Translational specificity of Bacillus stea-rothermophilus ribosomes
(initiation/secondary structure/ribosomal affinity/mRNA site selection)

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ABSTRACT
The translational specificity of Bacillus stea-rothermophilus ribosomes was studied by determining the effectiveness of various synthetic RNAs as templates at 37° and at higher temperatures. The effectiveness of poly(G,U) was maximal at a G:U ratio of 1:3; it declined with lower G content because of reduced ribosomal affinity for the RNA and, with higher G, because of interference by secondary structure. The effectiveness of poly(A,C,G,U) also declined when secondary structure was increased by increasing (G+C) content. Escherichia coli ribosomes exhibited a similar specificity for poly-
(G,U), but had a lower sensitivity to interference by RNA secondary structure. In both bacterial species, sensitivity to secondary structure was determined by the 30S ribosomal sub-unit.

The Bacillus stea-rothermophilus ribosomes translate only the A protein cistrons of f2 viral RNA, unlike Escherichia coli ribosomes, which translate all three cistrons of the RNA (1). This selectivity of translation was shown to be dependent on the 3OS ribosomal subunit of B. stea-rothermophilus (2) and was traced subsequently to the 16S rRNA and the S-12 protein of the small subunit (3). These observations have led to the proposal that the initiator site of mRNA is selected by base pairing of the 3′ terminus of the 16S rRNA with a leader sequence of the initiator site (4).

Because the studies carried out with the B. stea-rothermophilus ribosomes have greatly influenced our views on the initiation of protein synthesis, we decided to undertake a more thorough examination of the translational specificity of these ribosomes. In the present study, we examined the activity of B. stea-rothermophilus ribosomes with synthetic RNAs of varied base composition and compared it to that of E. coli ribosomes. A preliminary report on this study has been presented (5).

MATERIALS AND METHODS
Poly(G1,U3.1) and poly(G1,U6.1) were purchased from Pabst Laboratories; other synthetic RNAs were prepared with polynucleotide phosphorylase purified by a modification of the procedure of Singer and Guss (6). The base ratio given for an RNA represents the value obtained by base analysis (7) or, when indicated, by the input ratio of nucleoside diphosphates used in the synthesis of the RNA.

E. coli B fMet-tRNA and preincubated S-30 extracts of E. coli Q13 and B. stea-rothermophilus (NCA 2184, culture kindly provided by W. E. Welker) were prepared as described previously (8–10). Ribosomal subunits of both bacterial species were isolated by sucrose density gradient centrifugation of 70S ribosomes at 25° in a Beckman Ti 14 zonal rotor. The gradient medium contained 10–50% sucrose, 10 mM imidazole-HCl at pH 7.4, 1 mM MgCl2, 100 mM NH4Cl, and 2 mM 2-mercaptoethanol. The 70S ribosomes of both bacteria were isolated from the S-30 extracts by centrifugation through an underlay of solution containing 50 mM Tris-HCl at pH 7.8, 10 mM MgCl2, 5 mM 2-mercaptoethanol, 50 mM KCl, and 20% sucrose.

In preparing the initiation factors used throughout the study, we added an amount of EDTA equal to that of the Mg2+ in the 1 M NH4Cl extract of E. coli Q13 or B. stea-rothermophilus ribosomes, diluted the NH4Cl concentration to 0.1 M, and then mixed the extract with phosphocellulose. The adsorbed initiation factors were eluted from phosphocellulose with a solution containing 0.8 M NH4Cl, 0.02 M imidazole-HCl at pH 7.4, 0.01 M 2-mercaptoethanol, and 1 mM EDTA. The active fractions were combined and concentrated by dialysis against a buffer solution similar to that used in elution except that it contained 0.5 M NH4Cl and 65% glycerol. The concentrated factors were then stored at −50°.

Details of the assay procedures for fMet-tRNA binding, fMet-puromycin formation, and fMet incorporation were as described previously (8–10), except for the addition of 10 mM aprotinin to inhibit possible proteolytic activity. The general reaction mixture contained in 0.1 ml: 50 mM Tris-HCl at pH 7.8, 0.2 mM GTP, 1 mM ATP, 2 mM phosphoenolpyruvate, 9 mM MgCl2, 80 mM NH4Cl, 10 mM 2-mercaptoethanol, and, if not indicated otherwise, about 20 pmol of [f14C]Met-tRNA or [f9H]Met-tRNA, 50 μg of 70S ribosomes and saturating amounts of initiation factors. The assay solution for fMet-puromycin formation contained 0.2 mM puromycin, and that for fMet incorporation contained a nonradioactive mixture of the 20 amino acids and a saturating amount of dialyzed supernatant.

RESULTS
The template activities of poly(G1,U3.1) and poly(G1,U17) are strikingly different when assayed at 37° and 55° with B. stea-rothermophilus ribosomes (Fig. 1). The activity, measured by fMet incorporation into polypeptides, is higher at 55° than at 37° with poly(G1,U3.1), but lower at 55° than at 37° with poly(G1,U17).

The reason for this reversal in the effectiveness of poly-
(G1,U17) at the two temperatures is made clear by the temperature profile for fMet incorporation (Fig. 2A): while the optimal temperature with poly(G1,U3.1) is in a range that could be considered normal for the ribosomes of the thermophilic bacteria, the optimal temperature with poly(G1,U17) is much lower, with the activity at 55° lower than that at 37°. The temperature profiles for fMet-puromycin formation (Fig. 2B) and for fMet-tRNA binding (Fig. 2C) also showed differences in optimal temperature for the two RNAs, indicating that the initiation step of polypeptide synthesis is affected by a change in the G-U ratio of the RNAs. The latter two profiles differ from the first in that the optimal temperatures lie at the extremes of the range tested. Another difference is that the profile for

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fMet-tRNA binding with poly(G₁,U₁₇) declines less rapidly as the temperature increases, which may be related to the very short incubation period of the assay.

Because the preceding studies indicated that the template activity of poly(G,U) varies with its base ratio, especially at different temperatures, we prepared a series of poly(G,U)s with various G contents and tested their activities for incorporating fMet at 37° and at 55° (Fig. 3A). Saturating amounts of each RNA were used to minimize the effect of differences in initiator codon frequencies. The activities at both temperatures rose until the G:U ratio reached 1:3 and then fell with higher G content. The activities at the two temperatures were the same at a G:U ratio of about 1:8; below this ratio, activity at 37° was higher, and at higher values, activity at 55° was higher. When the ratios of the two activities are plotted as a function of the G:U ratio, a smooth curve is obtained (Fig. 3B), the ratios increasing with increasing amounts of G. Although the activity at either 37° or 55° measures, to some extent, the ribosomal affinity for the RNA, the ratio of the activities at these two temperatures should be a more sensitive measure because the effect of any variations in each RNA preparation is eliminated. Thus, we conclude that ribosomal affinity increases with increasing G, at least for the RNAs with a G content giving a G:U ratio lower than 1:3 and where activity increases at both temperatures. There is some indication that the ratio of activities may depend more generally on the ratio of purines to pyrimidines. For example, the ratios of activities obtained with poly(C₃₈,G₃,G₁, U₃₈) and poly(G₁₂,G₆,U₄) fit the curve better when they are plotted according to a ratio of purine to pyrimidine (Fig. 3B, points a and c, respectively) when plotted as a ratio of G to U (points b and d, respectively).

Further evidence for a difference in ribosomal affinity for poly(G₁₂,U₃₁) and poly(G₁₂,U₇) is presented in Fig. 4. The initiation complex of 70S ribosomes, fMet-tRNA, and poly(G₁₂,U₇), formed at 37°, is much less stable at 57° than a similar complex formed with poly(G₁₂,U₃₁); 71% of the former breaks down during a second incubation at 57°, whereas only 15% of the latter breaks down. Evidently, an initiation complex formed with a RNA containing a higher amount of G, for which the ribosomes have a greater affinity, can better withstand the destabilizing effects of the elevated temperature.

The requirement of a significantly higher concentration of Mg²⁺ for optimal activity at 55° with poly(G₁₂,U₇), but not with poly(G₁₂,U₃₁), also indicates that increasing the amount of G in poly(G,U) increases ribosomal affinity for the RNA (Fig. 5). A higher concentration of Mg²⁺ is required at 55°, probably to stabilize the ribosomal complex formed with poly(G₁₂,U₇). At 37°, this complex is sufficiently stable so that the optimal Mg²⁺ concentration is similar to that for the complex with poly(G₁₂,U₃₁).

The specificity observed with *B. stearothermophilus* ribo-
Fig. 4. Temperature stability of 70S initiation complexes. Binding of fMet-tRNA was carried out for 10 min at 37° under conditions described for Fig. 2, and then incubation was continued at 57° for 5 or 10 min. •, Poly(G1,U1); ○, poly(G1,U17).

Ribosomes for poly(G,U) with various G contents is not unique. E. coli ribosomes also respond similarly to various poly(G,U)s with different base ratios when activity is measured at two temperatures (Fig. 6). In this case, 47° was selected as the higher temperature instead of 55° to accommodate the E. coli system. The ratios of activities indicate that E. coli ribosomes also have an increased affinity for poly(G,U)s with high G content. The smaller slope of the E. coli system in the region of low G content implies that the E. coli ribosomes retain better relative affinity for poly(G,U) with very high U content. The ratios of activities with the B. stearothermophilus ribosomes are higher than those observed earlier (Fig. 3B), probably because of the use of the puromycin assay and a shorter incubation period.

The decline in activity observed earlier with a G:U ratio higher than 1:3 could be explained either by decreasing ribosomal affinity for RNAs with higher G content or, because RNAs rich in G would be expected to aggregate highly in solution (11), by increasing interference of RNA secondary structure. To assess the validity of these explanations, we compared fMet-puromycin formation by B. stearothermophilus ribosomes at 37° with that at 65°, a temperature too high even for endogenous incorporation and thus a severe test of ribosomal affinity. The results, in Fig. 7A, reveal a shift in peak activity from a G:U ratio of 1:3:1 at 37° to a ratio of 1:2 at 65°, and an increase in peak activity at 65° to about 3.6 times that at 37°. The shift in peak activity at high temperature toward RNA with a higher G content rules out a decrease in ribosomal affinity for the RNA; the shift would not have occurred if ribosomal affinity decreased with higher G content, because the higher temperature would have decreased the relative activity of an RNA with lower affinity. Indeed, such a decrease can be seen at 65° in the activity of the RNA with a G:U ratio of 1:6, which had previously shown an increase when measured at 55° (Fig. 3). Thus, the shift and the increase in the activity of the peak RNA at 65° suggest that ribosomal affinity for the RNAs continues to increase with higher G content and that the progressive decline in the activity of these RNAs is due to interference by secondary structure.

To confirm the above observations, we repeated the experiment with a series of poly(A,C,G,U)s in which secondary structure was increased by increasing the (G+C) content. As Fig. 7B shows, a shift occurred again in the peak activity upon raising the temperature from 37° to 65°; in this case, the shift was from an RNA with 10% (G+C) to one with about 30% (G+C). Because, in addition, the peak activity at 65° increased while the activity of the RNA with 10% (G+C) decreased, we
may conclude that ribosomal affinity for the RNAs increased with increasing (G+C) and that the decline in activity with high (G+C) content must be caused by interfering secondary structure. We obtained additional evidence that secondary structure caused the decrease in activity by showing that heating of poly(A,C,G,U)s of various (G+C) contents to 85° for 5 min prior to their addition to the reaction mixture stimulated fMet-tRNA binding at 37° over that in unheated controls, especially at levels of (G+C) that were inhibitory (data not presented).

Because our studies with synthetic mRNAs did not reveal any striking difference in specificity between B. stearothermophilus and E. coli ribosomes that could account for the difference in their translation of f2 RNA, we decided to test the possibility that the two species of ribosomes might differ in sensitivity to mRNA secondary structure. Ribosomes from B. stearothermophilus and E. coli were therefore tested for fMet-puromycin formation with poly(G,U)s that showed declining activity with increasing G content. As shown in Fig. 8A, E. coli ribosomes proved less sensitive to secondary structure in the mRNA. B. stearothermophilus ribosomes reached their maximal activity at a lower G content; at a G:U ratio of 1:2, their activity was already reduced by one-third, whereas activity was still maximal with E. coli ribosomes.

Poly(A,C,G,U)s of various (G+C) contents were also tested with ribosomes from B. stearothermophilus and E. coli, to determine whether the ribosomes differed in sensitivity to increasing secondary structure. Fig. 8B shows clearly that the relative activity of B. stearothermophilus ribosomes in forming fMet-puromycin declined more rapidly for the RNAs with increasing (G+C) content. The difference was maximal at a (G+C) content of about 40%. Similar results were obtained when fMet incorporation was used as an assay with isolated 70S ribosomes, which gave a more comparable maximal activity with both ribosomal species, and also when S-30 extracts were used without the addition of exogenous fMet-tRNA (data not presented).

Because the above experiments indicated that the different translational specificity of B. stearothermophilus and E. coli ribosomes for f2 RNA may be due to a difference in their sensitivity to RNA secondary structure, we tested the two ribosomal species to determine whether the sensitivity to secondary structure was related to the 30S subunit, as is the translational specificity (2). E. coli initiation factors were used throughout the experiment with both species of ribosomes, and fMet-puromycin formation by different combinations of ribosomal subunits was measured with poly(A,C,G,U) of various (G+C) contents (Fig. 9). The results, similar to those in Fig. 8B, clearly show that ribosomes containing 30S ribosomal subunits of B. stearothermophilus are more sensitive to high secondary structure than ribosomes with 30S subunits of E. coli.

**DISCUSSION**

The studies undertaken here show that the specificity of the B. stearothermophilus ribosomes for an RNA in polypeptide synthesis is influenced by the base composition of the RNA as expressed in its primary and secondary structures, which in turn determine its ribosomal affinity and the accessibility of its initiator codons. Because these studies mainly employed relatively simple synthetic RNAs with random base sequences, many containing only two bases, the specificity examined was necessarily imprecise. Nonetheless, the mere fact that these RNAs served as templates suggests that the requirement for specificity is not particularly stringent in the B. stearothermophilus ribosomes.

The effect of base composition on ribosomal affinity, and thus on ribosomal specificity, was observed separately from the effect of secondary structure in poly(G,U)s containing a G:U ratio of less than 1:3. Ribosomal affinity for the RNAs was determined by measuring activity at 55° or 65°, because any mRNA suitable for B. stearothermophilus must be expected to function in a temperature range that is optimal for the growth of the bacteria. The results showed that ribosomal affinity was the predominant factor affecting ribosomal specificity for the poly(G,U)s with G:U ratios up to 1:3, and that the affinity continued to increase with increasing G content. There was some indication that ribosomal affinity for an RNA might increase, more generally, with increasing purine content.

At a G:U ratio of about 1:3 of the poly(G,U), ribosomal affinity is sufficiently high so that the RNA can function effectively as a template at 55°. At higher ratios, however, interference by increasing secondary structure begins to set in, and the activity of the RNAs begins to decline. We were able to show that ribosomal affinity for the RNAs nonetheless still increased in this range of G content, and that the decline in ac-
tivity was due to interference by secondary structure. Ribosomal specificity for poly(G,U) with G:U ratios greater than 1:3 was thus affected primarily by RNA secondary structure. A similar inhibition of template activity by increasing secondary structure was also demonstrated with poly(A,C,G,U) of increasing (G+C) content.

The ribosomal affinity for the various synthetic RNAs exhibited by the *B. stearothermophilus* ribosomes did not differ much from that of the *E. coli* ribosomes. This affinity is not likely, therefore, to be the basis for the difference in the translational specificities of the two ribosomal species for the RNAs of *f2* and related RNA phages. A more likely basis is the differential sensitivity of the *B. stearothermophilus* and *E. coli* ribosomes to secondary structure that we have observed; initiator sites accessible to *E. coli* ribosomes may not be accessible to *B. stearothermophilus* ribosomes because of greater sensitivity to our observation that the differential sensitivity to RNA secondary structure resides in the 30S ribosomal subunits, similarly to the translational specificity of the two ribosomes for *f2* RNA (2). The difference in response of the two ribosomal species was fairly striking despite the fact that secondary structure was randomly increased in our experiments. Greater differences in the responses of the ribosomes could occur with the more highly ordered structure of natural mRNAs.

The importance of mRNA secondary structure in translation has been demonstrated by Lodish, who showed that three new sites in *f2* RNA, not normally initiator sites, become available to *E. coli* ribosomes as initiator sites when the RNA was treated with formaldehyde (12). He also showed that translation of the A protein cistron of *f2* RNA by *B. stearothermophilus* ribosomes was increased considerably when the RNA was treated with formaldehyde and the reaction was carried out at 65°C (13). The difference in the translational specificities of *B. stearothermophilus* and *E. coli* ribosomes, however, was never attributed to interference by the secondary structure. Instead, Shine and Dalgarno (4) proposed that the difference was due to a species difference in the base sequence at the 3' end of the 16S rRNAs, which participates in initiator site selection by base pairing with a leader sequence of the site. Although this proposal has been widely cited, it may not be tenable because the base sequences at the 3' ends of the 16S RNA of *B. stearothermophilus* and of *E. coli*, which were expected to base pair differently with the initiator site, are identical (14).

Finally, the results of the present studies, when extrapolated to natural mRNAs, also suggest that secondary structure may play the major role in determining ribosomal specificity for mRNAs. Because natural mRNAs contain approximately equal amounts of all four bases with a purine to pyrimidine ratio of about one and a (G+C) content of about 50%, ribosomal affinity for the RNAs should be sufficiently high, and specificity would thus be determined by secondary structure that affects accessibility of the initiator site. Moreover, if secondary structure restricted the accessibility of all internal and out-of-phase codons for fMet-tRNA (15, 16), selection of the correct initiator site would occur even when ribosomes interact randomly with mRNA, as we have suggested (17).

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