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Messenger Selection by Bacterial Ribosomes

(*Caulobacter crescentus*/bacteriophage Cb5 and MS2/initiation factor 3)

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ABSTRACT **The counterpart of *Escherichia coli* initiation factor 3(IF-3) was isolated from *Caulobacter crescentus*, purified to homogeneity, and used in comparative studies on *in vitro* translation of RNA from the *C. crescentus* RNA phage Cb5 and of coliphage MS2 RNA. The two phage RNAs are similar in physical properties and analogous in genetic content. The factor, C-IF-3, substitutes for *E. coli* IF-3 and promotes correct translation of MS2 RNA by *E. coli* ribosomes. Conversely, *E. coli* IF-3 substitutes for C-IF-3 in translation of Cb5 RNA by *C. crescentus* ribosomes. However, each phage RNA could be translated only by host ribosomes or by mixed ribosomes containing the host 30S subunit. C-IF-3 dissociates *C. crescentus* and *E. coli* 70S ribosomes into subunits. It binds phage, ribosomal, and, less efficiently, transfer RNA.**

Ribosomal binding and translation of natural mRNAs, such as the single-stranded genomes of RNA coliphages and T4 mRNA, specifically require the presence of initiation factor 3(IF-3) (1, 2), whereas translation of synthetic polynucleotides having an AUG initiation codon proceeds in the absence of this factor. This finding suggested that IF-3 may recognize messenger start signals, thus directing the ribosomes to the proper sites for correct initiation (for review see ref. 3). The nature of these start signals, whether nucleotide sequences, certain features of secondary and tertiary structure, or both, is unknown.

Subspecies of IF-3 from *Escherichia coli* can reportedly recognize different cistron initiation sites of polycistronic messenger (4–6). Two subspecies of IF-3 isolated and purified to virtual homogeneity in this laboratory (7, 8) exhibit high, although not absolute, selectivity towards two classes of messengers: IF-3α efficiently translates MS2 RNA and *E. coli* and early T4 RNA and has low activity with late T4 RNA, while the reverse is true for the other subspecies, IF-3β. The relative amounts of phage-specific proteins synthesized by translation of MS2 RNA with either IF-3α or β, analyzed by electrophoresis in Na dodecyl sulfate–polyacrylamide gels, were the same. These results indicate that the two IF-3 species do not discriminate between, or have the same relative affinities for, initiator sites of individual cistrons of the viral messenger.

As regards species specificity, previous work of this laboratory (9) showed that 1.0 M NH_4Cl-washed ribosomes of *Pseudomonas* sp. 412 and *Micrococcus cryophilus* translate coliphage RNA correctly with crude initiation factors from *E. coli*, *Pseudomonas*, or *M. cryophilus*. This finding indicated a relative lack of specificity of messenger recognition by ribosomes as well as by initiation factors. Such lack of specificity appears to be prevalent in eukaryotic systems (10, 11). On the other hand, whereas crude initiation factors from *E. coli* and *Bacillus stearothermophilus* are interchangeable in translation of coliphage RNA by *E. coli* ribosomes (12), in line with the above observations, *B. stearothermophilus* ribosomes recognize only the A-protein cistron of phage f2 RNA whichever the initiation factors used; this specificity resides in the *B. stearothermophilus* 30S subunit.

We here report on studies showing that homogeneous IF-3 preparations from either *E. coli* or *Caulobacter crescentus* cells are interchangeable in translation of (*a*) MS2 RNA by *E. coli* ribosomes or (*b*) RNA from *C. crescentus* phage Cb5 (13, 14) by *C. crescentus* ribosomes. However, MS2 RNA is not translated by *C. crescentus* ribosomes nor is Cb5 RNA translated by *E. coli* ribosomes, irrespective of the origin of the IF-3. As with *B. stearothermophilus* ribosomes, messenger specificity resides in the 30S subunit.

MATERIALS AND METHODS

Cells. C. crescentus Cb13 was kindly provided by Dr. L. Shapiro, Albert Einstein College of Medicine. Cells were grown in 180-liter containers at 30° in a medium containing 0.2% bactopeptone, 0.1% yeast extract, 0.1% glucose, and 0.5% $MgSO_4 \cdot 7H_2O$ in tap water and collected at mid-log phase (A_{600} = 0.600). Before harvesting, pH was adjusted to 8.3. This treatment prevented excessive cell lysis during centrifugation in a Sharples centrifuge. Cells were washed with a buffer containing 20 mM Tris·HCl (pH 7.8)–10 mM magnesium acetate–50 mM NH_4Cl–10 mM 2-mercaptoethanol (buffer A). *E. coli* Q13 was grown as described (15).

Ribosomes and Ribosomal Subunits from *C. crescentus* Cb13 were obtained by procedures established for *E. coli* (1, 16). Unfractionated ribosomes were washed with buffer A (containing 0.5 M NH_4Cl and 1 mM dithiothreitol instead of mercaptoethanol) for 12 hr, pelleted, and washed twice in buffer A containing 1.0 M NH_4Cl. The 0.5 M NH_4Cl wash was the source of IF-2a and b (molecular weight 65 $\times$ 10^3 and 84 $\times$ 10^3, respectively). The first 1.0 M wash was the source of IF-3. Ribosomes were fractionated into subunits by sucrose gradient centrifugation (10–30% sucrose, Spinco SW 25.2 rotor for 14 hr at 22,000 rpm at 2°) in a buffer containing 20 mM Tris·HCl (pH 7.8)–100 mM NH_4Cl–1 mM magnesium acetate–0.5 mM dithiothreitol

Preparation of C. crescentus IF-3 (C-IF-3). The factor was purified from the first 1.0 M NH_4Cl wash of *C. crescentus* ribosomes by fractionation with ammonium sulfate (step 2), followed by chromatography on phosphocellulose (step 3),

Abbreviation: IF, initiation factor.

DEAE–Sephadex (step 4), and Sephadex G-100 in 6 M urea (step 5). Na dodecyl sulfate–gel electrophoresis of C-IF-3 of step 5 (3–30 μg of protein) showed a single band. C-IF-3 of step 5 was used in all experiments reported here. Under the conditions of the standard assay (with *E. coli* ribosomes and MS2 RNA, see below), 1 mg of freshly isolated C-IF-3 of step 5 promoted incorporation of 700–1200 nmol of [^{14}C]lysine. This result means that the specific activity of C-IF-3 assayed in the *E. coli* system was about the same as that of *E. coli* IF-3 (see Table 1 in ref. 17). When kept at −70° in 50% glycerol in a buffer containing 20 mM Tris·HCl (pH 7.8)–200 mM NH_4Cl–0.5 mM dithiothreitol–0.1 mM EDTA, the factor lost about 50% activity in 2 months. Purification of IF-3 and IF-2 from *C. crescentus* will be described in detail in a subsequent publication.

Assays. C-IF-3 activity was assayed by incorporation of labeled amino acids into hot trichloroacetic acid-insoluble material with either Cb5 RNA or MS2 RNA as messenger using *C. crescentus* or *E. coli* ribosomes, respectively. The assay mixture (0.06 ml) contained 60 mM Tris·HCl buffer (pH 7.8); 60 mM NH_4Cl; 1.2 mM ATP; 0.2 mM GTP; 17 mM phosphocreatine; 1 μg of creatine kinase; 10 mM or 14 mM magnesium acetate with *C. crescentus* and *E. coli* ribosomes, respectively, or 12 mM with hybrid ribosomes; 5 mM dithiothreitol; 10 μg of *E. coli* W tRNA; 0.1 mM [^{14}C]lysine (specific radioactivity 25 or 50 Ci/mol); 0.1 mM each of the 19 remaining unlabeled amino acids; 2.5 A_{260} units of 1.0 M NH_4Cl-washed ribosomes of *C. crescentus* or *E. coli;* about 120 μg of protein of the 150,000 × *g* supernatant of *E. coli* Q13 (this supernatant was used throughout as a source of aminoacyl-tRNA synthetases, etc., since it was more active than the corresponding *C. crescentus* fraction); 0.6–0.8 A_{260} units of Cb5 RNA or MS2 RNA; 0.2–0.4 μg of C-IF-3 or 0.8–1.2 μg of *E. coli* IF-3 (homogeneous IF-3 of step 6, ref. 17); 0.1–0.3 μg of *C. crescentus* IF-2b (homogeneous protein, molecular weight 84 × 10^3) or 1.0–2.0 μg of *E. coli* IF-2 (purified IF-2, ref. 18), with *C. crescentus* or *E. coli* ribosomes, respectively; and 1.0–1.5 μg of *E. coli* IF-1 (purified IF-1, ref. 1). Initiation factor 1 was used to supplement only *E. coli* ribosomes because NH_4Cl-washed or run-off 70S and 30S ribosomes of *C. crescentus* show no requirement for IF-1 (Leffler and Szer, in manuscript). Incubation was for 20 min at 37°.

For the RNA binding assay, the samples (0.1 ml) contained 20 mM Tris·HCl buffer (pH 7.8); 50 mM NH_4Cl; 10 mM magnesium acetate; 0.5 mM dithiothreitol; 1.6–1.8 μg of RNA of the following specific radioactivities: 2600 cpm/μg of MS2 [^{3}H]RNA, 11,560 cpm/μg of *E. coli* W [^{14}C]-tRNA; 1320 cpm/μg of *E. coli* Q13 23S [^{14}C] RNA, and C-IF-3 of step 5 as indicated in Fig. 2. Samples were incubated for 15 min at 0°, diluted with the above buffer to 2 ml, and passed through Millipore filters, which were then washed three times with 2 ml of buffer. Radioactivity retained on the filters was measured.

For assay of ribosome dissociation, the samples (0.125 ml) contained the same buffer as above, except that magnesium acetate was 5 mM; 1.0 M NH_4Cl-washed ribosomes of *E. coli* MRE600 or *C. crescentus* Cb13, 1.0 A_{260} unit; and C-IF-3, 3.0 μg. Samples were incubated for 10 min at 37° without C-IF-3 and again 15 min after addition of factor. 0.1 ml was layered on 5 ml of a 5–20% sucrose gradient in the same buffer and centrifuged for 90 min at 42,000 rpm in a Spinco SW 50.1 rotor. Gradients were analyzed in an ISCO analyzer with a 10-mm light path flow cell.

Electrophoresis of Polypeptides Synthesized In Vitro upon Translation of MS2 RNA. The experiments were designed to test the effect of C-IF-3 on translation of MS2 RNA. The control sample contained the homologous *E. coli* system with [^{3}H]lysine; the experimental, with [^{14}C]lysine, was identical except for the substitution of C-IF-3 for *E. coli* IF-3. After phenol extraction, the samples were mixed and subjected to coelectrophoresis. The composition of the incorporation mixture was as in the standard assay, except that the sample size was increased to 0.3 ml and [^{14}C]lysine (specific radioactivity, 312 Ci/mol) or [^{3}H]lysine (5320 Ci/mol) was used. Incubation was for 30 min at 37°. Isolation of labeled proteins and polyacrylamide gel electrophoresis after treatment with sodium dodecylsulfate were as described by Viñuela *et al.* (19). The ratio of ^{3}H:^{14}C cpm layered on gels for coelectrophoresis was kept at about 5:1. Samples (0.1–0.2 ml) were heated for 15 min at 80° before layering to dissociate possible protein aggregates. The gel columns were 0.6 × 11 cm.

Miscellaneous. MS2 RNA was isolated as described by Weissmann and Feix (20). About 80–90% of the RNA sedimented as a sharp peak at 29 S [10 mM Tris·HCl (pH 7.4)–0.1 M NaCl]. This preparation was used for incorporation and binding experiments. For gel electrophoresis of phage-specific proteins, MS2 RNA was further purified by sucrose density gradient centrifugation (10–30% sucrose in the above buffer, 18 hr at 23,000 rpm in a Spinco SW 25.2 rotor at 2°). Cb5 RNA was isolated as described (14). About 70–80% of the RNA sedimented at 31 S. It was further purified as de-

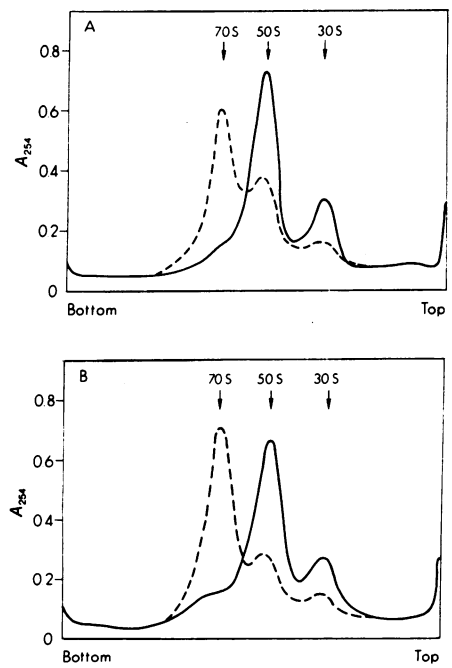

FIG. 1. Ribosomal dissociation activity of C-IF-3. (*A*) *C. crescentus* ribosomes; (*B*) *E. coli* ribosomes. *Dashed line,* control without C-IF-3; *solid line,* with 3.0 μg of C-IF-3. Composition of samples and conditions of assay are described in *Methods.* In an analogous experiment with *E. coli* IF-3 (not shown), the results were identical.

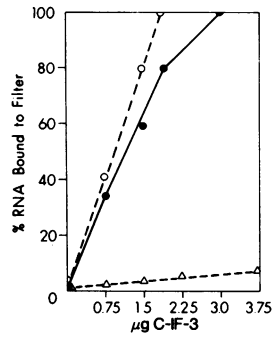

FIG. 2. RNA-binding activity of C-IF-3. O, MS2 [^{3}H]RNA; ●, *E. coli* Q13 23S [^{14}C]RNA; Δ, *E. coli* W [^{14}C]tRNA. Composition of samples and conditions of assay are described in *Methods*.

scribed for MS2 RNA. Cb5 phage is similar in physical properties (13, 14) and genetic content (13) to RNA coliphages.

Formaldehyde treatment of MS2 RNA [1.0 M formaldehyde in 10 mM phosphate buffer (pH 7.0), for 15 min at 37°] was as described by Lodish (21).

RESULTS

Properties of C. crescentus IF-3. The factor is a basic protein of molecular weight about 24,000 as estimated by Na dodecyl sulfate–polyacrylamide gel electrophoresis and by gel filtration on Sephadex G-100. The two methods give essentially the same results, indicating that C-IF-3 is a single polypeptide. Like *E. coli* IF-3 (7, 8), homogeneous C-IF-3 has ribosome dissociation activity. This activity is about the same with *C. crescentus* or *E. coli* ribosomes (Fig. 1) and copurifies with the activity detected by the assay for amino-acid incorporation. Loss of activity upon storage of the factor is evident in assays for both ribosome dissociation and amino-acid incorporation. When labeled RNAs, natural or synthetic, are mixed with IF-3, retention of label on Millipore filters indicates that the factor has RNA-binding activity in the absence of ribosomes (see ref. 17). Binding is proportional to the concentration of C-IF-3 (Fig. 2). The factor has low affinity for tRNA as compared to ribosomal or phage RNA.

Translation of Phage RNA. Experiments on translation of coliphage MS2 RNA and *C. crescentus* phage Cb5 RNA in the two ribosomal systems with either *E. coli* IF-3 or C-IF-3 are presented in Table 1. Both IF-3s are interchangeable in translation of phage RNAs by host ribosomes. There exists, however, a basic incompatibility between the phage messenger and nonhost ribosomes, which is evident in both systems. In other experiments (not shown), we have ascertained that Cb5 RNA will not direct the binding of fMet-tRNA to *E. coli* ribosomes with any combination of factors.

TABLE 1. *Translation of Cb5 RNA and MS2 RNA with E. coli and C. crescentus IF-3*

Unfractionated ribosomes	Phage RNA	Incorporation of [^{14}C]lysine (pmol*) with *C. crescentus* IF-3	Incorporation of [^{14}C]lysine (pmol*) with *E. coli* IF-3
C. crescentus	Cb5	57	46
C. crescentus	MS2	−3	−1
E. coli	Cb5	3	3
E. coli	MS2	82	78

* Net value with IF-3. Blanks in the absence of IF-3 (3–5 pmol) were subtracted.

In all experiments in Table 1, ribosomes were supplemented with homologous IF-2s. However, exchange of the IF-2s was without effect on the observed nontranslatability of phage messengers by nonhost ribosomes. Further experiments on the translation of the two phage RNAs with the use of mixed ribosomal subunits from *E. coli* and *C. crescentus* (Table 2) demonstrated that the ability to select the messenger resides specifically in the 30S subunit; the species from which the 50S subunit is derived is irrelevant.

Lodish has shown (21) that mild formaldehyde treatment of f2 RNA results in partial unfolding of the native molecule without loss of messenger activity. However, this treatment markedly changes the frequency of initiation at the three RNA cistrons and apparently exposes some ribosomal binding sites, leading to the synthesis of "nonsense" peptides. In addition, formaldehyde treatment decreases the requirement for IF-3 in binding of phage RNA to ribosomes (5). Table 3 shows that formaldehyde-treated MS2 RNA (HCHO-MS2 RNA) can be translated by *C. crescentus* ribosomes. As with *E. coli* ribosomes, there is fairly extensive incorporation of amino-acid label in the absence of C-IF-3, and this incorporation is further stimulated by addition of the factor.

Analysis of Polypeptides Synthesized In Vitro upon Translation of MS2 RNA with C. crescentus IF-3. The products of translation of MS2 RNA with *E. coli* IF-3 ([^{3}H]aminoacid label) and C-IF-3 ([^{14}C]aminoacid label) were coelectrophoresed on Na dodecyl sulfate–polyacrylamide gels. Reactions were run for 10 and 30 min at 37°, with labeled lysine and histidine, respectively (the latter does not occur in MS2 coat protein). In all four experiments the ^{14}C and ^{3}H electrophoretic patterns were indistinguishable. The results of a

TABLE 2. *Effect of addition of host ribosomal subunits on translation of Cb5 and MS2 RNA*

Fractionated subunits 30S + 50S	Additions	Phage RNA	Incorporation of [^{14}C]-lysine (pmol*)
C. crescentus	—	Cb5	27
C. crescentus	—	MS2	−1
C. crescentus	*E. coli* 30S	MS2	47
C. crescentus	*E. coli* 50S	MS2	2
E. coli	—	MS2	54
E. coli	—	Cb5	1
E. coli	*C. crescentus* 30S	Cb5	22
E. coli	*C. crescentus* 50S	Cb5	3

Composition of samples and conditions of assay as described in *Methods*, except that 0.5 A_{260} units of 30S and 1.0 A_{260} unit of 50S ribosomes were added as indicated.

* Net values with C-IF-3, which was used throughout this experiment. Blanks in the absence of C-IF-3 (2–4 pmol) were subtracted.

TABLE 3. *Translation of native and formaldehyde-treated MS2 RNA by E. coli and C. crescentus ribosomes*

Unfractionated ribosomes	Additions	Incorporation of [^{14}C]lysine (pmol)		
		Treatment of MS2 RNA		
		Untreated	15 min at 37°	15 min at 37° in 1.0 M HCHO
E. coli	None	3	7	36
E. coli	C-IF-3	48	44	76
C. crescentus	None	4	5	17
C. crescentus	C-IF-3	3	6	31

typical experiment with labeled lysine are presented in Fig. 3. It is clear that the use of "foreign" IF-3 does not affect the frequency and accuracy of translation of individual cistrons of MS2 RNA by *E. coli* ribosomes.

DISCUSSION

The properties of C-IF-3 and its activity in the *in vitro* systems studied by us make it clear that this protein is the *C. crescentus* counterpart of *E. coli* IF-3. The two IF-3s are interchangeable in the ribosome dissociation assay; they are also interchangeable in the amino-acid incorporation assay with coliphage MS2 RNA and with *C. crescentus* phage Cb5 RNA. Moreover, C-IF-3 promotes translation of late T4 RNA*, as does unresolved *E. coli* IF-3 (IF-3$\alpha\beta$, ref. 8). It is possible that *Caulobacter* IF-3, like *E. coli* IF-3, consists of subspecies.

The present work with highly purified IF-3 from *E. coli* and *C. crescentus* is consistent with earlier observations with crude initiation factors (9) suggesting that IF-3s from unrelated bacterial species have similar messenger recognition properties and lack of selectivity for different prokaryotic ribosomes.† This may be so regardless of the existence of two subspecies of IF-3 capable of discriminating between two broad classes of messengers (8). It would appear from the work of Lodish (12) and our present results that recognition of messenger initiation signals in prokaryotes may primarily be a function of the 30S ribosomal subunit, the subunit to which messenger binds. In addition, as suggested by the effects of formaldehyde treatment of coliphage RNA (ref. 21, this paper, and Sabol, Lee-Huang & Ochoa, unpublished), recognition of initiation sites is highly dependent on the secondary structure of the messenger.

This finding suggests a built-in complementary relationship between the 30S subunit and the messenger that makes possible the binding in which IF-3 assists. The nature of this complementarity is not known. In particular, it is not understood why *B. stearothermophilus* ribosomes translate only the

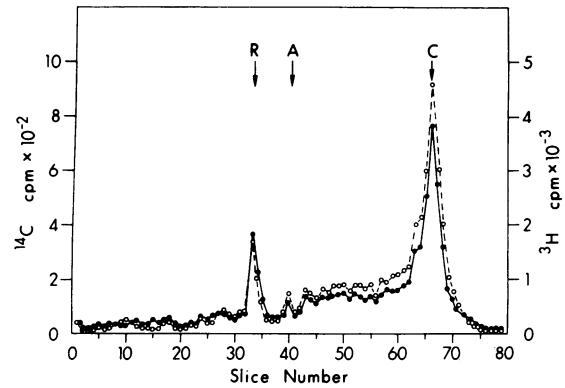

FIG. 3. Coelectrophoresis of labeled polypeptides separately synthesized *in vitro* upon translation of MS2 RNA by *E. coli* Q13 ribosomes with *E. coli* IF-3, (○, [^{3}H]lysine) and with *C. crescentus* IF-3, (●, [^{14}C]lysine). Incubation was for 30 min at 37°. *Arrows* indicate positions of MS2 replicase subunit (*R*), maturation protein (*A*), and coat protein (*C*).

maturation protein of f2 RNA (12) and those of *Pseudomonas* sp. 412 and of *M. cryophilus* translate correctly MS2 RNA (9), whereas *C. crescentus* ribosomes are inactive with MS2 RNA and *E. coli* ribosomes are inactive with phage Cb5 RNA. Reconstitution of 30S subunits from host and nonhost components (core particles, split proteins, etc.) may provide some understanding of this complementary relationship.

As regards IF-3, this work supports the notion that this factor recognizes over-all features of the ordered structure of mRNAs and, perhaps by inducing some subtle conformational changes, promotes ribosomal binding to sites determined by the 30S subunit–messenger complementarity. The conformations of the two phage RNAs used in this study may be quite similar as judged by sedimentation coefficients, thermal hypochromicities, and melting temperatures; their base compositions differ only slightly (14).

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* With *E. coli* ribosomes. Neither IF-3 promotes translation of late T4 RNA with *C. crescentus* ribosomes; however, a relatively high amount of translation independent of IF-3 is observed (experiments done with S. Lee-Huang).

† Similar observations with *Clostridium pasteurianum* and *Streptococcus faecalis* have been recently reported by Stallcup and Rabinowitz [(1973) *Fed. Proc.* **32**, 533 Abstr].

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