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Differential requirements for polypeptide chain initiation complex formation at the three bacteriophage R17 initiator regions

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ABSTRACT

The initiation specificity of washed E. coli ribosomes in the presence and absence of purified initiation factors and/or S1 protein has been examined in protection experiments using ^{32}P-labelled R17 RNA. We find that the three bacteriophage initiator regions do not exhibit equal requirements for either of these components during initiation complex formation. Specifically, both factors and S1 stimulate ribosome binding to the beginnings of the coat and replicase cistrons to a greater extent than they promote recognition of the A protein initiation site. The differential effects are therefore inversely correlated with the degree of mRNA·16S rRNA complementarity exhibited by the three initiator regions. We also observe that S1 suppresses ribosome binding to spurious sites in the R17 RNA.

INTRODUCTION

There now exists substantial evidence that an important component of messenger RNA recognition by E. coli ribosomes is an RNA·RNA interaction.[1,2] During formation of a polypeptide chain initiation complex, direct hydrogen bonding apparently occurs between the pyrimidine-rich 3' terminus of 16S ribosomal RNA and a polypurine stretch common to initiator regions in E. coli and bacteriophage mRNAs.

The initiation factors are other well-known contributors to the early stages of translation.[3,4] IF_2 is required for positioning the formylmethionyl tRNA on the ribosome and for subsequent GTP hydrolysis.[5-7] IF_3 is regarded as essential for initiation using natural mRNAs.[8] It is also required for ribosome binding of fMet-tRNA directed by initiator triplets[3,7,9] and will stimulate translation of all synthetic messengers.[10] IF_1 functions in the recycling of IF_2.[11]

Ribosomal protein S1 has likewise been implicated in the specific binding of natural mRNAs to the 30S ribosome. Whereas ribosomes lacking S1 can utilize AUG as messenger, intact phage RNA is bound and translated efficiently only by S1-containing ribosomes.[12-17] Chemical crosslinking data locate S1 in the mRNA binding site of the 30S ribosome,[18] directly adjacent

to the 3' end of 16S rRNA.[19,20] The work of Dahlberg and Dahlberg[21] suggests that this protein may interact specifically with the pyrimidine-rich terminal dodecanucleotide of the rRNA molecule. Physical[22-24] and other studies[17,25-30] indicate that S1 is a polynucleotide-binding protein with high affinity for pyrimidine-rich single-stranded regions in RNA.

Previously we observed that a crude ribosomal wash, which is now known to contain both initiation factors and S1,[31-33] differentially stimulates ribosome recognition of the beginnings of the three bacteriophage R17 cistrons.[34] Specifically, greatest dependence was exhibited by the coat protein gene, which is predicted to form the weakest mRNA·rRNA interaction; least dependence was observed at the A protein initiator region, which has the longest potential mRNA·rRNA complementarity (see Table 1).

To ask which component of the ribosomal wash is responsible for these differential effects in initiation complex formation, we have now repeated R17 RNA binding experiments using purified initiation factors and S1. We find that S1, by itself, does not influence the selection of the A protein initiator region by the ribosome; but in the presence of initiation factors, it stimulates recognition of the coat and replicase initiators relative to the A site. Conversely, initiation factors greatly enhance binding efficiency at the beginnings of the coat and replicase genes, but only when S1 is present. In addition, we observe that S1 decreases ribosome protection of spurious sites in the R17 RNA.

MATERIALS AND METHODS

A. Buffers:

A: 20 mM Tris, 10 mM Mg^{++} acetate, 100 mM NH_4Cl, 6 mM 2-mercaptoethanol, pH 7.5

B: A containing 850 mM NH_4Cl

C: B containing 18% sucrose

B. Ribosomes:

E. coli MRE600 was grown to late log phase in the medium of Weismeyer and Cohn.[35] Ribosomes were prepared according to Staehelin, Maglott and Monro.[36] Subunits were derived as described earlier[37] by 2 cycles of sucrose gradient centrifugation; after each cycle the subunits were concentrated by making the solutions 10 mM Mg^{++}, 20 mM Tris, 100 mM NH_4Cl and pelleting 4-5 hours at 60,000 rpm in a Spinco Ti60 rotor.

30S(-S1) particles were prepared by two methods.

a) Starting with 70S, the subunit preparation proceeded as for 30S ribosomes, except that before the concentration step (which follows each

Table 1. Complementarity of R17 Initiator Regions to 16S rRNA

A protein	GAU UCC UAG GAG GUU UGA CCU *AUG* CGA GCU UUU AGU
coat protein	CC UCA ACC GGG GUU UGA AGC *AUG* GCU UCU AAC UUU
replicase	AA ACA UGA GGA UUA CCC *AUG* UCG ACA ACA AAG
16S RNA 3' end	HO^{A} U U C C U C C A C U A $G_{5'}$

Sequences of the regions surrounding the three R17 initiator codons (italics) are from Steitz.[58] The 3' terminal sequence of 16S rRNA is from Shine & Dalgarno,[1] Noller & Herr,[59] Ehresmann et al.[60] and Sprague & Steitz.[61] Complementarity is indicated by underlining; dots denote G·U base pairs. A quantitative estimate of the relative stabilities of the mRNA·rRNA complexes is given by ΔG*, which is -15.8 kcal/mole for the A site, -9.0 kcal/mole for the replicase site and -8.4 kcal/mole for the coat protein initiator region. Here ΔG* is the free energy of formation of a double helical structure calculated according to Gralla & Crothers,[62] since the positive free energy of nucleation (resulting from the bimolecular nature of the mRNA·rRNA interaction) has not been included, these values should be considered only in a relative sense.

cycle of sucrose gradient centrifugation) the 30S solution was adjusted to 10 mM Mg^{++}, 20 mM Tris, 850 mM NH_4Cl and diluted with buffer B until the 30S ribosome concentration reached either 14 A_{260}/ml (after the first cycle) or 5-6 A_{260}/ml (after the second cycle). The diluted 30S particles were then pelleted (45,000 rpm for 16 hours in the Ti60 rotor) through 0.77 volumes of buffer C. The resulting 30S(-S1) preparation (which is stored in buffer A) contained 2-3% S1, as judged by comparing the area under the S1 peak with that in the S2-S21 peaks in an SDS polyacrylamide gel profile.

b) Alternatively, 30S(-S1) particles were prepared from 30S ribosomes by the method of Tal et al.[25] The 30S subunits were dialysed against 1 mM Tris HCl, pH 7.5, over a 24 hour period, during which the reservoir solution was changed 4 times. The resulting S1 content was 0.6%.

C. S1:

Two kinds of S1 preparations were used.

a) Crude S1 was obtained by fractionating LiCl-urea extracted 30S proteins on CM cellulose at pH 5.6 in 6 M urea, 30 mM methylamine-acetate, 6 mM 2-mercaptoethanol.[38] S1, which appears in the flow-through fraction, was further purified by filtration on Sephadex G100 in the same buffer. Finally, the S1 was dialyzed against 1 M NH_4Cl, 10 mM Mg^{++}, 10 mM Tris, pH 7.5, 3 mM 2-mercaptoethanol, and stored at -70°C.

b) S1 was isolated as described by Miller et al.[26] with the addition of a polyC cellulose column as detailed in the accompanying paper.[12]

Both types of S1 preparations were homogeneous and free of detectable protein contamination when run on SDS gels at high loading (10 µg protein per disc gel). It is also highly unlikely that ribonuclease contamination is significant, since S1 stimulates (rather than decreases[39]) ribosome recognition of the R17 coat and replicase initiator regions.

D. Initiation Factors:

Factors were prepared from the 1 M NH_4Cl ribosomal wash as described by Wahba & Miller[40] and the purity of each was assessed by SDS polyacrylamide gel analysis.

E. Cistron Specificity Assay:

R17 RNA was bound to ribosomes in 40 µl reaction mixtures containing: 100 mM Tris HCl, pH 7.5; 50 mM NH_4Cl; 9 mM Mg acetate; 0.25 mM GTP; 3.5 mM 2-mercaptoethanol; 1.0 A_{260} units of charged formylated mixed E. coli tRNA; 2.5 A_{260} units 50S ribosomal subunits; and 0.8 A_{260} units ^{32}P-labelled R17 RNA (specific activity = 1 - 4 x 10^6 cpm/µg). 1.3 A_{260} units of 30S ribosomes or 30S(-S1) particles and 0.4 µg of IF_1, 0.5 µg of IF_2, 2.3 µg of IF_3 or 70 µg of crude initiation factors were added as indicated. Optimal amounts of initiation factors per A_{260} unit of ribosomes were predetermined by assaying R17 RNA-directed fMet-tRNA binding. The molar ratio of ribosomes to R17 RNA in the initiation reactions was approximately 2:1, suggesting that there may be some competition of mRNA binding sites for available ribosomes. 30S(-S1) particles were reconstituted with S1 before addition to initiation reactions by incubating 2.6 A_{260} units of 30S(-S1) ribosomes prepared by method a) with 15 µg S1 (1:2 molar ratio) in 30 µl containing 20 mM Mg^{++}, 150 mM NH_4Cl, 15 mM Tris HCl, pH 7.5, 4 mM 2-mercaptoethanol for 4 min at 37°C.

After incubation of reaction mixtures at 38°C for 8 minutes, the initiation complexes were trimmed with pancreatic ribonuclease and fractionated on sucrose gradients as previously described.[39] The five fractions including the 70S peak, as determined by direct Cerenkov counting, were pooled and the RNA extracted. RNase T1 fingerprints were produced by pH 3.5 electrophoresis on cellogel in the first dimension and chromatography on PEI plates (Brinkmann, Cel 300 PEI) using homomix c[41] in the second. Oligonucleotides were quantitated by counting in toluene scintillation fluid and were subsequently analyzed by digestion with pancreatic RNase to confirm their identity and purity. Ratios of the three R17 initiator regions were calculated from the cpm/PO_4 in spot *1* (ACCUAUG) from the A site, spot *6* (CAUG) from the coat site, and spot *8* (AUUACCCAUG) from the replicase site.

All other oligonucleotides labelled in the figure were routinely quantitated to provide substantiating data and to insure that differential oligonucleotide blotting efficiencies did not greatly affect the results.

RESULTS

A. Factors and S1 Differentially Stimulate Recognition of the Three R17 Sites

To assess ribosome recognition of the three R17 initiator regions, we used a previously described assay.[39] It involves ribosome binding to ^{32}P-labelled phage RNA in a protein synthesis initiation reaction, trimming of the resulting 70S complexes with nuclease, and fingerprinting of the protected regions of mRNA. The relative yields of the three R17 initiation sites are calculated from the cpm/PO_4 in the initiator AUG-containing oligonucleotides (spots *1*, *6* and *8* in Figure 1); however, all other products labelled in the figure are quantitated and analyzed to substantiate the validity of the ratios obtained. The ^{32}P-mRNA utilized is somewhat degraded by autoradiolysis, allowing ribosome binding to the beginning of all three phage cistrons. Although this initiation assay does not monitor the same molecular events as fMet-dipeptide synthesis,[42] it has been previously demonstrated that the two assays yield similar results.[39,43,44]

Four separate experiments, each of which tested the initiation specificity of various combinations of 30S ribosomal subunits, initiation factors and S1 protein, are reported in Table 2. In experiments I, II and IV stoichiometric amounts of IF_2 were used, effectively substituting for the catalytic contribution of IF_1 to the formation of initiation complexes. All initiation factor and S1 preparations were judged to be at least 85% pure (see Materials and Methods). Representative fingerprints [from Experiment IV (Table 2)] are shown in Figure 1.

If we first examine the effect of purified initiation factors on reactions containing native 30S ribosomes (Ia compared to Ib, IIb versus IIc, and IIIa versus IIIb), we see that relative to the A site, binding to the coat protein initiator region is stimulated 4- to 18-fold (average 7-fold) and to the replicase site 2- to 10-fold (average 5-fold). Comparable results are observed with 30S(-S1) particles reconstituted with S1 before use (Ic versus Id, IId versus IIe, IVa versus IVb, and Figure 1a versus 1b). Thus we conclude that the initiation factors themselves make a substantial contribution to the differential stimulation of initiation at the three R17 sites first observed with the crude ribosomal wash (experiment IIa versus IIc).

Table 2 also documents the dependence on S1 for ribosomal recognition of the three R17 initiator regions. The native subunits (30S) contained

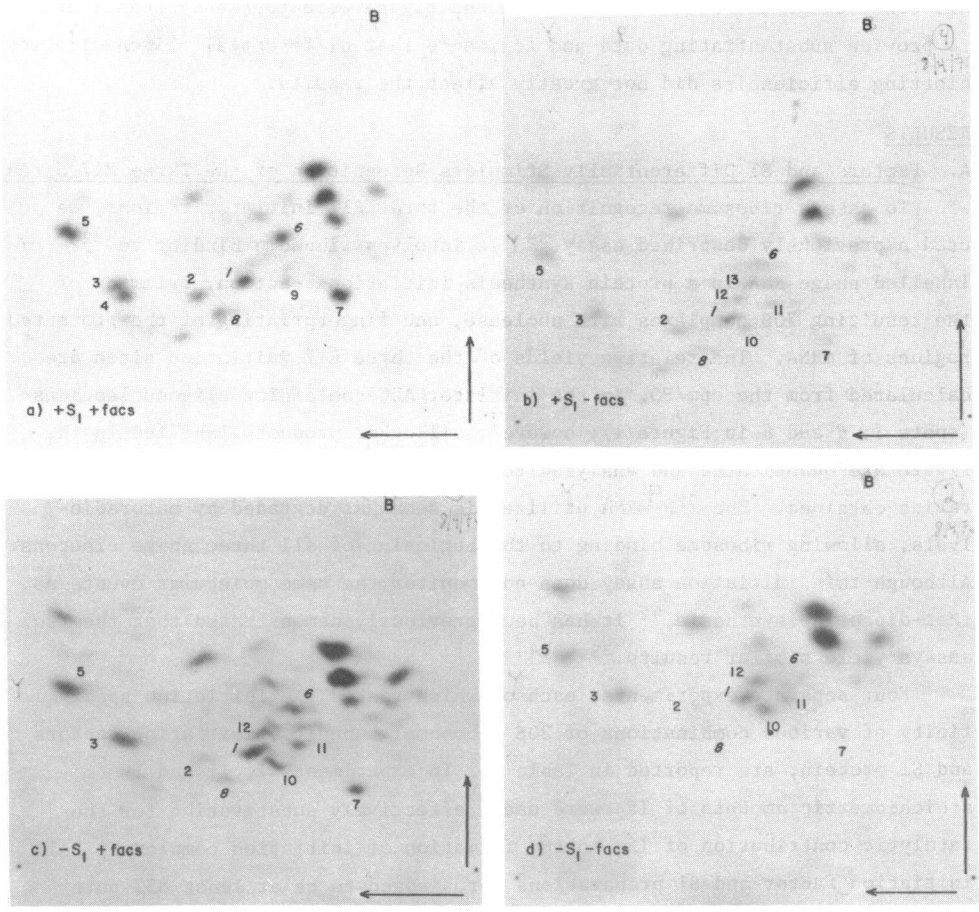

Figure 1. R17 sites protected by ribosomes in the presence and absence of initiation factors and S1. T1 RNase fingerprints are from Experiments IV, Table 2. Electrophoresis was from right to left and chromatography from bottom to top. B indicates the position of the blue marker dye. The three prominent unlabelled spots running vertically below the blue dye are G, AG, and AAG. Labelled oligonucleotides are: *1* = ACCUAUG, 2 = AUUCCUAG, 3 = CUUUUAG, and 5 = UUUG from the A site; *6* = CAUG, 4 = CUUCUAACUUU, 5 = UUUG, and 7 = CCCUCAACCG from the coat site; *8* = AUUACCCAUG and 9 = AAACAUG from the replicase site; and 10 = (AU,C_xU_x)AAG, 11 = (C,AU)AAG, 12 = ($C_{\sim 3}$,U_2)G, and 13 = AUAAG from unknown regions of the R17 RNA.

approximately 0.5 moles of S1 per particle. 30S(-S1) ribosomes were prepared by two different methods (see Materials and Methods) which gave comparable results. The mRNA recognition capacity of S1-containing versus S1-depleted ribosomes in the presence of factors is compared in reactions Ia versus Ie, IIb versus IIf, and IIIa versus IIIc. It is apparent that the S1 requirement is greater for binding at the coat and replicase initiator

Table 2. Effect of S1 and Factors on Recognition of R17 Initiator Regions

Experiment	Ribosomes	Factors	S1	Ratio A : coat : replicase	cpm/PO_4 in A site	total cpm in 70S peak
I a	30S	IF_2,IF_3	-	1 : 2.0 : 0.5	22	12,200
b	30S	-	-	1 : 0.2 : 0.05	33	9,400
c	30S(-S1)	IF_2,IF_3	+	1 : 1.3 : 0.4	31	15,000
d	30S(-S1)	-	+	1 : ∿0.2 : 0.1	17	9,500
e	30S(-S1)	IF_2,IF_3	-	1 : ∿0.3 : 0.04	36	11,200
f	30S(-S1)	-	-	1 : ∿0.4 : 0.03	18	8,900
II a	30S	crude	-	1 : 7.1 : 2.7	29	12,400
b	30S	IF_2,IF_3	-	1 : 3.6 : 0.8	19	7,700
c	30S	-	-	1 : ∿0.2 : 0.2	21	3,700
d	30S(-S1)	IF_2,IF_3	+	1 : 1.2 : 0.6	55	7,300
e	30S(-S1)	-	+	1 : ∿0.4 : 0.2	9	3,000
f	30S(-S1)	IF_2,IF_3	-	1 : 0.8 : 0.2	53	5,500
g	30S(-S1)	-	-	1 : ∿0.1 : 0.06	29	4,300
III a	30S	IF_1,IF_2,IF_3	-	1 : 0.8 : 1.0	100	26,300
b	30S	-	-	1 : ∿0.2 : 0.5	41	22,400
c	30S(-S1)	IF_1,IF_2,IF_3	-	1 : ∿0.1 : 0.2	87	20,100
d	30S(-S1)	-	-	1 : ∿0.1 : 0.2	11	6,400
IV a	30S(-S1)	IF_2,IF_3	+	1 : 1.5 : 0.4	105	9,100
b	30S(-S1)	-	+	1 : 0.3 : 0.1	28	3,100
c	30S(-S1)	IF_2,IF_3	-	1 : 0.9 : 0.15	80	6,500
d	30S(-S1)	-	-	1 : ∿0.2 : 0.06	44	4,900

Experiments were performed and analyzed as described in Materials and Methods. Experiments I, II and IV utilized 30(-S1) particles prepared by method a); in Experiment III, they were prepared by method b). S1 was prepared by method a) in Experiment I and method b) in Experiments II and IV. ∿ indicates that the CAUG spot (initiator oligonucleotide for the coat site) appeared contaminated by other oligonucleotides in the final analysis; the values given have therefore been adjusted accordingly. Note that the total cpm quantitated in the A site oligonucleotide is 7-fold the cpm/PO_4 value appearing in column 6, since ACCUAUG contains 7 phosphates. ACCUAUG (spot 1) was invariably greater than 90% pure. Machine background values ranging from 12-15 cpm were subtracted from the total before determining the cpm/PO_4.

regions than at the A site. Average 6- to 8-fold stimulations are obtained at the two former relative to the latter initiator region.

To rule out the possibility that the above effects resulted not from the absence of S1 but from the removal of other ribosomal components during the preparation of 30(-S1) subunits, we also examined mRNA binding to ribosomes which had been reconstituted by preincubation of 30(-S1) particles with purified S1 protein. Comparison of the data from reactions Ic with Ia, IId with IIb, and IVc with IVa (also see Figure 1a and 1c) shows that S1 can

significantly restore recognition of the coat and replicase sites relative to the A site. The differential stimulatory activity of S1 protein itself is thereby confirmed.

Finally, it is interesting to examine the interdependence of initiation factors and S1 in our reactions. Except in experiment II, the addition of factors in the absence of S1 produces much less relative stimulation of ribosome binding to the coat or replicase initiation sites than when factors are added in the presence of S1. Conversely the effect of S1 is usually more pronounced in the presence than in the absence of factors (here experiments I and IV are more representative than experiment II). With intact R17 RNA, stimulation of initiation (presumably at the coat cistron) by S1 is also observed only in the presence of factors.[12] Thus, it appears that factors and S1 contribute in different ways to ribosome recognition of mRNA initiator regions. One cannot substitute completely for the other; both are required to obtain maximal stimulation (see especially Figure 1a).

An alternative explanation for the altered ratios of the three R17 initiator regions recovered in the experiments of Table 2 is that S1 (or initiation factors) somehow actively depress recognition of the A site and thereby only apparently stimulate binding to the other two sites. Arguments presented in the next section make this possibility highly unlikely, although under the assay conditions of Table 2 competition between initiator regions for available ribosomes (see Materials and Methods) may in fact have produced lower absolute protection of the A site in some reactions.

B. Minimal Stimulation by Factors and S1 at the R17 A Site

Although the relative ribosome recognition of the three R17 initiator regions is easily assayed, it is much more difficult to determine the absolute magnitude of dependence on initiation factors and S1. Because several transfers are involved in the preparation of the protected sites for analysis, the cpm/PO_4 in A site oligonucleotides (Table 2, column 6) do not accurately reflect yields in the various reactions. Likewise the total radioactivity obtained in the trimmed 70S initiation complexes (column 7) does not provide a straightforward measure since the ratio of the three sites changes and spurious sites on the R17 RNA (see below) may be bound with varying efficiency. As an alternative, the ability of ribosomes to recognize the isolated A protein initiator region can be measured directly in separate experiments, either by fractionating initiation reactions in sucrose gradients[39] or by examining the appearance of an mRNA·rRNA complex on polyacrylamide gels.[2]

Previously, the ability of purified initiation factors to stimulate binding of the isolated R17 A protein initiator region to ribosomes containing S1 was examined by gradient fractionation of initiation reactions; 2- to 3-fold effects were observed.[39] Similarly, the addition of S1 has produced from 1- to 5-fold increases in ribosome rebinding of the A fragment in both gradient and gel experiments (J.A. Steitz, unpublished results). Since it seems likely that the isolated A site behaves comparably to the same region when it is a part of whole R17 RNA, we conclude that ribosome recognition of the A site is stimulated somewhat by initiation factors and by S1. Consequently, the absolute dependence on factors or S1 protein for ribosome binding to the R17 replicase and coat protein initiator regions may be several-fold higher than the relative values reported in Table 2.

C. S1 Decreases Ribosome Binding to Spurious Sites in R17 RNA

The fingerprints of Figure 1 contain not only spots derived from the three authentic initiator regions in the R17 RNA (see Table 1) but other large oligonucleotides as well (numbers 10,11,12,13). These are prominent only in the ribosome-protected RNA from reactions where S1 was not present (Figure 1c and d); they are barely detectable in maps from reactions containing either 30S(-S1) subunits reconstituted with S1 (Figure 1a and b) or native 30S subunits (not shown). Although these same oligonucleotides have been seen in variable yield in many previous ribosome protection experiments,[45] neither their location in the R17 RNA nor the reason why they are bound by ribosomes is known.

Does the presence of S1 actively depress recognition of these spurious sites, or does it simply increase relative ribosome binding at the three true initiator regions? Again, interpreting the data is not straightforward. Figure 1 (a and b versus c and d) shows that the yields of these oligonucleotides compared to those from the R17 A site are decreased in reactions containing S1. Since the total radioactivity bound is not stimulated greatly by S1 (Table 2, column 7), this suggests that the absolute amounts of the spurious nucleotides are lower in the presence than in the absence of S1. Alternatively competition for available ribosomes may again be a factor. In any case, it is clear that the addition of initiation factors does not suppress recognition of the spurious site(s) (Figure 1c) and that this activity of S1, is observable in the absence of factors (see Figure 1b versus 1d).

DISCUSSION

We have found that ribosome recognition of different mRNA initiator regions is not equally dependent upon the presence of initiation factors and

ribosomal protein S1. Specifically, binding to the phage R17 A protein initiator region, which is highly complementary to the 3' end of 16S rRNA, is nearly independent of both S1 and factors. In contrast, the addition of S1 and factors greatly enhances the recognition of coat and replicase initiator regions, which exhibit lower complementarity to rRNA. Neither S1 nor factors stimulate significantly in the absence of the other. S1 can also suppress binding to certain non-initiator regions in the R17 genome.

A. S1 Function

Our conclusion that S1 is differentially required for recognition of the three R17 initiator regions is in agreement with previous observations. In the cases where one copy of S1 per ribosome was reported to be essential for ribosome binding and translation of RNA phage messenger, intact RNA was used;[12-17] since such molecules direct coat protein synthesis almost exclusively,[39,46] it is not surprising that a large dependence on S1 was observed. In addition, Isono and Isono[47] recently reported that synthesis of the f2 replicase and coat protein by E. coli ribosomes is more dependent on S1 than translation of the f2 A protein, similar to our results.

Together with the many observations that S1 interacts strongly with RNA molecules, our data suggest that S1 may facilitate the formation of or stabilize hydrogen bonding between the 16S rRNA and the mRNA. Three different models can be considered. 1) S1 could use its "RNA unwinding" activity[22-24] to disrupt internal rRNA secondary structure, thereby exposing bases near the 3' terminus of the 16S molecule for interaction with the complementary sequence in the mRNA.[21] S1 would be expected to be most essential when the intramolecular rRNA structure is to be replaced by a relatively weak mRNA·rRNA interaction. 2) Alternatively, S1 could act directly on the mRNA, disrupting its secondary structure to allow subsequent interaction of the initiator region with various components of the ribosome.[48] Perhaps fortuitously, the sequences surrounding the R17 coat protein and replicase initiator regions can be folded into theoretically stable hairpin loops whereas no such structures can be drawn in the direct vicinity of R17 A protein initiation site.[39] Hence, our results are also consistent with this idea for S1 function. 3) Since an interaction of S1 with double-stranded polynucleotides as well as single-stranded RNA has been detected,[22] S1 could function to stabilize the mRNA·rRNA base pairs, once formed. Here too, the S1 requirement would be greatest for ribosome binding to initiator regions with minimal complementarity.

At this time, the weight of experimental evidence does not overwhelming

favor any one of the three above hypotheses. However, possibilities 1) and 3) appear most likely in light of recent physical studies on the isolated colicin fragment from the 3' end of 16S rRNA.[49] The intramolecular rRNA base pairs which would need to be disrupted exhibit a Tm of 21°C in buffers approximating physiological conditions and are in fact opened by the addition of S1 (R. Yuan, personal communication). Thus, although an external melting activity may not be required simply to expose this portion of the 16S sequences, S1 may align the bases in a specific configuration, facilitating stable interaction with the mRNA.

On the other hand, the observation that S1 can be photo-crosslinked at multiple sites to poly U on 70S ribosomes[18] would fit with an mRNA unwinding function for S1 (model 2). Van Dieijen et al.[48] interpret their results that S1 is not required for translation of formaldehyde-unfolded MS2 RNA in this way. However, their observations are also compatible with idea 1) or 3); formaldehyde may well expose internal initiator triplets which happen to be preceded by strong polypurine tracts and therefore can bind to ribosomes without S1. In addition, it is difficult to see how S1 could depress recognition of non-initiator regions simply by unfolding the phage mRNA.

Since physical studies suggest the existence of multiple independent polynucleotide binding sites on S1 protein,[23] perhaps it is most reasonable to assume that S1 fulfills two or more of the above roles in stimulating mRNA recognition by ribosomes.

B. Contribution of Initiation Factors

Differential requirements of the three R17 cistrons for initiation factors can also be interpreted in several ways. One attractive idea is that the phenomenon is related to the role of factors in facilitating the fMet-tRNA·initiator codon interaction in the P site of the ribosome.[3,4] IF_2 certainly functions in this capacity. Although IF_3 is often regarded to have some special role in recognizing natural mRNAs, its ability to promote translation of synthetic polynucleotides[10] or ribosome binding of fMet-tRNA in the presence of initiator triplets[3,7,9] underscores its general importance to all initiation events.

If we now consider the mRNA·rRNA interaction and the fMet-tRNA·anticodon interaction to be the two primary contributors to mRNA recognition by ribosomes, it is reasonable that the three R17 cistrons might be differentially dependent on initiation factors. The high degree of complementarity between the A site and the 16S 3' end may provide sufficient binding energy

and therefore substitute for the usual factor requirements. Conversely, the coat site with its weak complementarity might be expected to require the presence of initiator tRNA in order for stable interaction with the ribosome to occur. Indeed, the results of Jay and Kaempfer[50] suggest that fMet-tRNA must be positioned on the 30S subunit before intact R17 RNA (i.e., the coat initiator region) can be bound and phased correctly; by contrast, ribosome binding at the isolated R17 A protein initiator region is only slightly stimulated by fMet-tRNA.[39]

In this context it is also interesting to reexamine the contribution of another 30S ribosomal protein known to play a role in initiation. S12 has been identified by Held et al.[51] as at least partially responsible for species-specific translational differences between ribosomes of B. stearothermophilus and E. coli. In single replacement ribosome reconstitution experiments, inefficient R17 translation - which most likely represents synthesis of A protein only - was transfered from B. stearothermophilus 30S subunits to any reconstituted particle which contained thermophilic S12 protein. S12 is also one of the 30S proteins which can be chemically cross-linked to initiation factors IF_2[52] and IF_3[53,54] on the E. coli ribosome. Thus, the heterologous S12 could alter the association of the factors with the ribosome, producing in turn the same differential affects on recognition of initiator regions as we report here.

C. Specificity in Translational Systems

One source of specificity in ribosome recognition of mRNA initiator regions is presumably the extent of the complementarity between the mRNA and the 16S rRNA. Indeed, the finding of Shine and Dalgarno[55] that different bacterial species have slightly different pyrimidine tracts at their 16S 3' ends has lately been regarded as the key to understanding translational specificity differences among prokaryotes. However, our observations strongly suggest that proteins may be equally potent determinants in the selection of initiator regions by ribosomes. In fact, E. coli-B. stearothermophilus reconstitution studies[44] in which the ability of the ribosome to recognize the three R17 cistrons was assayed directly did show the protein fraction, rather than the 16S rRNA, to be the primary specificity determining component. Clearly, if B. stearothermophilus ribosomes differ from those of E. coli both in their S12[51] and as recently suggested[56] in their S1, it is no surprise that their ability to recognize R17 initiator regions is limited to the A site.

It is possible that our findings with the three R17 initiation sites can be generalized to other mRNAs. Since the initiator regions of various

messengers exhibit different extents of complementarity to 16S rRNA, it is reasonable that they should also have different S1 and factor requirements. Thus translational specificity could change upon an alteration in the physiological state of a single cell. For instance, it has been observed that as E. coli approaches stationary phase, initiation factors are recovered in reduced amounts;[57] if true in vivo, this situation would be expected to affect relative translation rates as cell growth ceases. The possibility clearly exists that an alteration in the amount or nature of the initiation factors, S1 protein, or any other ribosomal protein which affects the ribosome·fMet-tRNA·mRNA interaction could produce significant changes in the translation pattern of any cell. However, the physiological import of such phenomena remains unknown.

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