# Studies on the Nature of the G-Factor Binding Site on the 50S Ribosomal Subunit†

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ABSTRACT: The ability of the 50S ribosomal subunit to form a complex with G factor (the soluble translocation protein) and GDP in the presence of fusidic acid has been used to assess the features of the particle which are involved in its interaction with G factor. This activity, as distinguished from the peptidyl transferase activity of the subunit, does not require the presence of a monovalent cation. Similarly, it is quite refractory to complete inactivation by $T_1$ and pancreatic RNase, although mild treatment of the particle with pancreatic RNase appears to reduce either the affinity of the 50S subunit for the ligands or the stability of the complex. By contrast, the ability of the subunit to form this complex is readily destroyed by trypsin digestion, unfolding by $Mg^{2+}$ depletion, or removal of proteins by CsCl. Neither the CsCl core particles nor the split proteins can replace the 50S subunit in complex formation; however, this activity, again, as distinguished from peptidyl transferase, is readily restored by recombining these components at low temperature and low ionic strength. We conclude that G factor in the course of uncoupled GTP hydrolysis interacts with one or more of the surface proteins of the 50S subunit and that this interaction depends on the overall integrity of the particle. The structural requirements for G-factor binding to the 50S subunit are clearly distinct from those required for peptidyl transferase activity.

Translocation is one of three processes which are repeated during the course of protein chain elongation (for a review, see Lucas-Lenard and Lipmann, 1971). During translocation, G factor interacts with the ribosome so as to bring about the hydrolysis of GTP, and it is this hydrolysis which is presumed to provide the motive force for the movement of the ribosome along mRNA (Nishizuka and Lipmann, 1966; Erbe *et al.*, 1969). In order to develop a structural model for this and other steps in protein synthesis involving the participation of soluble factors, it is important to define the portion of the ribosome with which the factor interacts.

With respect to translocation, the problem is somewhat simplified because G factor and the ribosome are capable of GTP hydrolysis in the absence of protein synthesis (Nishizuka and Lipmann, 1966). In addition, this uncoupled interaction can be examined in a more-or-less all-or-none context with the aid of fusidic acid. This antibiotic stops the hydrolytic reaction after a single round by preventing the dissociation of G factor and GDP from the ribosome (Bodley *et al.*, 1970b; Brot *et al.*, 1971). By assessing the form of the ribosome which is essential to the formation of the GDP-containing complex, one is relieved of circumstances which might affect the rate of hydrolysis without altering the fundamental recognition process itself. Employing this technique, for example, it was possible to show that the interactions of uncoupled GTP hydrolysis are entirely restricted to the 50S subunit (Bodley and Lin, 1970). Subsequently, it was observed that catalytic hydrolysis on this subunit had been obscured by the facultative but nonessential role of the 30S subunit in this process (Modolell *et al.*, 1971).

In the present report, we have employed this complex to determine what gross structural features of the 50S subunit are required for its recognition by G factor.

## Materials and Methods

*Materials.* Cellular components were derived from either *Escherichia coli* B (Grain Processing Corp.) or A-19 (General Biochemicals). The preparation of G factor, high-salt-washed ribosomes, S-100, and tRNA was described previously (Highland *et al.*, 1971). Fusidic acid was generously provided by Miss Barbara Stearns of E. R. Squibb. The [$^3$H]GTP (5.66 mCi/mmole) and [$^{14}$C]phenylalanine (390 mCi/mmole) were from New England Nuclear Corp. and poly(U) was from Miles Laboratories.

*Ribosomal Subunits.* High-salt-washed ribosomes (0.5–1.5 g) were dissociated by dialysis against 10 mM Tris-Cl (pH 7.4), 50 mM $NH_4Cl$, 0.7 mM magnesium acetate, and 1 mM dithiothreitol and layered on a 1.5-l. (10–30% w/v) linear sucrose gradient containing the above buffer. Centrifugation was for 18 hr at 27,000 rpm in a Spinco 15 Ti zonal rotor. Following centrifugation, the gradient was displaced with continuous monitoring at 254 nm. The 30S and 50S peaks were pooled conservatively so as to minimize cross-contamination of subunits, and magnesium acetate was added to the pooled fractions to a final concentration of 10 mM. These fractions were then centrifuged overnight at maximum speed in the Spinco 42 rotor. The subunits were redissolved, dialyzed, and stored at −20° in the buffer used to dissociate them except that it contained 10 mM magnesium acetate and in some cases, 50% glycerol. Cross-contamination of subunits as measured by phenylalanine polymerization was <1%. Although some GDP binding activity was lost during the course of the preparation (Table I), the activity which remained was stable to storage.

*Assay Methods.* The ribosome–G-factor–[$^3$H]GDP complex was formed and assayed by the standard Millipore assay described previously (Bodley *et al.*, 1970a). The 50-μl reactions contained 7.7 units of G factor (Highland *et al.*, 1971), 13.4

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TABLE I: 50S CsCl Core Particles and the Binding of G Factor–GDP.[a]

| Ribosomal Component[b] | Moles of GDP Bound/ Mole of Ribosomal Particle or Particle Equiv |
|---|---|
| 70 S | 0.652 |
| 50 S | 0.402 |
| Core | 0.014 |
| SpA | <0.002 |
| SpB | <0.002 |
| SpA + SpB | <0.002 |
| Core + SpA | 0.083 |
| Core + SpB | 0.034 |
| Core + SpA + SpB | 0.215 |

[a] The binding of [$^3$H]GDP was conducted with limiting ribosomal particles or subribosomal components as described in Materials and Methods. For the purpose of these calculations, it was assumed that 1 $A_{260}$ unit corresponds to 25.5 pmoles of 70S ribosomes or 38.2 pmoles of 50S subunits or CsCl core particles. Split protein (1 pmole) was defined as that amount derived from 1 pmole of 50S subunit. Reconstitution of cores and split proteins was performed with a twofold excess of protein and calculation of binding efficiency of the reconstituted particle was based on the amount of core particles employed. All of the data are corrected for the dissociation of complex (50%) which occurs during filtration (Bodley *et al.*, 1970a). [b] Core refers to the CsCl core particle and SpA and SpB, the acidic and basic split proteins, respectively.

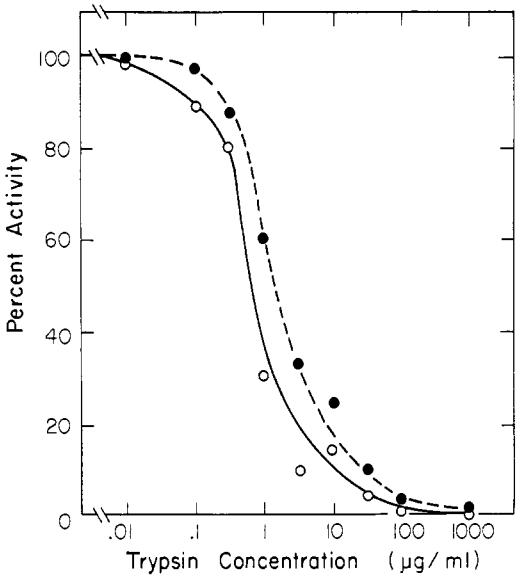


FIGURE 1: Amino acid polymerization and complex formation by trypsin-digested 50S subunits. 50S ribosomal subunits (8.5 mg/ml) were incubated for 15 min at 37° with the indicated concentrations of trypsin as described in Materials and Methods. Following termination of the digestion by incubation with soybean tyrpsin inhibitor, the activities of the subunit were assayed. Polymerizing activity (in the presence of excess 30S subunit), open circles; binding activity, closed circles.

pmoles of [$^3$H]GTP (78,500 cpm), and 3 mM fusidic acid. All of the assays performed here were intended to assess the activity of ribosomes in forming this complex. Consequently, they were performed with excess G factor and [$^3$H]GTP creating conditions where complex formation was linearly dependent on ribosomes (Bodley *et al.*, 1970a).

Polyphenylalanine synthesis with limiting ribosomes was conducted exactly as described by Highland *et al.* (1971).

*Modification of Ribosomes by Enzymatic Treatment.* All reactions were conducted at 37° with ribosomes (either 70S or 50S subunits) at 2.5–15 mg/ml in a buffer containing 10 mM Tris-Cl (pH 7.4), 10 mM magnesium acetate, 10 mM $NH_4Cl$, and 1 mM dithiothreitol. With trypsin, the reaction was terminated after 30 min by the addition to 0.5 mg/ml of soybean trypsin inhibitor, and the incubation was continued for 15 min prior to the assay of the ribosomes. RNase digestion was conducted for 60 min and terminated by simply chilling on ice and the samples were assayed immediately. Control experiments indicated in all cases that the effect of the enzymatic degradation was on the ribosome prior to the assay of its activity, not on some component of the assay mixture.

*Unfolding of Ribosomes by $Mg^{2+}$ Depletion.* 50S ribosomal subunits were first dialyzed overnight against a solution containing 10 mM Tris-Cl (pH 7.4), 1 mM magnesium acetate, 50 mM $NH_4Cl$, and 1 mM dithiothreitol. The concentration of the subunit was then adjusted to 3.5 mg/ml and placed in a 0.25-in. dialysis bag which was open at one end. Dialysis of 0.5 ml of solution was conducted with rapid mixing against 250 ml of a buffer identical to that above except that 1 mM EDTA replaced the magnesium acetate. At the indicated times, samples were withdrawn, added to magnesium acetate at a final concentration of 10 mM, and immediately assayed for activity.

*Preparation of CsCl Core Particles and Acidic and Basic Split Proteins.* The procedure was essentially that of Meselson *et al.* (1964). 50S subunits (20 mg) were placed in 5 ml of a solution containing 4.9 M CsCl, 20 mM Tris-Cl (pH 7.4), 1 mM dithiothreitol, and 8 mM magnesium acetate. Following centrifugation for 36 hr at 36,000 rpm in a Spinco SW-39 rotor, the gradients were fractionated and the region corresponding to the core particles was pooled and dialyzed against the buffer used in complex formation (10 mM Tris-Cl (pH 7.4), 10 mM magnesium acetate, 10 mM $NH_4Cl$, and 1 mM dithiothreitol) and stored at −20°. The upper portion of the gradient along with the film of split protein was dissolved in the above buffer containing 1 M LiCl and further fractionated into acidic and basic split proteins by chromatography on DEAE-cellulose as described by Traub and Nomura (1968). Finally, the split protein fractions were dialyzed against the buffer used in complex formation as specified above and stored at −20°.

## Results

*Effect of Degradative Enzymes on the Ability of the 50S Subunit to Bind G Factor and [$^3$H]GDP.* The ability of trypsin-degraded 50S subunits to participate in overall amino acid polymerization is compared in Figure 1 with their ability to bind G factor and [$^3$H]GDP. Loss of both activities occurs over a narrow range of trypsin concentrations. Moreover, the polymerizing activity of this subunit is only slightly more sensitive to trypsin digestion than is its ability to participate in GDP binding.

The inactivating effect of ribonucleases on the GDP binding activity of ribosomes is illustrated in Figure 2. Complete loss of this activity requires a high level of either pancreatic or $T_1$

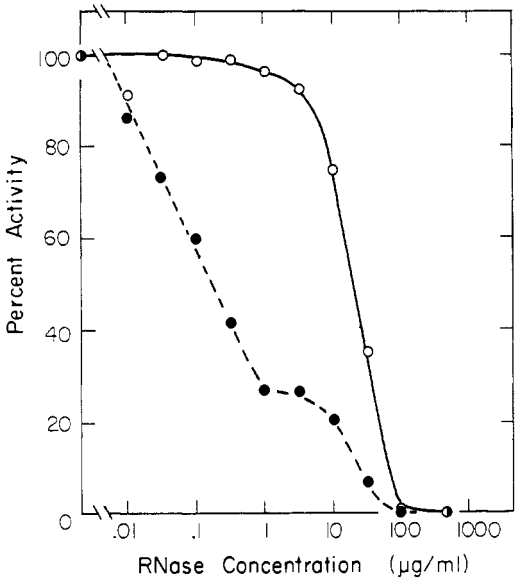


FIGURE 2: [$^3$H]GDP binding by 70S ribosomes digested with pancreatic or $T_1$ RNase. The treatment of 70S ribosomes (2.5 mg/ml) with varying levels of RNase was conducted for 60 min at 37° as described in the text. Pancreatic RNase, closed circles, $T_1$ RNase, open circles.

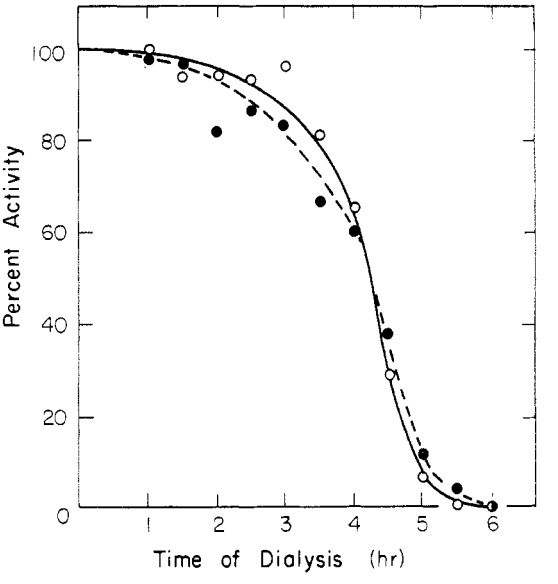


FIGURE 3: Loss of amino acid polymerizing and [$^3$H]GDP binding activities upon $Mg^{2+}$ depletion of 50S riboxomal subunits. The 50S ribosomal subunit was depleted of $Mg^{2+}$ by dialysis against EDTA as described in the text. Polymerizing activity (in the presence of excess 30S subunit), open circles, and GDP binding, closed circles.

ribonuclease. Other experiments have shown that in both cases, these rigorous digestion conditions are associated with gross structural alteration of the ribosome. For example, in some cases, GDP binding activity persists even after a significant portion of the ribosomal protein has visibly precipitated. In all cases, however, the residual binding activity was associated with ribosomes and not the low molecular weight or insoluble portions of the mixture.

As with trypsin treatment, inactivation of binding activity by ribonuclease $T_1$ was consistently observed to occur over a relatively narrow range of concentrations. The observation with pancreatic ribonuclease was quite different. In all cases, with several preparations of both 70S and 50S particles, inactivation was distinctly biphasic and occurred over a wide range of enzyme concentrations. For reasons that are not entirely clear, the degree of partial inactivation at lower pancreatic RNase concentrations was variable; the result shown in Figure 2 represents the most pronounced effect we have observed.

The peculiar response to pancreatic RNase could result from one of two circumstances. Either the ribosomes are heterogeneous with respect to the direct or indirect involvement of pancreatic RNase sensitive regions in the G-factor recognition site or mild digestion with this enzyme alters some property of all of the particles with respect to their ability to participate in complex formation. The most likely possibility in the latter regard would be a decrease in the affinity of the partly digested ribosome for its ligands or a reduction in the stability of the final complex. Experiments designed to test these alternatives (data not shown) suggested that mild treatment with pancreatic RNase decreased both the affinity and stability of the complex without reducing the number of active ribosomes. However, the variability in the RNase effect has precluded a quantitative examination of this problem. The most likely interpretation is that the ribosomal G-factor binding site is not heterogeneous and the effect observed under mild treatment with pancreatic RNase reflects indirect involvement of RNA in this interaction.

*G Factor–GDP Binding by $Mg^{2+}$-Depleted 50S Subunits.* The removal of magnesium ion from the 50S subunit is known to inactivate the polymerizing activity of the particle by causing it to irreversibly unfold without the detectable loss of soluble elements (Gesteland, 1966). This observation offers a means of determining the degree to which the overall conformation of the particle is involved in its recognition by G factor. As can be seen in Figure 3, unfolding of the 50S subunit by $Mg^{2+}$ depletion leads to a simultaneous loss in both polymerizing and GDP binding activities.

*Ionic Requirements for the Formation of the 50S–G-Factor–GDP Complex.* A monovalent cation ($NH_4^+$ or $K^+$) is not only required for peptidyl transferase activity of the 50S subunit but the removal of this ion is known to cause a reversible, presumably structural, alteration in the particle. Following removal of the monovalent cation, peptidyl transferase activity depends upon a time-dependent, high-temperature incubation of the particle with either $NH_4^+$ or $K^+$ (Miskin *et al.*, 1970). In examining the cation requirements for complex formation, we could establish a requirement only for $Mg^{2+}$ but the possibility of a low-level monovalent cation requirement remained (Bodley *et al.*, 1970a). More recent experiments employing exhaustively dialyzed ribosomes and G factor have confirmed this lack of a monovalent cation requirement. In addition, high temperature incubation of depleted ribosomes with $NH_4^+$ or $K^+$ did not significantly alter their binding activity (data not shown). Therefore, whatever the structural change that accompanies monovalent cation removal and inactivates the peptidyl transferase activity of the 50S subunit, it does not influence its recognition by G factor.

*G Factor–GDP Binding by CsCl Core Particles, Split Proteins, and Reconstituted Particles.* Treatment of the 50S subunit with CsCl yields a core particle and split proteins which are individually devoid of polymerizing and peptidyl transferase activities. Both of these activities can be restored by recombining the separated components but this reconstitu-

tion required prolonged incubation at high temperatures and ionic strengths (Traub and Nomura, 1968; Staehelin *et al.*, 1969).

The data shown in Table I summarize the G-factor–GDP binding activity of the components derived from the 50S subunit by treatment with CsCl. The activity of the 70S and 50S particles are included for comparative purposes. As pointed out previously (Bodley *et al.*, 1970a), the binding of GDP to the 70S ribosome is less than equimolar, presumably reflecting some inactivation during preparation. In fact, the preparation employed in these studies was the most active we have observed. Further partial inactivation results during the separation of subunits but this level of activity is not increased by the addition of 30S subunits (Bodley and Lin, 1970). The CsCl core particle retains <5% of the activity of the 50S subunit and no detectable activity was observed with either of the split protein fractions, individually or in combination. Partial restoration of activity was observed when either split protein fraction was combined with the core particle. Slightly higher activity was routinely observed with the acidic protein fraction. The recombination of all of the CsCl split components restored slightly more than half of the activity which was associated with the original 50S subunit preparation.

We have systematically examined the conditions which are necessary for reconstitution of activity from the three split components since the conditions employed here differ markedly from those which are required to restore polymerizing and peptidyl transferase activities (Traub and Nomura, 1968; Staehelin *et al.*, 1969). No reconstitution conditions were found which significantly increased the activity shown in Table I. Reconstitution was found to be rapid (complete in <2 min) at 0° and independent of the concentration of $NH_4Cl$ or KCl up to 0.5 M (data not shown).

## Discussion

The participation of the 50S subunit in the formation of the ribosome–G-factor–GDP complex represents a relatively simple ribosomal function. It requires the recognition of the particle by G factor and a single round of GTP hydrolysis and it occurs rapidly at 0°. Aside from magnesium ion, there appears to be no other requirement for the reaction. Yet, despite this simplicity, it is apparent that the binding of G factor–GDP places considerable structural demands on the 50S subunit. We had originally hoped to isolate an element of the subunit which could replace it in binding G factor–GDP. Clearly, we have not realized this expectation.

The nearly parallel loss in polymerizing and binding ability of the 50S subunit upon mild digestion with trypsin argues that a surface protein(s) is involved in the binding process. Similarly, the fact that binding activity is quite refractory to RNase digestion suggests that surface rRNA is not directly involved. In this context, however, should be noted that the integrity of the 23S RNA is not essential to the participation of the 50S subunit in overall polyphenylalanine synthesis (Cahn *et al.*, 1970). These authors demonstrate that all of the 23S RNA in the 50S subunit can be cleaved with pancreatic RNase without loss of its amino acid polymerizing activity. It is conceivable that such cleavage might loosen the subunit structure and indirectly alter its ability to interact with G factor–GDP and thus give rise to the partial effects we have observed with mild pancreatic RNase digestion. The importance of the gross structure of the subunit is demonstrated by the essentially identical loss in polymerizing and complexing activity of the subunit upon dialysis against EDTA. This would seem to suggest that G factor interacts with two or more surface proteins which must be maintained in a specific conformation.

The present experiments indicate a clear difference in the structural requirements for the peptidyl transferase and G-factor–GDP binding activities of the 50S subunit. The former activity not only requires the presence of a monovalent cation but the subunit itself must either be maintained in the presence of $K^+$ or $NH_4^+$ or thermally reactivated with one of these ions (Miskin *et al.*, 1970). G-factor–GDP binding, on the other hand, exhibits no requirement for a monovalent cation, either during the reaction or the preparation of the subunit. Similarly, the reconstitution of peptidyl transferase activity from CsCl core particles and split proteins is a slow, temperature-dependent reaction which requies high ionic strength (Taub and Nomura, 1968; Staehlin *et al.*, 1969). By contrast, G-factor–GDP binding activity is rapidly reconstituted from these components at low temperatures and ionic strength. These results complement the findings of Modolell *et al.* (1971). Employing ribosome-specific antibiotics, these authors observed a clear distinction between peptidyl transferase and translocation inhibitors. No compound inhibited both reactions and while peptidyl transferase inhibitors competed with each other in ribosomal binding, the binding of translocation inhibitors to the ribosome had no effect on the subsequent binding of peptidyl transferase inhibitors. Recently, we have presented evidence (Highland *et al.*, 1971) that G factor–GDP binds at the same site as one of these translocation inhibitors, thiostrepton. Thus, on the basis of these earlier results and those reported here, it would appear that these two reactions of the 50S subunit (peptidyl transfer and G-factor–GDP binding) occur on different regions of the particle and have different structural requirements as well.

The present experiments demonstrate that maximal restoration of G-factor–GDP binding activity to a CsCl core particle prepared in the presence of low magnesium ion (8 mM) requires at least one acid and one basic split protein. Recently, Kischa *et al.* (1971) demonstrated at least partial restoration of GTPase activity assayed in the presence of the 30S subunit when a single acidic protein is combined with a 50S core particle prepared in the presence of high magnesium ion (50 mM). A similar observation has also been made by Hamel and Nakamoto (1971). It will be of interest to determine if any of these proteins are directly involved in the interaction with G factor or whether they simply promote the correct ribosomal conformation.

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# Characterization of the 5′ and 3′ Ends of the 16S Ribonucleic Acid from $T_1$-Ribonuclease-Treated 30S Ribosomes*

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ABSTRACT: Three RNA fragments, obtained from $T_1$-RNase-treated $^{32}P$-labeled 30S ribosomes, were completely digested with $T_1$-RNase and separated on a two-dimensional electropherogram. One fragment is about 120 nucleotides long and comes from the 5′ end of the 16S RNA; it contains the sequence pAAAUUGp. One fragment about 25 nucleotides long comes from the 3′ end of the 16S RNA and contains an oligonucleotide with the following partial sequence: (AUC,C)-(AC, UC)UUCA$_{OH}$. A third RNA fragment, 65 nucleotides long, has been characterized. Its position in the 16S RNA chain is not known.

$T_1$-Ribonuclease treatment of 30S ribosomes hydrolyzes rRNA at a limited number of sites producing RNA fragments of a wide size range (Fellner *et al.*, 1970b; Santer and Székely, 1971). These RNA fragments must come from portions of the RNA which are inaccessible to nuclease action, probably because they are complexed with protein molecules and/or are buried in the "interior" of the ribosome. $T_1$-RNase treatment of ribosomes offers an easy way of obtaining RNA molecules for sequence study.

In this paper, we report on the isolation of three RNA fragments from $T_1$-RNase-treated 30S ribosomes. Two of the fragments are the 5′ and 3′ ends of the 16S RNA. The third RNA fragment arises from some as yet unknown portion of the 16S RNA. None of these fragments is contained in the large pieces isolated by Fellner *et al.* (1970b) or Ehresmann *et al.* (1970), although they do contain some of the $T_1$-RNase-generated oligonucleotide fragments which are produced from complete $T_1$-RNase digests of free 16S RNA (Fellner *et al.*, 1970a).

## Materials and Methods

*Ribosomes.* $^{32}P$-Labeled ribosomes were prepared from *Escherichia coli* MRE 600 cells grown in a low-phosphate medium in the presence of 10 mCi of [$^{32}P$]$P_i$ (Sanger *et al.*, 1965). $^{32}P$-Labeled cells were lysed by freezing and thawing in the presence of lysozyme followed by treatment with sodium deoxycholate, according to Ron *et al.* (1966). 70S ribosomes obtained from cell-free supernatant were dissociated into 50S and 30S units by suspending them in pH 7.8 buffer containing 0.01 M Tris–0.001 M magnesium acetate and 0.1 M $NH_4Cl$, and shaking in the cold for 24 hr (Atsmon *et al.*, 1969). 50S and 30S ribosomes were separated on 5–30% sucrose gradients, made up in the same buffer, centrifuged in a SW25 rotor at 21,000 rpm for 17 hr at 4°. Peaks were located by counting fractions. The 30S ribosome peak was collected, the $Mg^{2+}$ concentration brought to 0.005 M (the sucrose concentration thus diluted to one-half), and the ribosomes were centrifuged for 20 hr at 100,000*g*.

*Enzyme Treatment and Preparation of RNA.* The 30S pellet was resuspended in pH 7.8 buffer containing 0.01 M Tris, 0.005 M magnesium acetate, and 0.01 M KCl. These ribosomes were treated with $T_1$-RNase in a ratio of 1–2 μg of enzyme/ODU for 1 hr at 23°, in a volume of about 0.3 ml. At the end of the incubation period, the RNA was obtained from ribosomes as previously described (Santer and Székely, 1971). The final RNA precipitate was lyophilized to remove the last traces of alcohol which was found to interfere with the layering of the RNA on top of the gel prior to electrophoresis. The [$^{32}P$]RNA bands were separated by electrophoresis in flat gels according to Adams *et al.* (1969). The gel contained 10% acrylamide and 0.5% bisacrylamide in 0.04 M Tris (pH 8.4) with 7 M urea.

*Sequence Determination.* The methods used in sequence determination were those devised by Sanger and associates. An RNA band obtained from polyacrylamide gels was completely digested with $T_1$-RNase and the products were separated by two-dimensional fractionation techniques (Brownlee and Sanger, 1967). Radioautography was used to locate these products, as well as the products of all subsequent treatments.

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