Role of 16S Ribosomal Ribonucleic Acid and the 30S Ribosomal Protein S12 in the Initiation of Natural Messenger Ribonucleic Acid Translation†

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ABSTRACT: While *Escherichia coli* 30S subunits can initiate translation of the coat cistron of RNA phage R17 with high efficiency *in vitro*, 30S subunits from *Bacillus stearothermophilus* cannot. To identify the ribosomal components responsible for this difference, reconstitution was performed using *E. coli* 16S RNA and a mixture of purified *E. coli* proteins with *B. stearothermophilus* components, singly or in combination, substituted for the corresponding *E. coli* components. Using *E. coli* initiation factors, it was found that among 17 *B. stearothermophilus* proteins examined individually, only substitution of *B. stearothermophilus* S12 showed a significant decrease (50%) in the translation of R17 RNA relative to that of poly(U). None of the other proteins showed such an effect. In addition to S12, substitution of *B. stearothermophilus* 16S RNA also caused 40–50% reduction in this ability and substitution of both *B. stearothermophilus* S12 and 16S RNA showed a near complete reduction (about 85%). Similar reductions were also observed using *B. stearothermophilus* initiation factors. Although substitution of *B. stearothermophilus* S12 (but *not* of 16S RNA) resulted in a reduction of the general initiation activity (as judged by AUG-directed fMet–puromycin formation) in the presence of *E. coli* initiation factor, no significant reduction was observed using *B. stearothermophilus* initiation factors. Thus the effect on R17 RNA translation observed by substitution of *B. stearothermophilus* S12 or 16S RNA cannot be attributed to defects in the general initiation. These experiments indicate that S12 is not only crucial in the general initiation function but also plays an important role, in conjunction with 16S RNA, in determining the efficiency of initiation at the coat cistron of R17 RNA.

One of the most important functions of the 30S ribosomal subunit is the recognition of some signals on a messenger RNA, with the aid of initiation factors and fMet-tRNA, and the formation of the initiation complex as a first step in the translation of the mRNA. It is known that the efficiency of initiation of translation at different cistrons varies considerably (for a review, see Steitz (1974)). Experimental results obtained in several laboratories have indicated that initiation factors and the 30S ribosomal subunits as well as the structure of the mRNA itself contribute to the efficiency of initiation at a given cistron (see references cited below). Despite considerable experimental information accumulated, the exact mechanism of recognition of initiation signals by the ribosomes is not known.

Lodish was the first to demonstrate that the 30S ribosomal subunit plays an important role in determining the efficiency of initiation at a given cistron (Lodish, 1970a). He found that while *Escherichia coli* 30S subunits can initiate translation of the coat cistron of RNA phage f2 with a high efficiency in a cell-free protein synthesizing system, the ribosomes from *Bacillus stearothermophilus* could, under appropriate conditions, initiate only at the A cistron. The source of the 50S ribosomal subunits and initiation factors had no qualitative effect on this species difference in initiation (Lodish, 1969, 1970a). These results indicate that the 30S ribosomal subunits are involved in the specificity for initiation of certain mRNA cistrons.

In order to identify molecular components in the 30S subunit that are responsible for this specificity, we have utilized heterologous 30S ribosome reconstitution techniques developed in our laboratory (Nomura *et al.*, 1968; Higo *et al.*, 1973). Experimental results described below indicate that both 16S RNA and protein S12 are uniquely involved in the initiation of translation of the coat cistron of the RNA phage messenger.

Materials and Methods

E. coli strain Q13 and *B. stearothermophilus* strain 799 were used, as in previous work (Nomura *et al.*, 1968; Higo *et al.*, 1973). The methods of preparation of ribosomal subunits, ribosomal RNA, and total 30S ribosomal protein mixtures have been described elsewhere (Traub *et al.*, 1971; Fahnestock *et al.*, 1974). Purification of 21 *E. coli* 30S proteins and their purity have been described before (Held *et al.*, 1973). Many *B. stearothermophilus* 30S proteins were purified by phosphocellulose column chromatography at pH 8.0 followed by Sephadex G-100 column gel filtration as described previously (Higo *et al.*, 1973). Some proteins were further purified by a second phosphocellulose column chromatography at pH 6.5. All *B. stearothermophilus* proteins were prepared and kindly provided by K. Higo in this laboratory. Full details of the purification method will be published later.

Purity of the proteins was examined by two-dimensional polyacrylamide gel electrophoresis (Kaltschmidt and Wittmann, 1970) and functional correspondence of purified proteins to *E. coli* 30S proteins was confirmed by the reconstitution technique and/or the immunochemical technique as described before (Higo *et al.*, 1973). Seventeen *B. stearothermophilus* 30S proteins were used in this study. Among them, the following 13 *B. stearothermophilus* 30S proteins were essentially pure: S3, S4, S7, S9, S10, S11, S12, S13, S15, S16, S18,

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S19, and S20. The remaining four *B. stearothermophilus* proteins, S5, S8, S14, and S17, contained small amounts of other *B. stearothermophilus* proteins. *B. stearothermophilus* proteins corresponding to *E. coli* S1 and S6 have not been identified because of lack of assay for these proteins (Higo *et al.*, 1973). Omission of S1 or S6 in the standard *E. coli* 30S reconstitution system does not cause significant reduction in the activity of the reconstituted particles. *B. stearothermophilus* protein S2 corresponding to *E. coli* S2 was not examined because of unavailability of the protein preparation at the time of the experiments. Similarly, *B. stearothermophilus* S21 was not examined.

The following buffers were used. TMAI, 10 mM Tris-HCl (pH 7.4) at 24°–30 mM NH_4Cl–10 mM $MgCl_2$–6 mM 2-mercaptoethanol; "reconstitution buffer," 30 mM Tris-HCl (pH 7.4) at 24°–0.33 M KCl–20 mM $MgCl_2$–6 mM 2-mercaptoethanol.

Reconstitution of 30S subunits using unfractionated 30S proteins was done as previously described (Nomura *et al.*, 1968; Traub *et al.*, 1971), except that the Tris buffer system (Traub *et al.*, 1971) was used to make the final composition of salts identical with that of the reconstitution buffer described above, and incubation was done at 50° for 1 hr. Reconstitution using purified 30S proteins was done as described in detail before (Held *et al.*, 1973) except that incubation was done at 45° for 1 hr.

The functional assays of reconstituted particles for poly(U) directed polyphenylalanine synthesis and R17 RNA directed [^{14}C]valine incorporation were performed as described before (Traub *et al.*, 1971). The former assay was done using [^{14}C]-phenylalanine (50 Ci/mol) at 37° for 10 min. The latter was done using [^{14}C]valine (50 Ci/mol) at 37° for 30 min. Incorporation of amino acids continued linearly under these conditions, and the rate of incorporation was proportional to amounts of the 30S subunits. AUG-dependent fMet–puromycin formation was done as described before (Held and Nomura, 1973), using unfractionated initiation factors either from *E. coli* or from *B. stearothermophilus*. Again, it was confirmed that the assay measures the initial rate of the reaction which is proportional to the amount of ribosomes. Unfractionated initiation factors were prepared as described before (Traub *et al.*, 1971), and optimum amounts to be used for the R17-dependent incorporation assay or the fMet–puromycin assay were predetermined. The fMet–puromycin reaction was mainly used to assess the ability of various reconstituted 30S particles to initiate at the AUG codon. This assay method has given more reliable results than the AUG-dependent fMet-tRNA binding assay when using crude preparations of initiation factors which are the only preparations of *Bacillus* initiation factors that we have. In addition, the fMet–puromycin reaction measures the initial *rate*, whereas fMet-tRNA binding usually measures the *extent* of binding.

The assay for AUG-directed binding of fMet-$tRNA_f^{Met}$ to 30S particles in the presence of *E. coli* initiation factor IF-2 was performed as described before (Held *et al.*, 1973), except that the Mg^{2+} concentration was 10 mM. Under the conditions used, the assay measures the extent of binding which is proportional to the amount of ribosomes. Purified initiation factor IF-2 from *E. coli* was generously supplied by J. W. Hershey (*cf.* Hershey *et al.*, 1971).

Results

(1) *Analysis of Hybrid 30S Subunits Reconstituted from 16S RNA and Unfractionated Total 30S Ribosomal Proteins.* Translation of intact R17 phage RNA by *E. coli* ribosomes starts mainly at the coat protein, and the translation of the replicase cistron follows only after the translation of at least the first part of coat cistron (Engelhardt *et al.*, 1967; Lodish, 1970b; Kozak and Nathans, 1972). Presumably, the initiation site for the replicase cistron is "masked" by the secondary or tertiary structure of the RNA and is "opened" by ribosomes which have traveled through the preceding coat cistron (Lodish, 1970b). Under our assay conditions using *E. coli* ribosomes (at 37° with intact R17 RNA), the major product was found to be the coat protein (about 90% of the total protein synthesized), and only a small fraction (about 10% or less) was the RNA replicase protein. No significant amount of the maturation protein was detected. These results were obtained by analyzing the products by sodium dodecyl sulfate polyacrylamide gel electrophoresis (data not shown) and are in general agreement with the results obtained by previous workers (Viñuela *et al.*, 1967; Lodish and Robertson, 1969). Thus, the R17 RNA directed [^{14}C]valine incorporation activity used in this study reflects mainly the production of the R17 coat protein or incomplete coat proteins very similar to it.

As described by Lodish (1970a), *B. stearothermophilus* 30S subunits are very inefficient in initiating at the coat cistron of R17 phage RNA, and hence the rate of R17 RNA directed [^{14}C]valine incorporation by *B. stearothermophilus* 30S is much less than that by *E. coli* 30S (see Table I). The rate of poly(U) directed polyphenylalanine synthesis by *B. stearothermophilus* 30S is also somewhat less than that by *E. coli* 30S subunits. In addition, the absolute activities in these assays are somewhat variable depending on ribosome preparations as well as efficiency of reconstitution. Therefore, we have used the ratio of the activity in the assay with R17 RNA to that with poly(U) as a measure of the ability of various 30S particles to initiate translation of R17 coat cistron. This ratio is normalized to the value obtained with the 30S subunits reconstituted with all the *E. coli* components.

Initial reconstitution experiments were done using unfractionated total 30S protein and 16S RNA from *E. coli* and *B. stearothermophilus*. Table I, experiment 1 shows the activity of four different kinds of reconstituted 30S ribosomes in poly(U) and R17-directed polypeptide synthesis. Whenever 30S proteins from *B. stearothermophilus* were used, the relative ability to translate R17 RNA was very much reduced (about 3–5% of the control 30S subunits reconstituted using both RNA and proteins from *E. coli*). This reduction correlates well with the weak activity of *B. stearothermophilus* 30S subunits in R17 RNA translation (7% of the control). Thus, replacement of all the *E. coli* proteins with *B. stearothermophilus* proteins has an effect which fully accounts for the difference between *E. coli* and *B. stearothermophilus* ribosomes in R17 RNA translation activity.

However, hybrid particles containing *B. stearothermophilus* 16S RNA and *E. coli* proteins also had significant decrease in their relative ability to translate R17 message, although the decrease was not as drastic as that found with particles having the opposite combination. This reduction was originally observed in our earlier work (Nomura *et al.*, 1968) and was confirmed in the present study.

In experiment 2 of Table I and all subsequent experiments to be described, hybrid particles were reconstituted using a mixture of 21 purified *E. coli* proteins (ΣSi). As can be seen in Table I, hybrid particles reconstituted from *B. stearothermophilus* 16S RNA and a mixture of purified *E. coli* proteins also showed a reduced level of translation of R17 RNA as indicated by the ratio of R17 to poly(U) activities. Although

TABLE I: Translation of R17 Message by *E. coli–B. stearothermophilus* Hybrid 30S Ribosomal Subunits.[a]

Expt No.	Source for "30S" RNA	Source for "30S" Proteins	Activity Poly(U) (%)	Activity R17 (%)	R17/Poly(U) Av	R17/Poly(U) (Range)
1	E	E	100	100	1.00	
	E	B	104	5	0.05	(0.03–0.06)
	B	E	83	54	0.65	(0.58–0.74)
	B	B	64	2	0.03	(0.01–0.06)
	E. 30S		121	123	1.02	(0.87–1.25)
	B. 30S		86	6	0.07	(0.06–0.07)
2	E	E (Σ*Si*)	100	100	1.00	
	B	E (Σ*Si*)	95	54	0.59	(0.48–0.72)

[a] Reconstitution was performed using 16S RNA and unfractionated 30S proteins either from *E. coli* (E) or from *B. stearothermophilus* (B). In experiment 2, a mixture of 21 purified 30S proteins (Σ*Si*) was used instead of unfractionated proteins. Reconstitution was done as previously described (Nomura *et al.*, 1968; Held *et al.*, 1973; Traub *et al.*, 1971), except that incubation of the reconstitution mixtures was done for 60 min at 50° (expt 1) or at 45° (expt 2). After the incubation, reconstituted particles were sedimented in reconstitution buffer (100,000*g*, 15 hr). The sedimented particles were suspended in TMAI, and incubated at 50° for 20 min to "activate" [*cf.* Zamir *et al.* (1971)] before assays. Aliquots containing 0.3 A_{260} unit of particles were then assayed for activities in poly(U) dependent polyphenylalanine synthesis (poly(U)) and in R17 RNA directed [^{14}C]valine incorporation (R17). Both 50S subunits and initiation factors from *E. coli* were used. In experiment 1, average values from three experiments are given. The range of the values for R17/poly(U) is shown in parentheses. In the poly(U) assay, 100% corresponds to 19,132 cpm. In the R17 assay 100% corresponds to 10,693 cpm. In experiment 2, average values from six experiments are given and the range of the values for R17/poly(U) is also given in parentheses. In the poly(U) assay, 100% corresponds to 8439 cpm and in the R17 assay, 100% corresponds to 8087 cpm. In these assays blank values obtained in the absence of reconstituted particles were subtracted. The difference between the 100% values for the poly(U) assays in the two experiments is probably due to the use of different poly(U) preparations, [^{14}C]phenylalanine, 50S subunit preparations, and "S100 enzyme" preparations in the two experiments. With all these assay components being the same, the control values, which are similar to that for reference *E. coli* 30S (*E.* 30S) usually give about the same incorporation value. It should be noted that the proteins synthesized by the four kinds of hybrid ribosomes in experiment 1 were analyzed by polyacrylamide gel analysis. In all the cases, the major protein product (>90%) behaved like coat protein. However, the amounts of coat protein synthesized by "EB," "BB," or *B. stearothermophilus* 30 S (*B.* 30S) particles were very low as expected.

not shown in the table, particles reconstituted with a mixture of purified proteins have activities usually similar to particles reconstituted with unfractionated 30S proteins or derived 30S subunits (Held *et al.*, 1973; see also Tables II and IV).

The results suggest that one or more *B. stearothermophilus* proteins are mainly responsible for the reduced capacity to translate R17 mRNA. In addition, *B. stearothermophilus* 16S RNA also appears to affect translation of R17 RNA significantly. It should be noted, however, that a decreased capacity to translate R17 RNA relative to poly(U) could be the result of a general defect in initiation due to some degree of incompatibility of *E. coli* initiation factors with *B. stearothermophilus* ribosomes or hybrid ribosomal particles. In the experiments shown in Table I, only *E. coli* initiation factors were used. This problem will be discussed below.

The large difference in translation of R17 phage RNA between reconstituted particles containing *B. stearothermophilus* protein mixtures and control particles containing only *E. coli* components cannot be explained by an inactivation of the coat cistron of R17 RNA by some components contained in *B. stearothermophilus* protein fraction. After incubation of R17 RNA with such reconstituted particles containing *B. stearothermophilus* proteins, the control particles were added and R17 RNA directed [^{14}C]valine incorporation was measured. Normal high incorporation was observed (data not shown).

(2) *Hybrid 30S Subunits Reconstituted from 16S RNA and a Mixture of Purified 30S Proteins with One Component from B. stearothermophilus.* Since it appeared that the major effect on R17 translation could be attributed to the protein components of *B. stearothermophilus* ribosomes, we examined the effect of substituting for an individual *E. coli* protein, the corresponding *B. stearothermophilus* protein. In order to distinguish between effects on translation of R17 RNA and possible general effects on the ability to initiate at the AUG codon (such as defects in AUG-dependent fMet-tRNA binding due to possible incompatibility of initiation factors and ribosomal particles), hybrid particles were also examined for activity in AUG-directed fMet-tRNA binding using purified *E. coli* initiation factor IF-2.

Reconstitution was performed using *E. coli* 16S RNA and a mixture of purified *E. coli* proteins in which a single *B. stearothermophilus* protein was substituted for the functionally equivalent *E. coli* protein. Seventeen *B. stearothermophilus* proteins were individually tested in this way; 13 of the proteins were essentially pure, and the remaining four contained small amounts of other *B. stearothermophilus* proteins. In most cases, the results shown in Table II are the average of two or three separate experiments.

As can be seen from the table, the only *B. stearothermophilus* protein which drastically reduced the ratio of R17 to poly(U) activity was S12, the protein coded for by the *str* gene in *E. coli* (Ozaki *et al.*, 1969). Substitution of another protein, *B. stearothermophilus* S18, showed a slight reduction. This slight reduction has not been studied further. Substitution of each of the other *B. stearothermophilus* proteins had no significant effect on the relative ability to translate R17 RNA. The effects of substitution of the *B. stearothermophilus* proteins S1, S2, S6, and S21 were not determined (see Materials and Methods).

It should be noted that substitution of *B. stearothermophilus* S12 also significantly reduced the relative activity in the AUG-directed fMet-tRNA binding assay using IF-2 from *E. coli*. Thus the reduced efficiency of hybrid particles containing *B. stearothermophilus* S12 in R17 RNA translation could possibly be attributed to poor interaction with *E. coli* initiation factors. In contrast, as can be seen in the table, substitution of *B. stearothermophilus* 16S RNA for *E. coli* 16S RNA did not cause such a decrease in AUG-directed fMet-tRNA binding activity, even though the relative efficiency of the translation of R17 RNA decreased by about

40% as described above. Thus, the reduced capacity of *B. stearothermophilus* 16S RNA hybrid particles to translate R17 cannot be attributed to a general defect in initiation.

It should also be noted that *B. stearothermophilus* 30S subunits also showed reduced activity in AUG-directed fMet-tRNA binding using *E. coli* IF-2.[1] This reduced activity was about the same as that of the hybrid 30S subunits containing *B. stearothermophilus* S12 (about one third of the control *E. coli* reconstituted or native 30S subunits). Thus the presence of *B. stearothermophilus* S12 in the 30S subunits is sufficient to explain the large difference in AUG-directed fMet-tRNA binding activity observed between *E. coli* and *B. stearothermophilus* 30S subunits. These observations strongly suggest that S12 is an important component in the ribosome structure with which *E. coli* IF-2 interacts.

Two other observations should be noted. Substitution of *B. stearothermophilus* S7 appeared to increase translation of R17 as well as the efficiency of fMet-tRNA binding. Hybrid 30S particles containing *B. stearothermophilus* S10 showed somewhat reduced fMet-tRNA binding although there was little effect on translation of R17 RNA. These observations have not been studied further.

(3) *Hybrid 30S Subunits Reconstituted from E. coli Components and Two Substituted B. stearothermophilus Components.* The above results show that, of the *B. stearothermophilus* components tested singly, only S12 and 16S RNA appear to have a major influence on the translation of R17 RNA.

In order to further assess this effect, substitution experiments were performed in which several *B. stearothermophilus* proteins were substituted individually for their *E. coli* counterparts along with *B. stearothermophilus* 16S RNA (Table III). As described above, substitution of *B. stearothermophilus* 16S RNA reduces the capacity of hybrid particles to translate R17 by a factor of 2. The additional substitution of either *B. stearothermophilus* S4, S7, S11, S15, S16, or S19 did not reduce R17 translation any further than *B. stearothermophilus* 16S RNA alone.

However, hybrid particles containing both *B. stearothermophilus* 16S RNA and *B. stearothermophilus* S12 had a drastically reduced capacity to translate R17 RNA as compared with translation of poly(U). Thus, the effect of *B. stearothermophilus* 16S RNA and *B. stearothermophilus* S12 on R17 RNA translation appears to be additive. Combinations of other proteins with 16S RNA have not been examined. As pointed out earlier, it is possible that the reduced capacity of hybrid 30S subunits containing both *B. stearothermophilus* 16S RNA and *B. stearothermophilus* S12 to translate R17 RNA reflects some general defects in initiation function.

In preliminary experiments, we have also examined the possibility that some other *B. stearothermophilus* protein acts cooperatively with *B. stearothermophilus* S12 in reducing the R17 RNA translation activity of reconstituted particles containing *E. coli* 16S RNA. Thus, in addition to *B. stearothermophilus* S12, each of 16 *B. stearothermophilus* proteins were substituted for their corresponding *E. coli* proteins individually (S1, S2, S6, and S21 were not tested). *E. coli* RNA 16S was always used for the reconstitution. The contribution of any additional *B. stearothermophilus* protein in reducing R17 translation should be reflected in further reduction of R17 translation below the level observed when only *B. stearothermophilus* S12 is substituted. None of the proteins tested

TABLE II: Activities of Hybrid 30S Ribosomal Subunits Containing a Single *B. stearothermophilus* Component.[a]

Bacillus Component	No. of Expt	Poly(U) (%)	R17/ Poly(U)	fMet/ Poly(U)
None (Σ*Si*, *E.*)		100	1.00	1.00
S3	3	76	0.80	1.18
S4	2	104	0.90	0.93
S5	2	86	0.81	1.08
S7	2	87	1.40	1.50
S8	2	74	1.01	0.98
S9	2	78	0.91	0.76
S10	2	93	0.91	0.55
S11	2	91	1.06	1.22
S12	*7*	*111*	*0.46*	*0.34*
S13	2	82	0.88	0.97
S14	2	66	1.06	0.87
S15	2	101	0.97	0.86
S16	2	96	0.95	1.00
S17	1	57	1.03	1.06
S18	1	83	0.76	1.14
S19	3	89	0.88	1.02
S20	2	97	0.92	0.95
16S RNA	*6*	*95*	*0.59*	*0.99*
E. 30S		111	1.11	0.93
B. 30S		69	0.09	0.36

[a] Reconstitution was performed using *E. coli* 16S RNA and a mixture of 21 purified *E. coli* proteins in which a single *E. coli* protein was omitted and the corresponding *B. stearothermophilus* protein added as indicated. Reconstituted particles were isolated and "activated" by heating at 45° for 20 min. Crude *E. coli* initiation factors were used for R17 RNA directed incorporation assays. The values given for poly(U) and R17/poly(U) activity are the averages obtained from the indicated number of experiments. The activity of the reconstituted particles in fMet-tRNA binding was not determined in all experiments. The values given for activity in fMet-tRNA binding/poly(U) (fMet/poly(U)) are those obtained in one experiment except for the following substitution experiments: *B. stearothermophilus* S3, *B. stearothermophilus* S10, *B. stearothermophilus* S15, *B. stearothermophilus* S19, and *B. stearothermophilus* 16S RNA (two experiments), and *B. stearothermophilus* S12 (three experiments). Aliquots containing 0.3 A_{260} unit of reconstituted particles were assayed for activity in poly(U) (100% = 9360 cpm) and R17-directed polypeptide synthesis (100% = 6740 cpm). Aliquots containing 0.1 A_{260} unit were assayed for activity in fMet-tRNA binding using purified *E. coli* IF-2 (100% = 16,170 cpm; approximately 1 pmol of fMet bound/6.7 pmol of reconstituted particles). Blank values obtained in the absence of reconstituted particles (poly(U) and R17 assays) or in the absence of AUG (fMet binding) were subtracted.

[1] Although Lodish's work (1970a) suggests that *E. coli* crude initiation factor can stimulate protein synthesis by *B. stearothermophilus* ribosomes, there have been no quantitative studies on this problem. In fact, Steitz's experiments in a similar system have shown that recognition of the A cistron (as analyzed by the "ribosome protection technique") by *B. stearothermophilus* ribosomes is not stimulated by *B. stearothermophilus* initiation factors to any significant extent, although it is stimulated weakly by *E. coli* initiation factors (Steitz, 1973). Present results clearly show that *E. coli* initiation factors work less efficiently in combination with *B. stearothermophilus* 30S subunits than with *E. coli* 30S subunits as judged by AUG-dependent fMet-tRNA binding as well as AUG-dependent fMet–puromycin formation. As described in the text, this poor "compatibility" between *E. coli* initiation factors and *B. stearothermophilus* ribosomes is related to the origin of protein S12.

TABLE III: Translation of R17 mRNA by Hybrids Containing *B. stearothermophilus* 16S RNA and Single *B. stearothermophilus* Proteins.[a]

Source			
RNA	*B. stearothermophilus* Protein	Poly(U) (%)	R17/ Poly(U)
E	None (*E.* ΣS*i*)	100	1.00
B	None (*E.* ΣS*i*)	119	0.48
B	S4	120	0.49
B	S7	105	0.75
B	S11	102	0.52
B	*S12*	*133*	*0.15*
B	S15	101	0.57
B	S16	107	0.52
B	S19	102	0.53
E. 30S		195	1.10
B. 30S		104	0.04

[a] Hybrid particles containing the indicated *B. stearothermophilus* components were reconstituted from 16S RNA and a mixture of purified *E. coli* proteins with single *B. stearothermophilus* protein substitutions. The particles were isolated and "activated" at 45° for 20 min. Aliquots containing 0.3 A_{260} unit of reconstituted particles were assayed for poly(U) and R17 RNA directed polypeptide synthesis using *E. coli* initiation factors. The values given are those obtained in one experiment except for *B. stearothermophilus* S12 which is an average of four experiments. The range of R17/poly(U) activity in four experiments in which *B. stearothermophilus* 16S RNA and *B. stearothermophilus* S12 were substituted was from 0.07 to 0.26. The activity for poly(U) assay, 100% = 3910 cpm. For R17 RNA assay, 100% = 4960 cpm.

resulted in any substantial reduction in contrast to substitution of both *B. stearothermophilus* 16S RNA and *B. stearothermophilus* S12 (data not shown). Thus, simultaneous substitution of the two components, *B. stearothermophilus* 16S RNA and *B. stearothermophilus* S12, appears to be unique in reducing R17 RNA translation activity.

(4) *General Initiation Activity of Hybrid 30S Particles as Measured by the AUG-Dependent fMet–Puromycin Reaction.* Experiments were performed to determine whether reduced R17 RNA translation by hybrid 30S particles containing *B. stearothermophilus* S12, *B. stearothermophilus* 16S RNA, or both, could be explained by some defects in the general initiation function of these particles. We have used the fMet–puromycin reaction to assess the ability of various reconstituted 30S particles to initiate at the AUG codon (see Materials and Methods for reasons of choice of this reaction). The formation of fMet–puromycin from fMet-tRNA and puromycin requires both the initiation codon, AUG, and initiation factors (Bretscher and Marcker, 1966; Zamir *et al.*, 1966; our unpublished experiments).

Assay for both R17 RNA translation and fMet–puromycin reaction were done in two ways, using *E. coli* initiation factors; or using *B. stearothermophilus* initiation factors. Table IV shows the results. The following points should be noted.

(1) Chain elongation of all hybrid particles as measured by activity in polyphenylalanine synthesis was similar to the activity obtained with control *E. coli* reconstituted or reference *E. coli* 30S subunits. (Reference *B. stearothermophilus* 30S subunits were always somewhat lower in this activity, as mentioned before.)

(2) Hybrid particles containing *B. stearothermophilus* S12 with either *E. coli* or *B. stearothermophilus* 16S RNA had reduced activity in AUG-directed initiation as measured by fMet-tRNA binding with purified *E. coli* IF-2 or the fMet–puromycin reaction with crude initiation factors from *E. coli*. Thus, reduction of the activity of these hybrid particles in R17 RNA translation using *E. coli* crude initiation factors could be explained by some defects in the general initiation function of these particles.

(3) As mentioned previously, substitution of *B. stearothermophilus* 16S RNA had no effect on AUG-directed initiation, but reduced activity in R17 RNA translation. This reduction, therefore, cannot be easily explained in terms of reduced initiation capabilities with *E. coli* initiation factors.

(4) The relative ability of all four reconstituted particles to translate R17 RNA using crude *B. stearothermophilus* initiation factors was essentially the same as that obtained using crude *E. coli* initiation factors; substitution of *B. stearothermophilus* S12 alone or *B. stearothermophilus* 16S RNA alone caused an activity decrease by a factor of about 2. Substitution of both together reduced the activity to 18% of the control *E. coli* reconstituted particles. This weak residual activity is about the same as that seen for the reference *B. stearothermophilus* 30S subunits. Yet all the hybrid particles containing *B. stearothermophilus* S12, *B. stearothermophilus* 16S RNA, or both showed no or very slight reduction in AUG-directed chain initiation activity analyzed by the fMet–puromycin reaction in the presence of *B. stearothermophilus* initiation factors. In fact, the hybrid with *B. stearothermophilus* 16S RNA and all other *E. coli* proteins showed considerably higher activity than the control particles with all *E. coli* components. When only *B. stearothermophilus* S12 was substituted without substituting *B. stearothermophilus* 16S RNA and assays were done using *B. stearothermophilus* initiation factors, the ability to translate R17 RNA was reduced by 41% whereas the general initiation function assayed by fMet–puromycin reaction was reduced by 10% (average of five experiments, see the legend to Table IV). We conclude that the 41% reduction in the R17 RNA translation cannot be explained by a 10% reduction in the general initiation function, especially because increasing the degree of reduction in the general initiation function to as much as 76% (by the use of *E. coli* initiation factors) still resulted in only 58% reduction in the R17 RNA translation (*cf.* Table IV), a figure not so different from the 41% reduction obtained with *B. stearothermophilus* initiation factors. Thus the reduced activity of these hybrid particles containing S12, 16S RNA, or both in translation of R17 RNA cannot be attributed to some general defects in initiation, nor defects in chain elongation. We conclude that both S12 and 16S RNA are specifically involved, either directly or indirectly, in the initiation of translation of the coat cistron of R17 phage RNA.

Discussion

(1) *Molecular Components Involved in the Initiation of Coat Protein Cistron Translation.* In the present work, we sought to identify those molecular components responsible for the difference between *E. coli* and *B. stearothermophilus* 30S subunits with respect to their ability to translate the coat cistron of R17 phage RNA. Under our conditions (at 37°) *E. coli* 30S subunits showed 10–20 times higher activity than *B. stearothermophilus* 30S subunits when *E. coli* initiation

TABLE IV: Activity of Hybrid 30S Ribosomal Particles Having *Bacillus stearothermophilus* S12, 16S RNA, or Both Substituted for *E. coli* Components.[a]

Hybrid 30S Source			*E. coli* IF			*B. stearothermophilus* IF	
RNA	S12	Poly(U) (%)	R17/Poly(U)	fMet–PM/Poly(U)	fMet/Poly(U)	R17/Poly(U)	fMet–PM/Poly(U)
E	E	100	1.00	1.00	1.00	1.00	1.00
E	B	126	0.42	0.24	0.33	0.59	0.90
B	E	90	0.61	1.48	0.99	0.47	1.68
B	B	133	0.15	0.27	0.17	0.18	1.18
E. 30S		114	1.30	1.16	0.91	1.38	0.94
B. 30S		70	0.08	0.69	0.38	0.24	2.32

[a] Hybrid particles containing the indicated *B. stearothermophilus* components were reconstituted, isolated, and "activated" at 45° for 20 min, and their functional activities were determined. The values shown are the averages obtained from several reconstitution experiments. The number of independent determinations of functional activity of the hybrid particles is as follows: poly(U), R17, and fMet–puromycin formation (fMet–PM), three experiments. (Two additional experiments with the "E, B" hybrid were done and the assays using *B. stearothermophilus* IF were performed. Therefore, the values R17/poly(U) and fMet–PM/poly(U) with *B. stearothermophilus* IF for the "E, B" hybrid are the averages of five independent experiments.) fMet-tRNA binding, two experiments. Aliquots containing 0.3 A_{260} unit were used in all assays except fMet-tRNA binding (0.1 A_{260} unit). The values corresponding to 100% for each functional assay were as follows: poly(U), 7967 cpm; R17 (*E. coli* IF), 6876 cpm; fMet–PM (*E. coli* IF), 8162 cpm; fMet-tRNA binding (*E. coli* IF-2), 24,195 cpm; R17 (*B. stearothermophilus* IF), 2675 cpm; fMet–PM (*B. stearothermophilus* IF), 9473 cpm.

factors were used, and five to ten times higher activity when *B. stearothermophilus* initiation factors were used. We cannot discuss our data in relation to the efficiency of the translation of the maturation protein cistron (A cistron). Under the conditions we used, even the system with reference *B. stearothermophilus* 30S subunits produced only a very small amount of maturation protein (unpublished experiments) and, therefore, our assay was not sensitive enough to determine the efficiency of translation of the A cistron.[2]

The results presented in this paper show that the difference in the ability to translate the coat cistron can be ascribed mainly to the protein fraction. Hybrid particles reconstituted from *E. coli* 16S RNA and unfractionated *B. stearothermophilus* 30S proteins behaved like *B. stearothermophilus* 30S subunits. The same conclusion was also obtained by Goldberg and Steitz (1974).

However, none of the proteins individually examined could account for the large difference between *E. coli* and *B. stearothermophilus* in coat cistron translation. The only protein uniquely identified in this regard is S12, the protein coded for by the *str* gene in *E. coli* (Ozaki *et al.*, 1969). However, even in this case, substitution of *B. stearothermophilus* S12 for *E. coli* S12 only resulted in about 50% reduction in the efficiency. Obviously, by examining the effects of substituting two or more *B. stearothermophilus* 30S proteins in various combinations, we should be able to find the minimum number of *B. stearothermophilus* 30S proteins required to cause the full effect. However, preliminary experiments in this direction have failed to find any protein which increases the effect of S12 when substituted together with S12. It is likely that at least more than two, and perhaps many, proteins are necessary to cause the full effect. On the other hand, we have found that substitution of *B. stearothermophilus* 16S RNA alone causes a significant effect and that substitution of both *B. stearothermophilus* 16S RNA and *B. stearothermophilus* S12 together is sufficient to cause almost the full effect with respect to the reduction in ability to translate the coat cistron. It is difficult, at the moment, to decide whether 16S RNA is directly involved in determining the efficiency of translation of the coat cistron or is involved only indirectly by affecting conformation or arrangements of several proteins. According to the former view, the full effect observed by substitution of all the *B. stearothermophilus* 30S proteins without substitution of the 16S RNA may imply that the structure of *E. coli* 16S RNA can be altered to that resembling the *B. stearothermophilus* 16S RNA structure by binding many *B. stearothermophilus* 30S proteins.

(2) *Specificity in the Recognition of Initiation Signals on Natural mRNA.* As discussed above, the hybrid 30S particles consisting of *E. coli* components with substitution of *B. stearothermophilus* S12, *B. stearothermophilus* 16S RNA, *B. stearothermophilus* S12 and *B. stearothermophilus* 16S RNA together, or all the *B. stearothermophilus* 30S proteins, show a partial or near complete reduction in the translation of the coat cistron. Because these hybrid particles show about the same activity in poly(U) directed polyphenylalanine synthesis and in AUG-directed fMet-puromycin formation (with the aid of initiation factors from *B. stearothermophilus*) as the control *E. coli* 30S subunits or reconstituted 30S subunits with all *E. coli* components, the defect in the coat cistron translation cannot be explained by defects in chain elongation or defects in the ability to form the initiation complex using the AUG-trinucleotide codon and fMet-tRNA. Therefore, as shown by previous workers (Lodish, 1970b; Steitz, 1973) with respect to the inefficient translation of the coat cistron by *B. stearothermophilus* 30S subunits, inefficiency of the above mentioned hybrid 30S subunits in the coat cistron translation must in some way be due to in-

[2] Under the conditions we used, even the system with reference *B. stearothermophilus* 30S subunits produced somewhat more coat protein than maturation protein (unpublished experiments). Our observation thus differs from previous observations that cell-free systems containing *B. stearothermophilus* 30S subunits mainly translate the A cistron (Lodish, 1969, 1970a,b). The reason for this discrepancy has not been determined. However, under certain conditions, Lodish also observed more translation of the coat cistron than the A cistron using *B. stearothermophilus* ribosomes and *E. coli* S100 fraction [see Table I in the paper by Lodish (1969)].

efficient recognition of some special structure of mRNA at the initiation site of the coat cistron.

Direct evidence for the above conclusion was provided by Goldberg and Steitz (1974) in the case of the hybrid 30S subunit consisting of *E. coli* 16S RNA and unfractionated *B. stearothermophilus* 30S proteins. They have shown that such a hybrid 30S subunit, together with other appropriate components, makes the initiation complex preferentially at the A cistron initiation site rather than the coat cistron initiation site. In this respect, the hybrid resembles *B. stearothermophilus* 30S, and differs from *E. coli* 30S subunits. On the other hand, the same workers have failed to detect reduction in the relative efficiency of initiation at the coat cistron by the hybrid particle consisting of *B. stearothermophilus* 16S RNA and unfractionated *E. coli* 30S proteins. It is possible that their assay procedures, the ribosome binding site assay by RNA sequencing and the dipeptide synthesis assay, are not appropriate to reveal the partial reduction we have observed; their assays probably measure the *extent* of the reactions and may not reflect the *rate* at which the ribosomes bind at the initiation sites. It is also possible that the reduced efficiency of translation observed with such hybrid 30S subunits is due to a block in a process subsequent to the formation of the initiation complex but required for *starting* ribosome movement along the mRNA chain, *i.e.*, polypeptide chain elongation. Although not absolutely excluded, the presence of some defects in the chain elongation step is difficult to accept in view of the results obtained in the poly(U) dependent polyphenylalanine synthesis assay. Finally, our assay for R17 translation was done at 37°, whereas Goldberg and Steitz (1974) did their assays at 49°. It is known that heating of R17 RNA alters its physical structure as well as its property as a messenger RNA (Lodish, 1971). Thus some possible subtle structural difference in R17 RNA or 30S subunits might be responsible for the discrepancy between their experimental results and our results.

It is not known whether the 30S subunits directly recognize some special initiation signal at the coat cistron. Although inefficiency of translation of the coat cistron by *B. stearothermophilus* and several hybrid 30S subunits cannot be explained by defects in general initiation at the AUG codon, as mentioned above, it is still possible that the observed inefficiency is due to "incompatibility" between the above mentioned 30S subunits and some nonribosomal factors needed for coat cistron translation, such as *E. coli* initiation factor IF-3. IF-3 has been implicated in determination of the efficiency of translation of natural mRNAs (Revel *et al.*, 1970; Pollack *et al.*, 1970; Lee-Huang and Ochoa, 1971; Yoshida and Rudland, 1972). The above mentioned inefficiency of coat cistron translation could be explained if one assumes that *E. coli* IF-3 or some other nonribosomal factors is strongly required for initiation at the coat cistron, but is less required for A cistron initiation,[1] and that these factors poorly interact with *B. stearothermophilus* and the various hybrid 30S subunits which were shown to be inefficient in coat cistron translation.

(3) *Role of S12 in the Initiation of Protein Synthesis.* The present work has demonstrated that protein S12 is uniquely involved in the initiation of translation of the coat cistron. Single substitution of *B. stearothermophilus* S12 for *E. coli* S12 caused a reduction of coat cistron translation by a factor of 2. No other protein caused such an effect; in this regard, S12 is a unique protein. Several points should be noted. As described above, decrease in the coat cistron translation caused by *B. stearothermophilus* S12 substitution takes place without significant decrease in the activity of the AUG-dependent fMet–puromycin reaction when *B. stearothermophilus* initiation factors are used. That is, the reduction cannot be accounted for by defects in the AUG-dependent fMet-tRNA binding function. However, the hybrid 30S subunit containing *B. stearothermophilus* S12 has rather low activity in both the AUG-dependent fMet-tRNA binding reaction using pure *E. coli* IF-2 and the AUG-dependent fMet–puromycin reaction using crude *E. coli* initiation factors. No other protein examined (except possibly S10) shows such properties (Tables II and IV; other data not shown). Apparently, *E. coli* initiation factors work less efficiently in combination with *B. stearothermophilus* 30S subunits than with *E. coli* 30S subunits.[1] This poor "compatibility" between *E. coli* initiation factors and *B. stearothermophilus* 30S subunits can be ascribed to the presence of *B. stearothermophilus* S12. Thus the simplest model is that S12 is a key protein in the structure which interacts with initiation factors (especially with IF-2), and that the same 30S structure containing S12 and possible other components such as some specific regions of 16S RNA (see below), in conjunction with the bound initiation factor proteins, plays an important role in the recognition of natural mRNA initiation signals.

The specific role of S12 in initiation has already been suggested from our previous experiments. It has been shown that the "30S" particles reconstituted in the absence of S12 have very severe defects in the translation of phage RNA, the AUG-directed fMet–puromycin reaction as well as the fMet-tRNA binding reaction, while the same particles show only moderate reduction in poly(U) directed polyphenylalanine synthesis and only slight reduction in nonenzymatic tRNA binding reactions directed by several synthetic polynucleotides (Ozaki *et al.*, 1969). The present results confirm and extend our previous conclusions about the role of S12 in the initiation of protein synthesis.

(4) *Functional Role of 16S RNA.* Previous reconstitution experiments have established that intact 16S RNA is essential for the reconstitution of functional 30S subunits (Traub and Nomura, 1968). Chemical modification experiments have also shown that integrity of 16S RNA is important for the assembly or function of 30S subunits (Nomura *et al.*, 1968; Nomura, 1970; Noller and Chaires, 1972; our unpublished experiments). However, in these experiments, most of the functions tested were affected more or less to the same extent. Thus, although there are several previous observations suggesting that the RNA has a role other than just holding ribosomal proteins in a proper arrangement in the functional ribosome (Bowman *et al.*, 1971; Helser *et al.*, 1971, 1972; Lai *et al.*, 1973; Noller and Chaires, 1972), there has been no direct experimental demonstration that ribosomal RNAs have some unique functional role in protein synthesis.

In earlier chemical modification studies, Moore suggested that 16S rRNA might be involved in the binding of poly(U) (Moore, 1966). However, experimental support for this conclusion was indirect, and, in addition, the choice of poly(U) as mRNA was unfortunate because nonspecific binding of poly(U) to basic proteins is rather common (our unpublished experiments), and such nonspecific binding could not be distinguished from specific binding.

Experimental results described in the present paper clearly demonstrate that a modification of 16S RNA, *i.e.*, substitution by heterologous 16S RNA, in the 30S subunits specifically affects one function without affecting others. In this case, the ability to translate a natural mRNA is greatly reduced without any damaging effect on other functions tested, such as the general initiation function using the AUG triplet and the

chain elongation function. As mentioned before, we cannot exclude the possibility that the unique participation of 16S RNA in the initiation of coat cistron translation is an indirect one mediated through some protein components. However, it is tempting to speculate that direct interaction of some parts of 16S RNA with mRNA is involved in recognition of initiation signals on natural mRNAs.

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