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Interaction of rabbit reticulocyte ribosomes with bacteriophage f1 mRNA and of *Escherichia coli* ribosomes with rabbit globin mRNA

(ribosome binding/protein synthesis/^{125}I-labeled mRNA)

STEVE LEGON*, PETER MODEL, AND HUGH D. ROBERTSON

The Rockefeller University, New York, New York 10021

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ABSTRACT **We have compared the behavior of a prokaryotic mRNA in a eukaryotic ribosome binding system and of a eukaryotic mRNA in a prokaryotic ribosome binding system. Using ^{32}P- and ^{125}I-labeled bacteriophage f1 mRNA, we have shown that rabbit reticulocyte 80S ribosomes can protect specific sequences from pancreatic RNase digestion, including those sequences protected by *Escherichia coli* ribosomes. We have also found that *E. coli* ribosomes fail to protect any region of ^{125}I-labeled globin mRNA. Iodination of the mRNA appeared to have little or no effect on the specificity of binding or protection by the ribosomes of either system.**

The eukaryotic and prokaryotic systems differ markedly in the ability of the small ribosomal subunits to protect mRNA from nuclease digestion. The regions of phage f1 mRNA protected by *E. coli* 30S subunits are virtually identical to those protected by the 70S ribosomes. By contrast, rabbit reticulocyte 40S subunits protect substantially larger fragments of mRNA from nuclease digestion than do the 80S ribosomes. These 40S-protected fragments are specific in the case of globin mRNA and overlap the shorter region protected by the 80S ribosomes. However, the 40S-protected fragments of phage f1 mRNA were found to be extremely heterogeneous, reflecting perhaps an important difference between the initial interactions made by these two mRNAs with the ribosomes.

The interaction of ribosomes with mRNA has been the subject of intensive investigation. In bacterial systems, one approach that has yielded a considerable amount of information has been the study of the nucleotide sequences within mRNA protected from nuclease digestion by initiating ribosomes (1–3). We have recently shown (4) that nuclease digestion of globin mRNA bound to ribosomes under these conditions allows the isolation of a specific mRNA fragment containing the initiation codon (5). The availability of the *Escherichia coli* and reticulocyte systems thus enables us to study the behavior of a prokaryotic mRNA in a eukaryotic ribosome binding system and of a eukaryotic mRNA in a prokaryotic system. In this paper we will demonstrate that rabbit reticulocyte ribosomes will respond to initiation signals in bacteriophage f1 mRNA, whereas *E. coli* ribosomes appear incapable of responding to the corresponding signals in rabbit globin message.

MATERIALS AND METHODS

Eukaryotic Components. The preparation and incubation of the rabbit reticulocyte lysate were as previously described (6, 7). All incubations of the lysate were at 30° in the presence of 20 μM hemin. Amino acids were present at one-fifth the concentrations described before (8)—i.e., a final concentration of about 20 μM. ^{14}C-Labeled reconstituted amino acid mix ("Schwarz Mixture" from Schwarz BioResearch) was added at 100 μCi/ml. Reaction mixtures were 2 mM in adenine. Rabbit globin mRNA was prepared essentially as described (6) and was kindly iodinated to high specific activity with ^{125}I by W. Prensky, Sloan-Kettering Institute, New York, NY (9). Labeled mRNA was bound to reticulocyte ribosomes in the presence of sparsomycin and diphtheria toxin, and those regions protected from pancreatic RNase digestion were extracted from the ribosomes. Full experimental details of these procedures have been presented elsewhere (4).

Prokaryotic Components. *E. coli* ribosomes, fMet-tRNA, and ^{32}P-labeled bacteriophage f1 *in vitro* mRNA were prepared as before (3). f1 mRNA for translation studies was freed of double-stranded RNA by two successive passages through Sigma-cell Type 20 cellulose in the manner described by Franklin (10). Unlabeled f1 *in vitro* mRNA was iodinated to high specific activity with ^{125}I by W. Prensky (9). fMet-tRNA-dependent protection of mRNA initiator regions (1) was carried out as described (3). Alternatively, a translation-blocked ribosome binding system was used. This consisted of all the components necessary for protein synthesis as described (11) except that, after 8 min of preincubation at 37°, thiostrepton (12, 13), a gift from J. Sy, was added (final concentration, 10 μM). Messenger RNA was added 2 min later and the reaction mixture was treated as before (3, 4).

Analytical Methods. Reticulocyte and *E. coli* ribosomes were analyzed on sucrose gradients as described (6, 14). RNA species were analyzed on 10–20% acrylamide gradient gels (4). RNA fingerprinting and secondary analyses were performed according to standard techniques (3, 4, 15, 16).

RESULTS

Ribosome Protection of mRNA. Fig. 1 *upper* shows that *E. coli* ribosomes will efficiently protect ^{32}P-labeled or ^{125}I-labeled f1 mRNA, but not ^{125}I-labeled rabbit globin mRNA. The major peak of protected f1 mRNA sedimented at about 70 S, but there was a secondary peak of protected material sedimenting at about 30 S. In addition, ^{125}I-labeled globin mRNA, which does not give a 70S peak in the bacterial system, yielded a small 30S protected peak. This lack of complete initiation complex formation with globin mRNA is in accord with other reports which show no proper initiation with the dipeptide assay (17). The profiles shown in Fig. 1 *upper* were obtained by using classical fMet-tRNA-dependent ribosome protection methods (1–3). When similar experiments were performed under translation conditions but in the presence of thiostrepton (12) (see *Materials and Methods*), a profile for ribosome protection of f1 [^{32}P]-mRNA similar to that in Fig. 1 *upper* was obtained, except that the yield of ribosome-protected fragments was enhanced 3- to 5-fold (data not shown).

Fig. 1 *lower* shows that all three sorts of mRNA tested yielded protected fragments in rabbit reticulocyte lysates. As previously demonstrated for globin mRNA (4), all three mRNAs gave protected peaks at 80S and 40S.

Size of Protected mRNA Fragments. Fig. 2 (slots 1 and 2) shows that fragments of f1 mRNA protected by *E. coli* 70S ri-

* Present address: Imperial Cancer Research Fund, P.O. Box 123, Lincoln's Inn Fields, London WC2A 3PX, England.

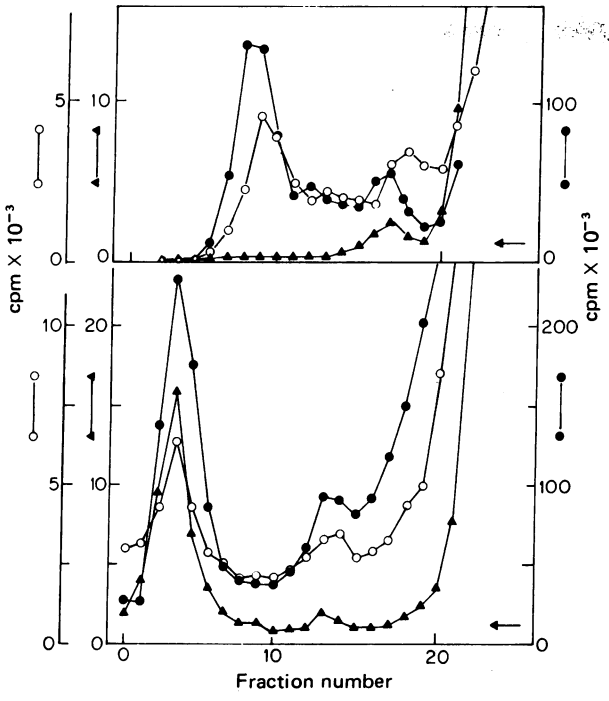

FIG. 1. Sucrose density gradient sedimentation analysis of mRNA protected by ribosomes. (*Upper*) Sedimentation profiles of *E. coli* ribosomes after interaction with mRNA and treatment with pancreatic RNase (see refs. 1–3 and *Materials and Methods*). (*Lower*) Sedimentation profiles of ribosome-protected mRNA fragments after incubation in the reticulocyte lysate and treatment with pancreatic RNase (4, 5). ●, ^{32}P-Labeled f1 mRNA, 96×10^6 cpm (specific activity 10^8 cpm/μg); ○, ^{125}I-labeled f1 mRNA, 5×10^6 cpm (specific activity 3×10^7 cpm/μg); ▲, ^{125}I-labeled globin mRNA, 3.8×10^6 cpm (specific activity 2.2×10^7 cpm/μg). Sucrose gradients were run, collected, and analyzed as described in *Materials and Methods*.

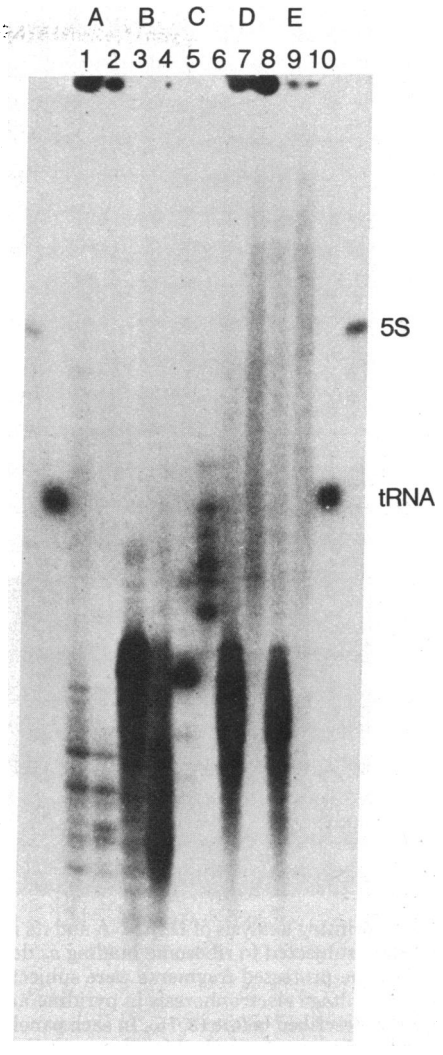

FIG. 2. Polyacrylamide gel analysis of ribosome-protected fragments. Analytical amounts (about 2×10^4 cpm) of ribosome-protected fragments were extracted from small subunits or ribosomes as before (4) and analyzed by 10–20% polyacrylamide gradient gel electrophoresis as described (5). The origin is at the top. In each case, fragments protected by the small ribosomal subunit were run next to those protected by ribosomes recovered from the same sucrose density gradient run. Five different such experiments are shown. In each case, the fragments recovered from small subunits are depicted to the right of those recovered from 70S or 80S ribosomes. ^{125}I-Labeled marker RNA species (HeLa cell 5S RNA and *E. coli* tRNA^{fMet}) were those used before (5). (*A*) Fragments of ^{32}P-labeled phage f1 mRNA protected by *E. coli* 70S ribosomes (slot 1) or 30S subunits (slot 2). (*B*) Fragments of ^{32}P-labeled phage f1 mRNA derived from translation-blocked 70S ribosomes (slot 3) or 30S subunits (slot 4) as described in the *text*. (*C*) Fragments of ^{125}I-labeled globin mRNA protected by reticulocyte 80S ribosomes (slot 5) or 40S subunits (slot 6). (*D*) ^{32}P-Labeled f1 mRNA protected by reticulocyte 80S ribosomes (slot 7) or 40S subunits (slot 8). (*E*) ^{32}P-Labeled f1 mRNA derived from 80S ribosomes (slot 9) or 40S ribosomal subunits (slot 10) after a reaction in which *E. coli* Met-tRNA^{fMet} was added at a concentration of 20 μg/ml.

bosomes or 30S subunits have similar size distributions [between 23 and 32 bases in length (3)]. Another sample of ^{32}P-labeled f1 protected fragments and a sample of ^{125}I-labeled f1 protected fragments were subjected to two-dimensional analysis and both gave patterns identical to those seen before (data not shown; see ref. 3, plate II). Fig. 2 (slots 3 and 4) shows that fragments of f1 [^{32}P]mRNA derived from translation-blocked *E. coli* ribosomes are larger and more heterogeneous than those derived from the usual binding system. This effect may simply be due to the milder pancreatic RNase digestion conditions. Fig. 2 (slots 5 and 6) shows fragments of ^{125}I-labeled globin mRNA protected by reticulocyte 80S ribosomes or 40S subunits; 80S ribosomes protected a major fragment about 40 bases long, while 40S subunits protected several fragments 50–75 bases long. Fig. 2 (slots 7 and 8) shows f1 [^{32}P]mRNA protected by 80S ribosomes or 40S subunits in a reticulocyte lysate. Whereas 80S ribosomes protected fragments similar in size to those protected by 70S ribosomes, 40S subunits protected material that was highly heterogeneous with a size spread including fragments larger than 5S RNA (Fig. 2, slot 8).

Fingerprinting Analysis of f1 mRNA Fragments. Fig. 3 *a–d* shows RNase T1 fingerprints of f1 mRNA protected by *E. coli* ribosomes or subunits. In each case, appropriate oligonucleotides were eluted and subjected to secondary and tertiary analysis as described in Table 1. These analyses yielded results in agreement with the published sequences for the three *E. coli* ribosome-protected fragments of f1 mRNA (3). These results and the similarity of the patterns shown in Fig. 3 *a–d* strongly suggest that: (*i*) *E. coli* 30S subunits and 70S ribosomes protect the same mRNA sequences; (*ii*) iodination does not change the specificity of the ribosome–mRNA interaction; and (*iii*) this same specificity is shown by translation-blocked ribosomes.

Fig. 3*e* shows f1 [^{32}P]mRNA fragments protected by 80S reticulocyte ribosomes. The oligonucleotides indicated, which comprised 30–50% of the total radioactivity protected by ribosomes, were eluted and analyzed together with those indicated in Fig. 3*a* (Table 1). They were found in each instance to be identical, demonstrating that reticulocyte 80S ribosomes

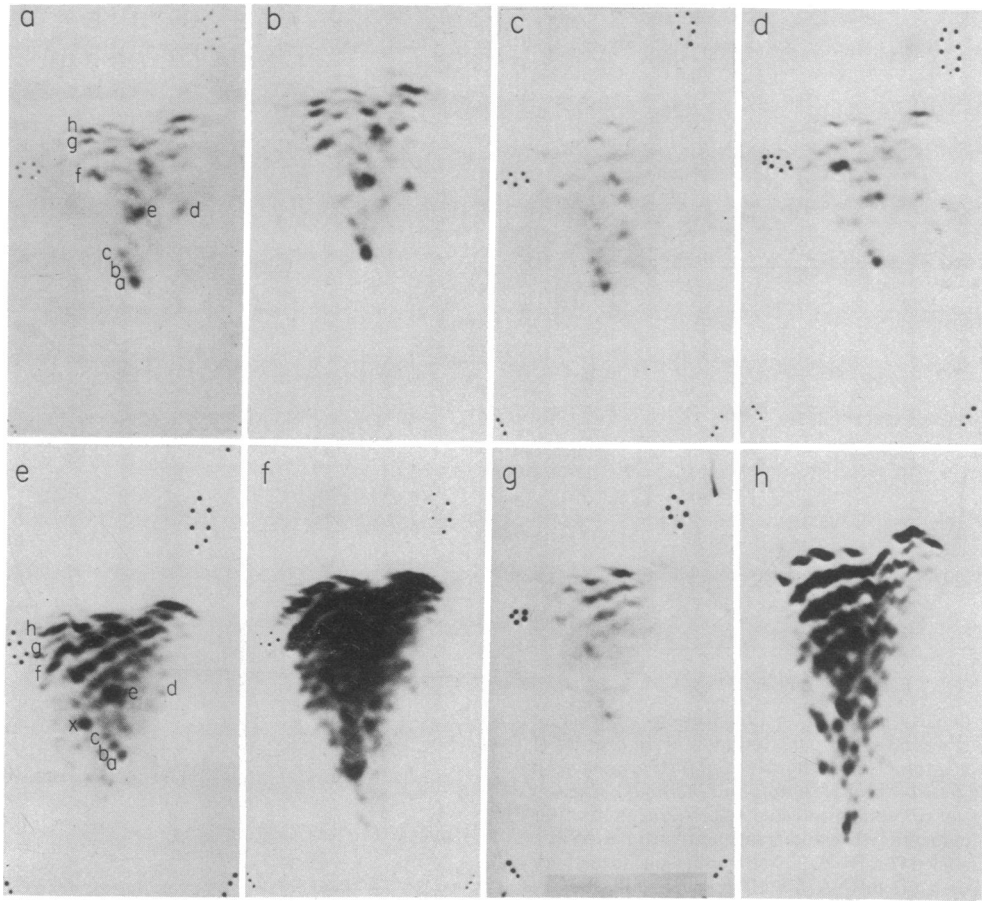

FIG. 3. Fingerprinting analysis of f1 mRNA and its ribosome-protected fragments. ^{32}P-Labeled f1 mRNA was synthesized and aliquots of about 10^8 cpm were subjected to ribosome binding as described in *Materials and Methods* and in the legend to Fig. 1. Aliquots of mRNA or the purified ribosome-protected fragments were subjected to RNase T1 digestion and two-dimensional fingerprinting analysis using a first dimension of high-voltage electrophoresis in pyridine/acetate/7 M urea, pH 3.5, on cellulose acetate and a second dimension of RNA homochromatography as described before (3, 15). In each panel an autoradiograph is shown in which the origin is at the lower right. The circle of black dots in the upper right represents the blue dye marker (xylene cyanol FF). (*a*) ^{32}P-Labeled f1 fragments protected by *E. coli* 70S ribosomes. The previously identified (3) spots a–h were eluted and subjected to secondary analysis (Table 1). (*b*) ^{32}P-Labeled f1 mRNA fragments protected by *E. coli* 30S ribosomes. Spots a–h were eluted and subjected to secondary analysis. (*c*) ^{125}I-Labeled f1 mRNA fragments protected by *E. coli* 70S ribosomes. In this case 5×10^6 cpm of ^{125}I-labeled mRNA was subjected to the ribosome protection reaction as described in the legend to Fig. 1. Fragments were purified from the 70S position of the sucrose density gradient and subjected to fingerprinting analysis. Oligonucleotides were eluted and subjected to secondary analysis as described (16). Of spots a–h, only a, b, c, and g contain C (Table 1) and are therefore subject to iodination. (*d*) ^{32}P-Labeled f1 mRNA fragments by use of translation-blocked *E. coli* ribosomes. The autoradiograph depicted here represents fragments of f1 mRNA protected by 70S *E. coli* ribosomes in the presence of thiostrepton. Spots a–h were located, eluted, and subjected to secondary analysis. (*e*) ^{32}P-Labeled f1 mRNA fragments protected by rabbit reticulocyte 80S ribosomes. Spots a–h were located, eluted, and subjected to further analysis (Table 1) together with spots a–h from *a*. The prominent spot labeled "x" has recently been shown also to map within one of the f1 ribosome binding sites (J. Ravetch, K. Horiuchi, and P. Model, unpublished data). (*f*) ^{32}P-Labeled f1 mRNA fragments protected by rabbit reticulocyte 40S ribosomal subunits. Spots a–h could not be located in this case. (*g*) ^{125}I-Labeled f1 mRNA fragments protected by rabbit reticulocyte 80S ribosomes. mRNA was added to this reaction in an amount identical to that used in *c*. However, the level of protection in this case was too low to allow secondary analyses. (*h*) ^{32}P-Labeled f1 mRNA. An aliquot (5×10^6 cpm) of unfractionated f1 mRNA was subjected to RNase T1 digestion and fingerprinting analysis as before (3).

select sites also recognized by *E. coli* ribosomes at least 20 times more frequently than would be expected on a random basis. In addition to the prominent series of oligonucleotides listed in Table 1, the protection of f1 mRNA by reticulocyte 80S ribosomes (Fig. 3*e*) yielded additional spots not found when *E. coli* ribosomes were used (Fig. 3*a*). This may represent some nonspecific protection or may result from the specific protection of the authentic initiator regions of cistrons that do not give rise to protected fragments with *E. coli* ribosomes. Whatever the origin of the extra spots, the major point of Fig. 3*e* and Table 1 is that reticulocyte 80S ribosomes recognize and specifically protect initiator regions for f1 genes IV, V, and VIII with an efficiency approaching that for globin mRNA.

Fig. 3*f* shows ^{32}P-labeled f1 mRNA fragments protected by reticulocyte 40S subunits. This fingerprint had a complexity approaching that of the fingerprint of intact f1 RNA (Fig. 3*h*) and, unlike the case of Fig. 3*e*, we cannot discern the specific spots indicated in Fig. 3*a*. Fig. 3*g* shows f1 [^{125}I]mRNA fragments protected by 80S reticulocyte ribosomes. This pattern has similarities to Fig. 3*c* (both contain spots a, b, c, and g), suggesting that iodinated f1 mRNA and f1 [^{32}P]mRNA bind similarly to eukaryotic ribosomes.

f1 mRNA in Rabbit Reticulocyte Lysates. In our hands, the f1 mRNA preparation used here stimulated little, if any, synthesis of completed f1-specific protein under conditions of protein synthesis in reticulocyte lysates (data not shown). This is not an inherent attribute of f1 mRNA in eukaryotic systems, because it, like other phage mRNAs (18–21), is properly

Table 1. RNase T1-resistant oligonucleotide sequences from bacteriophage f1 ribosome binding sites protected by *E. coli* 70S or rabbit reticulocyte 80S ribosomes

Spot; sequence[a]	f1 gene[b]	Pancreatic RNase products[c]		RNase U2 products[c]	
		E. coli 70 S	Rabbit 80 S	*E. coli* 70 S	Rabbit 80 S
(a) AAAC*U*UC*C*UC*AUG[A]	VIII	AAAC* A*U C, U	AAAC* A*U C, U	C*U*UC*C*UCA*[d]	C*U*UC*C*UCA*[d]
(b) UAA*U*UCACA*AUG[A]	V	AA*U*[e] AA*U[e]	AA*U*[e] AA*U[e]	*UUCA A	*UUCA A
(c) UAA*U*UCAA*AUG[A]	IV	AAA*U AA*U*	AAA*U AA*U*	*UUCA A	*UUCA A
(d) AAAAAG*[U]	VIII	AAAAAG*[f]	AAAAAG*[f]	G	G
(e) A*U*UAAAG*[U]	V	AAAG*[g] A*U*	AAAG*[g] A*U*	*UUA[h] A, G	*UUA[h] A, G
(f) U*U*UAA*UG[G]	VIII	AA*U[i] U	AA*U[i] U	U*U*UA[j] A	U*U*UA[j] A
(g) UC*U*U*U[A]	VIII	U, C	U, C	UC*U*U*U[k]	UC*U*U*U[k]
(h) *UUG[A]	V	U	U	*UUG[l]	*UUG[l]

[a] Oligonucleotides are identified by the letters indicated in Fig. 3 *a* and *e*. Sequences are as determined by Pieczenik *et al.* (3) with asterisks indicating those bases that should be labeled by a nearest neighbor U (because RNA was labeled with UTP).

[b] The f1 genes that encode these ribosome binding sites are genes VIII [the major coat protein (3)]; V [the DNA binding protein (3)]; and IV (J. V. Ravetch, K. Horiuchi, and P. Model, unpublished data). Spots a, b, and c are the three largest RNase T1-resistant oligonucleotides protected by ribosomes in f1 mRNA. They are 13, 12, and 11 bases long, respectively, and each contains the AUG initiator triplet for a different gene mRNA (underlined), and the recovery of spots *a–c* in good yield is thus a reliable assay for ribosome protection of the gene IV, V and VIII binding sites.

[c] Oligonucleotides were eluted as indicated from the fingerprints shown in Fig. 3*a* (*E. coli* 70S ribosomes) and Fig. 3*e* (rabbit reticulocyte 80S ribosomes) and subjected to pancreatic RNase digestion and fractionation by electrophoresis at pH 3.5 on DEAE-paper (3, 15) or RNase U2 digestion and fractionation by electrophoresis at pH 1.9 on DEAE-paper (3, 15). The resulting products were in turn eluted and their base compositions were determined (3, 15). The nucleotide residues found to be labeled are indicated by asterisks.

[d] This RNase U2 product was identified by mobility on electrophoresis at pH 1.9 and by labeling pattern. By comparison to the xylene cyanol FF blue marker, both the *E. coli* 70S oligonucleotide and that from rabbit 80S ribosomes had an R_F of 0.11.

[e] AAU was identified by mobility on electrophoresis at pH 3.5; the presence of 1 mol each of AA*U* and AA*U in both the *E. coli* 70S and rabbit 80S sites was confirmed by the finding of a 2:1 ratio of A to U after base composition analysis of the AAU pancreatic RNase product.

[f] AAAAAG was identified by mobility with respect to the blue marker (0.025 for the 70S *coli* sites; 0.03 for the rabbit 80S sites). The number of As was previously determined by Pieczenik *et al.* (3).

[g] AAAG* was identified by labeling pattern and relative mobility on electrophoresis at pH 3.5 (R_F = 0.10 with respect to the blue marker for the 70S sites; R_F = 0.095 for the 80S sites).

[h] U*UA was identified by labeling pattern and relative mobility at pH 1.9 (0.49 for 70S sites; 0.52 for 80S sites).

[i] AA*U was identified by labeling pattern and relative mobility (0.312 for 70S fragments; 0.288 for 80S fragments).

[j] U*U*UA was identified by composition and by relative mobility (70S sites, 0.177; 80S sites, 0.18).

[k] UCUUU was identified by relative mobility (70S sites, 0.067; 80S sites, 0.071). The sequence was previously determined (3).

[l] UUG was identified by relative mobility (70S sites, 0.27; 80S sites, 0.28).

translated by some eukaryotic cell extracts (P. Model and G. Blobel, unpublished observations). Because the authentic initiator regions of f1 mRNA have been identified directly by RNA sequence analysis (ref. 3 and Table 1), the experiments described here depend only upon ribosome recognition of these RNA regions and not upon translation.

DISCUSSION

In this paper we have used a prokaryotic mRNA (phage f1 mRNA) and a eukaryotic mRNA (globin mRNA) in ribosome binding systems from *E. coli* and rabbit reticulocytes. The globin mRNA was labeled with ^{125}I but controls with ^{125}I-labeled f1 mRNA indicated that iodination was without effect on ribosome binding in either system. We chose to assay for initiation by looking for ribosome-protected fragments rather than by assaying the formation of initiation dipeptides, as others have done (17).

mRNA Protection by the Small Ribosomal Subunits. We noted previously (4, 5) the ability of reticulocyte 40s subunits to protect a larger fragment of the initiation region of globin mRNA than did the 80S ribosomes, and subsequent work with reovirus mRNA and wheat germ ribosomes (22) suggests that this may be a general phenomenon with 40S subunits. In contrast, we have found that *E. coli* 30S subunits can protect initiation regions in f1 mRNA that are almost identical in size and composition to the corresponding regions protected by the 70S ribosomes. Most strikingly, when the regions of f1 mRNA protected by reticulocyte 40S subunits were analyzed, they were found to be much larger and more heterogeneous (Fig. 2, slot 8) compared with 80S-protected regions. If these fragments are generated by the same mechanism as the specific 40S-protected fragments of globin mRNA, this extra protection may be produced either by the binding of additional proteins (initiation factors?) or may result from the ability of the bound RNA to wind around the small subunit in some fashion.

Ribosome Recognition Signals in mRNA. We have found that reticulocyte ribosomes can bind to and protect authentic initiation regions in f1 mRNA whereas *E. coli* ribosomes do not respond to rabbit globin mRNA. This requires that we recognize a number of features (at least two) within an RNA that can lead to ribosome attachment and that at least one of these is common

to prokaryotes and eukaryotes. Such features and their possible relevance to our findings are as follows:

(*i*) A genuine initiation site must contain an initiation codon, and the structure of the RNA must be such as to make the initiation region available to initiator tRNA and ribosomes (23). Further requirements are defined by the failure of *E. coli* ribosomes to recognize the globin mRNA initiation site.

(*ii*) Many eukaryotic mRNAs, including rabbit globin mRNA (24), are "capped" and this appears to have an important influence on ribosome attachment (ref. 24, but see also ref. 25). This might restrict initiation to a 5′-terminal region and would, if it were the only specific initiation signal in globin mRNA, account for the failure of *E. coli* ribosomes to bind to this mRNA. It is clear that a "cap" is not essential for binding by eukaryotic ribosomes because some mRNAs are not "capped" [e.g., polio mRNA (26, 27)], and our data suggest that a nearby 5′ terminus also is not essential because one of the three initiation sites in f1 mRNA to which the reticulocyte ribosomes bind (gene V) is at least 500 bases from the nearest strong promoter (28–31). However, we cannot exclude limited cleavage of the mRNA to expose new 5′ termini.

(*iii*) Some other form of interaction must be invoked to explain the specific binding of reticulocyte ribosomes to initiation sites in f1 mRNA. For example, as has been postulated for prokaryotic 16S rRNA and mRNA (32, 33), the 3′ end of 18S rRNA (A-U-C-A-U-U-A_{OH}, ref. 34) may interact with some sequence in the initiation site. In three eukaryotic initiation sites (5, 35, 36), this sequence is complementary to the initiation codon and surrounding bases, giving a potential five or six base pairs if G:U pairs are included. Each of the three major f1 initiation regions could make this interaction, although its importance, if any, to the initiation process is as yet uncertain. The other possible interaction with the 3′ end of 18S rRNA would invoke base pairing to a U–A–A–U sequence in the mRNA. This sequence, contained in all three f1 ribosome-protected fragments (3), is present in brome mosaic virus species 4 RNA (35), and a related sequence was noted in simian virus 40 (36).

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