrates more slowly than the wild-type synthetase protein during electrophoresis in SDS-polyacrylamide gels. Figure 6 shows this result using strain FM6 (Rasse-Messenguy and Fink, 1973), which carries the *SUP*4-1 ochre suppressor. The new polypeptide is a read through product from the synthetase gene, since both it and the wild-type protein disappear when RNA from the amber mutant is used (Figure 6). Apparently, the normal terminator for the synthetase is UAA, which when suppressed gives translation up to the next in-phase nonsense triplet. We can thus take the amount of this read through protein as an indication of the level of ochre suppression.

Densitometric analysis in Figure 7 shows that the *SUP*4-1 suppressor gives 65% suppression of the Qβ synthetase terminator codon.

Translation of the amber mutant RNA in the ochre extract shows no amber suppression. Conversely, the amber-suppressing extracts do not synthesize the read through protein from wild-type RNA (Figures 3d and 3c).

Rasse-Messenguy and Fink (1973) have isolated mutants of FM6 that are temperature-sensitive for the ochre suppressor, and the new mutation maps very close to or within the *SUP*4 gene. The suppressor is active in vivo at 23°C but not at 37°C; how-

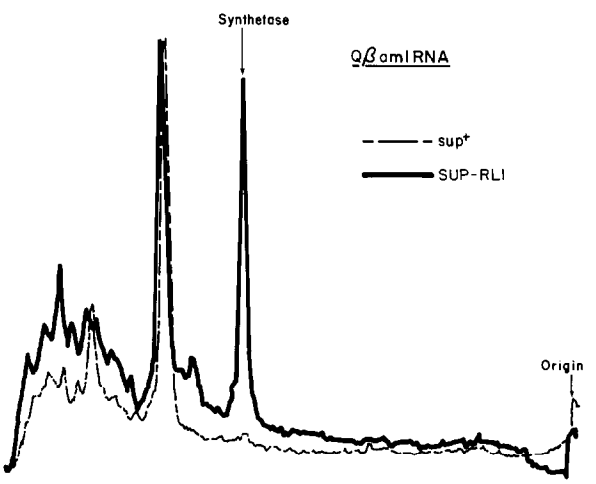

Figure 5. Densitometer Tracing of Autoradiogram in Figure 4

Tracks (b) and (d) of Figure 4 were scanned with a Joyce-Loebl Chromoscan; however, a 1.5 day exposure was used to insure linearity. Peak areas were estimated by cutting and weighing or by integration.

ever, to observe the suppressor even at the low temperature, it is necessary to have the *psi* determinant present. Extracts from strain FM811C, which is *psi*$^+$ and temperature-sensitive for suppression and *psi*$^+$, do give ochre suppression in vitro (Figure 6, g), although the level is lower than with strain FM6. Preliminary results suggest that this suppression is temperature-sensitive in vitro.

Internal Starts

In addition to the synthetase protein, the wild-type Qβ RNA directs the synthesis of a prominent polypeptide of molecular weight 50,500 (Figure 6, a,c,e, and g), and like the wild-type protein, this also disappears when RNA from the amber mutant is used, which implies that it too comes from the synthetase gene. With the amber RNA product, a 39,600 dalton polypeptide is seen migrating faster than the 55,600 dalton amber fragment, and neither of these is seen in the wild-type RNA product. Thus the two polypeptides made from wild-type RNA differ by 16,500 daltons, whereas those from amber RNA differ by 16,000 daltons (Figure 6, b,d,f, and h). This similarity suggests that both fragments are missing the same sized piece, which can only be from the amino-terminal end of the protein, since the amber mutation prevents synthesis of the carboxy-terminal end; that is, the additional polypeptides could be due to ribosomal initiation at an internal position in the synthetase gene: in one case, the polypeptide made is terminated at the normal site, and in the other, at the amber site. This model predicts that ochre suppression of the normal terminator should result in elongation of the "restart" fragment from wild-type RNA by the same 2700 daltons seen with the protein of normal size, and that no such elongation should be seen with the restart fragment from the amber mutant RNA. This is borne out by the experiment shown in Figure 6 c, where a read through polypeptide from the wild-type restart is seen with ochre suppression. This polypeptide has a calculated molecular weight of 58,500 daltons, or 2600 daltons longer than the normal restart.

We cannot rule out the alternative possibility that these shorter fragments are due to proteolytic cleavage at a specific site in the synthetase. This seems improbable, however, because of the specificity required and because specific cleavage or degradation in cell-free systems has not yet been observed.

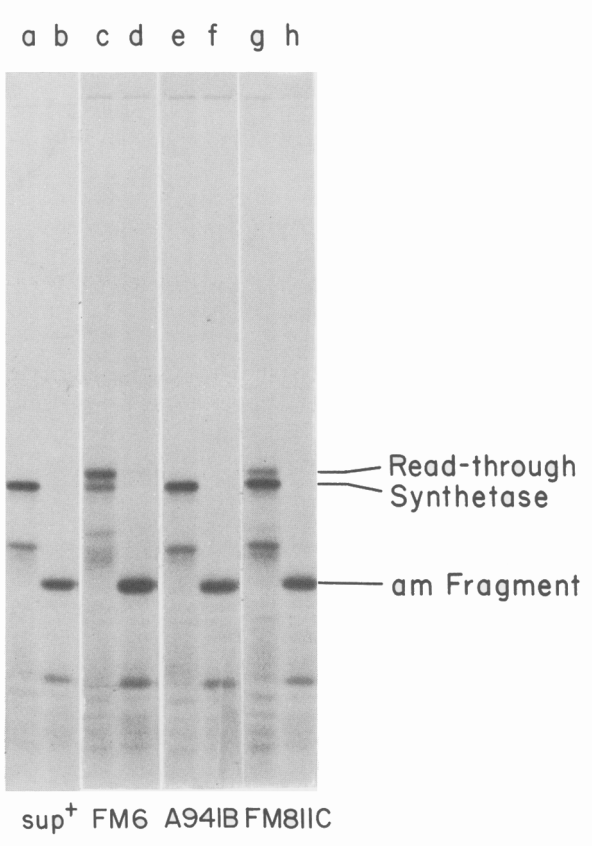

Figure 6. Suppression by *SUP*4-1 Ochre Suppressor

The autoradiogram is a 7 day exposure of a 10% polyacrylamide gel. The samples were 10 μl aliquots from cell-free protein synthesis, as follows:

S-100 Source	mRNA
(a) wild type, S288C	Qβ^+
(b) wild type, S288C	Qβ *am*l
(c) FM6, *SUP*4-1 suppressor, *psi*$^-$	Qβ^+
(d) FM6, *SUP*4-1 suppressor, *psi*$^-$	Qβ *am*l
(e) A941B, nonsuppressing, *psi*$^+$	Qβ^+
(f) A941B, nonsuppressing, *psi*$^+$	Qβ *am*l
(g) FM811C *SUP*4-1 temperature-sensitive suppressor, *psi*$^+$	Qβ^+
(h) FM811C *SUP*4-1 temperature-sensitive suppressor, *psi*$^+$	Qβ *am*l

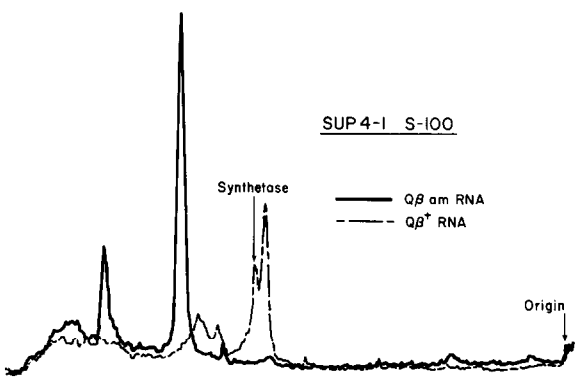

Figure 7. Densitometer Tracing of Autoradiogram in Figure 6

Tracks c and d of Figure 6 were scanned, but a 2 day exposure was used to insure linearity.

Suppression Is Mediated by tRNA

We can ask which component of the yeast extract is responsible for suppression activity by adding components from a suppressing extract to the cell-free mixture made from a nonsuppressing strain. The results in Figure 8 show that the tRNA fraction isolated from suppressing cells by phenol extraction, salt extraction, and DEAE-chromatography contains the active suppressor. The tRNA from *SUP*4-2 (tyrosine-inserting amber suppressor), when added to the cell-free system prepared from the parental nonsuppressing strain, gives amber suppression at a level of about 15% (Figure 8, c). This is approximately the same level seen if the S-100 fraction is prepared from *SUP*4-2 (Figure 3, c). Addition of tRNA from the nonsuppressing strain does not give suppression (Figure 8, b), and addition of either tRNA to the suppressing extract does not substantially increase or decrease the observed level of suppression (Figure 8, f and g).

We have also found that the suppressor activity is in the tRNA fraction for the RL-1 amber suppressor (Figure 8, i, j, and k) and for FM6, the *SUP*4-1 ochre suppressor (Figure 8, l, m, and n).

In all cases, the maximum levels of suppression obtainable are about the same as the level seen when the S-100 fractions from the corresponding strains are used in the hybrid system.

Discussion

Although we do not understand the details of the biochemistry of this cell-free system that uses yeast crude extracts supplemented with partially purified mammalian components, we can study certain aspects of eucaryotic translation that were inaccessible until now. One example is given by our results implicating tRNAs in suppression of nonsense mutations. It may also be possible to study the biochemistry of the *psi* factor that affects ochre suppression levels (Cox, 1965, 1971) and perhaps the biochemistry of some of the mutants of yeast cells that are temperature-sensitive for protein synthesis (Hartwell and McLaughlin, 1968).

The level of nonsense suppression in the cell-free system is surprisingly high, especially in the case of the *SUP*4-1 ochre suppressor, which suppresses in vivo at a level of only 4–12% (Gilmore et al., 1971) compared to the 65% seen in vitro. The most probable explanation for this is that the termination or release factors are not functioning efficiently, perhaps because of incompatibility in the mixed system. This would allow suppression to gain a relative advantage over chain termination. An analogous situation has been observed in E. coli, where it has been shown that inactivation of the release factors by specific antibodies increases the observed level of suppression of termination at a nonsense codon (Capecchi and Klein, 1970; J. F. Atkins and R. F. Gesteland, unpublished observations; J. Manley, unpublished observations).

The suppressing activity is in the tRNA fraction for at least one representative of the class I tyrosine-inserting amber and ochre suppressors. The same will probably hold true for all the suppressors of this class (which map at eight distinct genetic loci). The simplest interpretation is that eight genetically distinct loci code for functionally identical tyrosine tRNAs, and that each tRNA can be independently altered by mutation to allow recognition of UAA. Each of these can in turn be altered by mutation to allow reading of UAG. It is possible, however, that the suppressing tRNA is not originally a tyrosine-accepting species, and that when the mutation which allows UAA reading is acquired, the tRNA simultaneously acquires the ability to be charged with tyrosine. A UAG suppressor in E. coli provides an example of this possibility (Soll, 1974; Yaniv, Folk, and Berg, 1974).

Although the results presented here argue that suppression is mediated by the tRNA, they do not prove that the mutation allowing suppression alters the primary sequence of nucleotides in the tRNA. The mutation may affect an enzyme that modifies tRNA, but this seems improbable, since it would require that all eight loci giving rise to these suppressors be involved in modification in very similar ways. By far the most probable explanation is that the mutations have occurred in the DNA encoding the tRNA. We are currently purifying the suppressing tRNA species to examine their nucleotide sequences.

The same arguments concerning the mutational event leading to suppression hold for the recessive lethal amber suppressor. In this case, however, the cell cannot survive without a wild-type copy of the gene involved, which suggests that there may be only one or a few copies of the gene for this tRNA, so that the cell cannot afford to give up one copy for suppression.

From the analysis of cytochrome c mutants (Stewart et al., 1972) and from the work reported here and by Capecchi et al. (1975), it seems clear that chain termination in yeast can occur at single UAA or UAG codons (and probably also UGA, but proof is lacking). Modified tRNAs from strong nonsense-suppressing strains can suppress this termination both in vivo and in vitro. This is directly analogous to the situation in bacteria, except that the UAG and UAA suppressors are totally codon-specific; that is, UAA suppressors in yeast recognize UAA only, not both UAA and UAG as in bacteria.

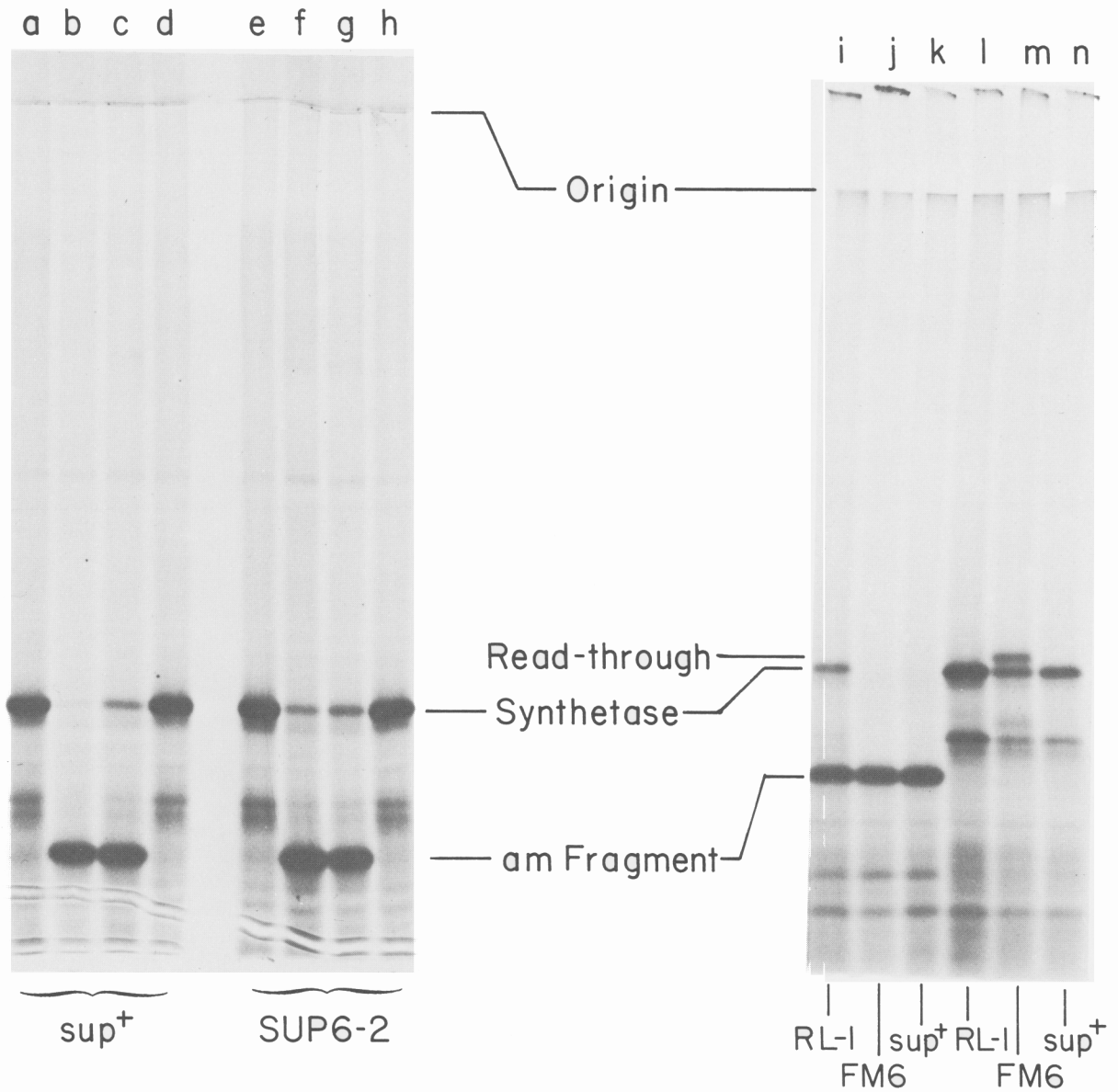

Figure 8. Suppression by tRNA

(a–h) are from one experiment; (i–n) are from another. Both autoradiograms were 5 day exposures.

S-100 Source	Added tRNA	mRNA
(a) S288C, wild type	S288C, (*sup*+)	Qβ+
(b) S288C, wild type	S288C, (*sup*+)	Qβ *am*l
(c) S288C, wild type	CY3, *SUP*6-2	Qβ *am*l
(d) S288C, wild type	CY3, *SUP*6-2	Qβ+
(e) CY3, *SUP*6-2	S288C (*sup*+)	Qβ+
(f) CY3, *SUP*6-2	S288C (*sup*+)	Qβ *am*l
(g) CY3, *SUP*6-2	CY3, *SUP*6-2	Qβ *am*l
(h) CY3, *SUP*6-2	CY3, *SUP*6-2	Qβ+
(i) S288C, wild type, *sup*+	RL-1	Qβ *am*l
(j) S288C, wild type, *sup*+	FM6, *SUP*4-1	Qβ *am*l
(k) S288C, wild type, *sup*+	*sup*+	Qβ *am*l
(l) S288C, wild type, *sup*+	RL-1	Qβ+
(m) S288C, wild type, *sup*+	FM6, *SUP*4-1	Qβ+
(n) S288C, wild type, *sup*+	*sup*+	Qβ+

"*sup*+" is the wild-type parent of the RL-1 suppressor, and the RL-1-suppressing tRNA used here was extracted from the aneuploid strain. The tRNA concentration added in all cases was about 100 μg/ml. Higher concentrations generally led to inhibition of total protein synthesis.

Will it be possible to extend such a suppressing system to other eucaryotes, particularly mammalian cells, where so far no examples of nonsense mutants or suppressors are known? Protein synthesis in cell-free extracts from mammalian cells obeys UAA and UAG termination signals at the normal ends of bacteriophage RNAs and at internal mutant sites (Aviv et al., 1972; Schreier et al., 1973; Morrison and Lodish, 1973, 1974; Capecchi et al., 1975). At least in vitro, the termination mechanism recognizes nonsense codons. The only data available on stop signals used in vivo in mammalian cells is for human hemoglobin. Comparison of the amino acid sequences of the Constant Spring (Clegg, Weatherall, and Milner, 1971) and Wayne (Seid-Akhavan et al., 1972) variants of the globin chain leads to the conclusion that UAA is the terminator of translation. This is corroborated by partial nucleotide sequence data (Marotta et al., 1974). Our preliminary data indicate that if globin mRNA is translated under conditions of UAA suppression, read through proteins can be detected that are not found with *sup*$^+$ or UAG suppression conditions. Thus at least the UAA codon is used to signal termination, and the existing cell-free systems can be used to establish the identity of stop signals in normal mRNA.

We know that the yeast-suppressing tRNAs can also suppress amber (Capecchi et al., 1975) and ochre (our unpublished results) codons in bacteriophage RNA when added to an otherwise fully mammalian cell-free system. Thus the biochemical data so far available do not in any way rule out the possibility of the existence of nonsense mutants and suppressors in mammalian cells. It appears that the appropriate tools are available to assay potential suppressors and potential nonsense mutants in any gene (viral or cell) whose wild-type mRNA can be translated in vitro into a recognizable product.

Experimental Procedures

Strains

The following yeast strains were used: S288C wild type (haploid), FM6, *ade*2-1, *lys*1-1, *can*1-100, *SUP*4-1, *psi*$^-$ (Rasse-Messenguy and Fink, 1973); FM811C, *ade*2-1, *lys*2-1, *SUP*4-2 (*ts*), *psi*$^+$ (Rasse-Messenguy and Fink, 1973); A941B, *sup*$^+$ *trp*5-7 *psi*$^+$ (Fink, unpublished data); RL1 aneuploid (DBA316), $\frac{\alpha\ leu2\text{-}1\ SUP\text{-}RL1\ trp1\text{-}1\ tyr7\text{-}1}{\alpha\ leu2\text{-}1\ sup^+}$ (similar to DBA317, Brandriss et al., 1975); RL1 diploid (DBD339), $\frac{a\ his1\ +\ leu2\text{-}1\ trp1\text{-}1\ tyr7\text{-}1\ CAN^s\ sup^+}{\alpha\ +\ ade1\ leu2\text{-}1\ trp1\text{-}1\ tyr7\text{-}1\ can^r\ SUP\text{-}RL1}$ (Brandriss et al., 1975); parent type of RL1 (DBD195), $\frac{a\ his1\ +\ leu2\text{-}1\ trp1\text{-}1\ tyr7\text{-}1\ CAN^s\ sup^+}{\alpha\ +\ ade1\ leu2\text{-}1\ trp1\text{-}1\ tyr7\text{-}1\ can^r\ sup^+}$ (Brandriss et al., 1975); *SUP*6-2 (strain SL75-5B), a *cyc*1-179, *trp*1-1, *tyr*7-1, *leu*2-1, *lys*1-1 *SUP*6-2 (provided by F. Sherman).

Growth and Extracts

All yeast strains were grown at 30°C in minimal medium (yeast nitrogen base without amino acids, from Difco, 6.7 g/l; glucose, 20 g/l) supplemented with the appropriate components to satisfy nutritional requirements of each strain (amino acids at 50 μg/ml). The recessive lethal diploid and aneuploid strains segregate wild-type, *sup*$^+$, cells during growth. This was prevented by growing them in the absence of leucine and lysine so that the suppressor activity was required for growth. Cells were harvested in mid-log phase at an OD_{550} of 1–3 in a Zeiss spectrophotometer for preparation of cell-free extracts, or at OD_{550} of 5 for preparation of tRNA. In each case, several clones of the cultures as grown up were scored for nutritional phenotype to verify their suppressor genotype. For extracts, cells were cooled rapidly by addition of ice to the culture, collected by centrifugation for 5 min at 2000 × g, and washed twice by sequential suspension in 20 mM Tris, 5 mM magnesium acetate, 100 mM KCl, 6 mM mercaptoethanol (pH 7.2), and centrifugation. The yield of cells was approximately 1 g/l/OD_{550}. The final cell pellet was quick-frozen by submersion in liquid nitrogen and stored at -70°C. The cells were thawed and dispersed in twice their weight of Y buffer [20 mM Hepes, Calbiochem; 5 mM magnesium acetate; 100 mM KCl; 1 mM dithiothreitol (pH 7.2)]. 4 times the cell weight of glass beads (0.45–0.5 mm) were added, and the cells were disrupted by a 40 sec treatment in the Braun homogenizer cooled with CO_2 to maintain the temperature at about 4°C. The mixture was centrifuged for 10 min at 23,000 × g, and the top three fourths of the supernatant fraction were taken as the S-23 fraction. (This was dialyzed against Y buffer if the S-23 was to be used for protein synthesis.) This fraction was centrifuged for 1 hr at 100,000 × g and the top three fourths were taken, dialyzed overnight against Y buffer at 4°C, quick-frozen in small aliquots with liquid nitrogen, and finally stored at -70°C. This S-100 fraction was stable under these conditions for months, but each aliquot was thawed only once.

Yeast tRNA was prepared by a modification of the method published by Capecchi (1966). Frozen cells were thawed in about one half their weight of 0.1 M NaCl, 5 mM magnesium acetate, 20 mM Hepes, 1 mM dithiothreitol (pH 7.4), 0.1% SDS, and an equal volume of phenol (distilled under nitrogen, with 8-hydroxyquinoline, and saturated with the same buffer) was added. After shaking for 1 hr, the mixture was centrifuged for 15 min at 8000 × g. The top phase was saved and the bottom phase was extracted with buffer again; after centrifugation, this top phase was combined with the first, and a second phenol extraction was carried out, with one half the original volume of phenol and shaking for 15 min. The top phase was removed and the RNA was precipitated by addition of 2 vol of ethanol. Extraction of the tRNA with salt, stripping of the tRNA at pH 8.8, and chromatography on DEAE were carried out according to Capecchi (1966). The final product was dissolved in sterile, deionized water at a concentration of 10 mg/ml and was stored at -20°C.

Cell-Free Protein Synthesis

The cell-free protein synthesis mixture is essentially that described by Schreier and Staehelin (1973), as modified by Anderson et al. (1974) and Atkins et al. (1975), but with the pH 5 fraction from mouse ascites cells replaced by the S-100 fraction from yeast cells. The reaction mixtures contained: 100 mM KCl; 30 mM Hepes (pH 7.2); 2.2 mM magnesium acetate, unless otherwise noted; 2 mM ATP; 0.4 mM GTP; 20 μg/ml creatine kinase; 20 mM creatine phosphate; 1 mM dithiothreitol; 0.1 mM 19 amino acids minus methionine; 0.8 mM spermidine (or 8 mM putrescine as noted); about 1 mCi/ml ^{35}S-methionine (made as described previously—each batch was titrated to determine the amount of methionine to give maximal incorporation of radioactivity; Crawford and Gesteland, 1973); plus the biological components: ascites ribosomal subunits and rabbit reticulocyte initiation factor fractions prepared as described previously (Anderson et al., 1974), the yeast S-100 as described above, and mRNAs described below. The ribosome concentration was 0.25 OD_{260} per 50 μl reaction mixture, and the 30–40% and 40–60% ammonium sulfate cuts of the high salt wash of rabbit reticulocyte ribosomes were titrated to give the optimal conditions for each batch. This was approximately 1 μl (20 μg protein) of the 30–40% fraction per 50 μl reaction mixture, and ranged

from 2 to 5 μl of the 40–60% fraction. The yeast S-100 fraction was similarly titrated, and 5 μl were normally used per 50 μl reaction mixture, but up to 20 μl was not usually inhibitory and many extracts already gave optimal activity with as little as 1 μl. The incubation was carried out at 30°C, and synthesis was linearly for 1 hr but continued at a progressively slower rate for up to 2 hr. Samples were taken after 90 or 120 min for analysis.

After incubation, 5 μl samples were removed to measure the radioactivity incorporated into protein, and the remainder was treated with RNAase and EDTA (Crawford and Gesteland, 1973), precipitated with 80% acetone, and dissolved in the sample buffer for SDS-polyacrylamide gel electrophoresis as described previously (Anderson et al., 1974). For autoradiography, Kodak SD-54 film was used.

The preparation of globin mRNA was described by Lingrel (1972), and that of late Ad2 mRNA by Anderson et al. (1974). The yeast cytoplasmic RNA was made by first making an extract from cells as described above for the S-23 fraction and then subjecting this to phenol, isoamyl alcohol, and chloroform extraction as described by Anderson et al. (1974) for Ad2 RNA. The following amounts of each RNA were used in a standard 50-μl reaction mixture: Ad2 cytoplasmic RNA, 10 μg; globin mRNA, 1 μg; yeast cytoplasmic RNA, 10 μg; Qβ RNA, 10 μg.

Qβ RNA

Qβ wild-type bacteriophage was grown on the nonsuppressing E. coli strain K-38, and the mutant Qβ *am*1 was grown on the isogenic but suppressing strain K-37, both obtained from K. Horiuchi and N. Zinder. Growth and purification of the phages, and extraction of the RNA with distilled phenol, were as previously described (Gesteland and Boedtker, 1964; Kolakofsky, 1971). Growth of the amber mutant phage was according to Capecchi (1966) to minimize revertant phage in the population. The preparations used here contained 2.1% wild-type revertants.

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