Articles

Function of the Repeating Homologous Sequences in Nucleic Acid Binding Domain of Ribosomal Protein S1†

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ABSTRACT: Ribosomal protein S1 contains in its RNA binding domain four repeating, homologous stretches of sequences. Its functionally active mutant form m1-S1 [Subramanian, A. R., & Mizushima, S. (1979) *J. Biol. Chem. 254*, 4309] contains only three repeating stretches. In order to assess the functional importance of this repeating sequence, we cleaved S1 at its reactive SH group on Cys-349 and isolated a fragment (S1-F4) that has lost two of the homologous stretches but retains all other essential elements. We find that ribosomes reconstituted with S1-F4 instead of S1 are functionally active in translating poly(U) and poly(A) but totally inactive in translating phage MS2 RNA. The significance of this result is discussed vis-à-vis the initiation step in translating natural mRNA, and a functional role for the tetrarepeat of S1 is suggested.

Ribosomal protein S1[1] is a large, multidomain, RNA-binding protein with important functions in protein biosynthesis and phage RNA replication, and a wide variety of methods have been used to determine its structure, function, and mechanism of action [reviewed in Subramanian (1983)]. We have isolated and studied several truncated forms and internal fragments of S1 that are active in partial functional reactions and therefore would contain intact functional domains (Subramanian & Mizushima, 1979; Suryanarayana & Subramanian, 1979; Giorginis & Subramanian, 1980; Subramanian et al., 1981). These studies revealed that two different domains of S1 are involved in the binding of S1 to the 30S ribosomal subunit and in the binding of messenger RNA to S1.

The amino acid sequence of S1 contains several repeating stretches of internal homology (Schnier et al., 1982; Kimura et al., 1982; Doolittle et al., 1982; Wittmann-Liebold et al., 1983; Subramanian, 1983). When the previously studied fragments and trimmed forms of S1 were precisely located within the S1 sequence, it became evident that four of the most significant homologous stretches are contained in the RNA binding domain of S1 (Subramanian, 1983). The homologies among these four stretches [designated R1, R2, R3, and R4; see Subramanian (1983)] are between 33 and 55% if only identical residues are considered or 50 and 70% if conservative amino acid replacements are also included. Lower but statistically significant homologies are also found in the remaining portions of S1 sequence.

S1 contains two –SH groups (Cys-292 and Cys-349). Both of these SH groups are located on the homologous stretch R2 (see Figure 1, Results). Cys-349, which has the reactive –SH group of native S1 (Subramanian, 1980), is located near the carboxyl end of R2. By cleavage of S1 at Cys-349, a truncated form of S1 with the sequence from 1 to 348 containing the NH_2-terminal ribosome binding domain together with only two of the repeating sequences can be generated. In this paper, we report the isolation and functional properties of this fragment and discuss the significance of the result.

Materials and Methods

Materials. ATP, GTP, phosphoenolpyruvate, poly(U), poly(A), deoxyribonuclease I, and phage MS2 RNA were purchased from Boehringer. [5-^{3}H]Poly(U) was from Miles. Uniformly ^{14}C-labeled lysine, phenylalanine, and valine were from Radiochemical Center, Amersham; *N*-[1-^{14}C]ethylmaleimide was from New England Nuclear. Labeled phage MS2 RNA was isolated from MS2 phage grown in *Escherichia coli* HfrC in the presence of [5,6-^{3}H]uridine (New England Nuclear) as described previously (Suryanarayana & Subramanian, 1983). Poly(U)–Sepharose was purchased from Pharmacia; 2-nitro-5-thiocyanobenzoic acid was from Serva. Commercial MS2 RNA was precipitated with ethanol and redissolved in distilled water before use.

Bacterial Strains and Growth Conditions. Escherichia coli strains MRE600 and A19 were grown at 37 °C with vigorous aeration and harvested in midlogarithmic phase as described by Minks et al. (1978).

Preparation of Ribosomes, Supernatant Fraction, and Initiation Factors. Cells were ground with alumina and extracted with buffer (10 mM Tris-HCl, pH 7.6, 50 mM KCl, 10 mM magnesium acetate, 7 mM 2-mercaptoethanol) containing 1 μg/mL DNase and further processed to obtain the supernatant fraction (S-100), 1 M NH_4Cl washed ribosomes, and initiation factors as previously described (Subramanian et al., 1976; Minks et al., 1978). Subunits were prepared by zonal centrifugation (Beckman Ti15 rotor, 25000 rpm, 16 h) of the A19 cell extract in 10 mM potassium phosphate, 1 mM magnesium acetate, and 7 mM 2-mercaptoethanol, pH 7.6. The subunits from pooled gradient fractions were pelleted and dissolved in buffer containing 10 mM Tris-HCl, 10 mM magnesium acetate, 50 mM KCl, 1 mM dithiothreitol, and 10% glycerol, pH 7.6. The 30S subunits prepared in this manner contain their cellular complement of IF-3 (Suryanarayana & Subramanian 1983). The subunits were heat activated by the procedure of Zamir et al. (1974).

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[1] Abbreviations: r protein, ribosomal protein; S1, ribosomal protein S1 of *Escherichia coli*; m1-S1, a mutant form of S1 with sequence 1–437; S1-F1, F2a, F3, and S1-F4, functional fragments of S1 with sequences 172–557, 1–193, 224–309, and 1–348, respectively; P site, ribosomal site for peptidyl tRNA; Tris, tris(hydroxymethyl)aminomethane; DTT, dithiothreitol; NTCB, 2-nitro-5-thiocyanobenzoic acid.

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Purification of Protein S1. S1 was purified from *E. coli* MRE600 by a procedure described recently (Subramanian et al., 1981) using poly(U)–Sepharose chromatography. Protein estimation was according to Lowry et al. (1951) with bovine serum albumin as standard.

Removal of S1 from 30S Subunits, S-100, and Initiation Factors. This was accomplished by passing the solutions through a poly(U)–Sepharose column. Details are given elsewhere (Suryanarayana & Subramanian, 1983).

Poly(U) Binding. The binding of S1 or S1-F4 to [^{3}H]-poly(U) was carried out in 0.1 mL of 10 mM Tris-HCl, pH 7.6, 100 mM NH_4Cl, 10 mM magnesium acetate, and 1 mM dithiothreitol containing 0.05 μCi of [5-^{3}H]poly(U) (512 μCi/μmol of P). The mixture was incubated at 0 °C for 5 min, diluted to 1 mL with the above buffer, and filtered through an alkali-treated Millipore filter (Smolarsky & Tal, 1970). The filter was washed with 3 mL of cold buffer, dried, and counted in the presence of 0.5% diphenyloxazole in toluene in a Beckman LS8000 scintillation counter.

MS2 RNA Binding to 30S Subunits. This was carried out in a 0.1-mL mixture containing 50 mM Tris-HCl, pH 7.6, 100 mM NH_4Cl, 10 mM magnesium acetate, 1 mM dithiothreitol, indicated amounts of S1-lacking 30S subunits preincubated with S1 or S1-F4 in a 1:1 molar ratio, and 42 000 cpm (6.5 μg) of [^{3}H]MS2 RNA. The mixture was incubated at 37 °C for 10 min, chilled to 0 °C, diluted with cold binding buffer (same salt concentration as the reaction mixture), and filtered through a Millipore filter. The filter was washed twice with cold buffer, and the radioactivity retained on the filter was determined as given above.

Binding of S1 or S1-F4 to 30S Subunits. Heat-activated 30S subunits lacking S1 (1.1 A_{260} units) were incubated in 0.5 mL of 10 mM Tris-HCl, pH 7.6, 6 mM magnesium acetate, 100 mM NH_4Cl, and 1 mM dithiothreitol with a 10-fold molar excess of S1 or S1-F4 at 37 °C for 10 min. The mixtures, after being chilled, were layered on sucrose gradients (5–20%) in 10 mM Tris-HCl, pH 7.6, 10 mM magnesium acetate, 100 mM NH_4Cl, and 0.5 mM ethylenediaminetetraacetate (EDTA) and centrifuged at 20 000 rpm for 16 h. The gradients were fractionated on an Isco gradient analyzer, and the presence of S1 or S1-F4 was determined by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (Laemmli, 1970) of the fractions.

Competition between S1 and S1-F4 for the 30S subunit was tested by using ^{14}C-labeled S1 and unlabeled S1-F4. 30S subunits lacking S1 (138 pmol) were incubated (37 °C, 10 min) either with an equimolar amount of labeled S1 or with the same amount of labeled S1 plus 5 times the molar amount of unlabeled S1-F4 in a final volume of 250 μL and with an ionic composition of 10 mM Tris-HCl, pH 7.6, 100 mM NH_4Cl, 10 mM magnesium acetate, and 1 mM DTT. The samples were chilled and passed through a column (30 × 0.5 cm) of Sephadex G-200 at 4 °C. Fractions (0.32 mL) were collected, and radioactivity was determined in the presence of Instagel (Packard). Labeled S1 for this experiment was prepared by reacting *N*-[1-^{14}C]ethylmaleimide with S1 under native conditions as described previously (Guerrier-Takada et al., 1983). Laughrea & Moore (1978) have shown that the *N*-ethylmaleimide derivative of S1 binds to 30S subunits with the same binding constant as S1.

Assay for Protein Synthesis. Poly(U)-dependent protein synthesis was carried out in 0.1 mL of 50 mM Tris-HCl, pH 7.6, 100 mM NH_4Cl, 18 mM magnesium acetate, 1 mM dithiothreitol, 1.6 mM ATP, 0.3 mM GTP, 8 mM phosphoenolpyruvate, 30 μM each of 19 unlabeled amino acids, 32 μM [^{14}C]phenylalanine (31 Ci/mol) containing 5 μg of pyruvate kinase, 0.25 mg of *E. coli* tRNA, 41 μg of 30S subunits lacking S1, 84 μg of 50S subunits, 200 μg of S-100 protein free of S1, and 2 μg of poly(U). For study of the stimulation by S1 or S1-F4, the reaction mixtures were preincubated with S1 or S1-F4 at 37 °C for 5 min before addition of poly(U). The mixtures were incubated at 37 °C for 20 min, and hot trichloroacetic acid insoluble radioactivity was determined after Millipore filtration.

Poly(A)-dependent polylysine synthesis was also carried out as described for the poly(U) system with the exception that 16 mM magnesium acetate, 33 μM [^{14}C]lysine (30 Ci/mol), and 2 μg of poly(A) were used. At the end of the incubation, hot trichloroacetic acid–sodium tungstate insoluble radioactivity was determined.

Phage MS2 RNA directed protein synthesis was carried out in a 0.1-mL reaction mixture containing 12 mM magnesium acetate, 30 μM each of 19 unlabeled amino acids, 18.9 μM [^{14}C]valine (123 Ci/mol), the other components as given for the poly(U) system, and 40 μg of initiation factors free of S1. The reaction was started with MS2 RNA (0.5 A_{260} unit) after a preincubation step (10 min at 37 °C) to reconstitute the system with S1 or S1-F4. The mixture was incubated at 37 °C for 20 min and processed as in the poly(U) system to determine incorporated radioactivity.

Reaction of S1 with 2-Nitro-5-thiocyanobenzoic Acid (NTCB). The protein (1 mg/mL) in 0.1 M Tris-HCl, pH 7.6, and 1 mM 2-mercaptoethanol was reacted with 10 mM NTCB at 37 °C for 30 min. In analytical experiments, the reaction was terminated with 10% trichloroacetic acid. The precipitate was collected, washed once with ether and dissolved in 0.1 M sodium tetraborate, and incubated at 37 °C for 16 h for cleavage of the cyanylated protein.

For preparative purpose (purification of S1-F4), the protein after cyanylation was dialyzed at room temperature for 48 h against 1000 volumes of 0.1 M sodium tetraborate with four changes. The cleaved reaction mixture was chromatographed on a Sephadex G-100 column (2 × 160 cm). Equilibration and elution were carried out with 10 mM Tris-HCl, pH 7.6, 100 mM NH_4Cl, 1 mM dithiothreitol, and 1 mM EDTA. Peak fractions containing fragment S1-F4, as determined by electrophoresis (Laemmli, 1970), were pooled and concentrated with dry Sephadex G-100. The concentrated material was dialyzed against 10 mM Tris-HCl, pH 7.6, 100 mM NH_4Cl, 1 mM dithiothreitol, and 10% glycerol and used for the studies described.

Antisera. Antibodies to S1 and F2a were raised in rabbits, and Ouchterlony double immunodiffusion assays were performed as described by Stöffler & Wittmann (1971).

Results

–SH Groups and the Repeating Sequences of S1. The four most significant repeating homologous sequences of S1 are between positions 190 and 530 of S1's primary structure. The approximate positions of these stretches and of the –SH groups are shown in Figure 1. It is evident that cleavage of S1 at Cys-349 will produce an NH_2-terminal fragment that has lost the repeating stretches R3 and R4 and a few of the terminal residues of R2. Figure 1 also shows that the m1-S1 molecule lacks R4 and a few residues of R3. This mutant form of S1 is active in binding and translation of mRNA (Subramanian & Mizushima, 1979; Thomas et al., 1979) and in the transcription of Qβ phage RNA (Guerrier-Takada et al., 1983).

NTCB Cleavage of S1: Isolation and Properties of S1-F4. When S1 was treated with NTCB under native conditions, as described under Materials and Methods, about half the amount

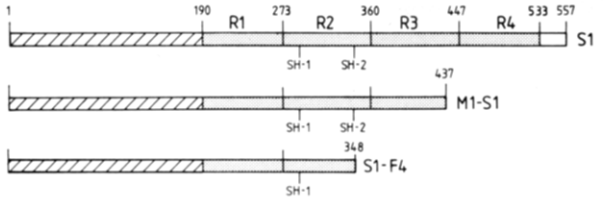

FIGURE 1: Linear representation of S1, m1-S1, and S1-F4. The four repeating homologous stretches are shown stippled. SH-2 is the "reactive" SH group of S1. S1-F4 is produced by the cleavage of S1 at SH-2 with 2-nitro-5-thiocyanobenzoic acid.

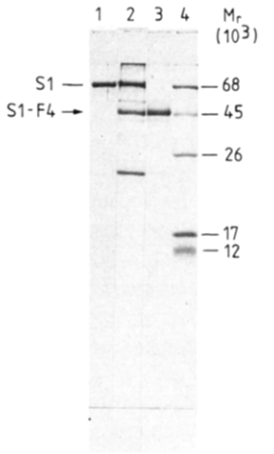

FIGURE 2: Sodium dodecyl sulfate–polyacrylamide gel electrophoresis of S1 (3 μg, lane 1), NTCB-treated S1 (5 μg, lane 2), purified S1-F4 (2 μg, lane 3), and molecular weight markers (bovine serum albumin, 68 000; ovalbumin, 45 000; chymotrypsinogen, 26 000; myoglobin, 17 000; cytochrome *c*, 12 000).

of the protein underwent cleavage, producing essentially two fragments in approximately equimolar amounts (Figure 2, lanes 1 and 2). The cleavage therefore occurred only at the reactive Cys residue, which, from our previous work (Subramanian, 1980), is located at Cys-349 in the S1 sequence (Schnier et al., 1982; Kimura et al., 1982). The cleavage mixture was fractionated on a column of Sephadex G-100, and this procedure yielded, in one step, both fragments in electrophoretically pure form.

NTCB cleaves a polypeptide at the aminopeptide bond of the reacted cysteine residue (Degani & Patchornik, 1974; Stark, 1977). The fragments produced in the case of S1 would therefore be sequence positions 1–348, with a calculated M_r of 38 670, and C-terminal fragment 349–557, with an M_r of 22 500. The larger, NH_2-terminal fragment (designated S1-F4) will contain the previously described ribosome binding domain (Giorginis & Subramanian, 1980) and the repeating stretches R1 and R2.

In sodium dodecyl sulfate gel, S1-F4 migrated with an apparent M_r of about 45 000 (Figure 2, lanes 3 and 4). This type of anomalously low electrophoretic mobility is, from our previous data (Subramanian 1983), a characteristic feature of S1 and all of its fragments that contain the repeat segments R1 and R2.

Double immunodiffusion tests of S1-F4 and S1 against anti-S1 serum and anti-F2a serum (F2a is the ribosome binding fragment of S1 with the amino acid sequence from 1 to 191) are shown in Figure 3. Fragment S1-F4 gave precipitin lines with both sera as expected, with no visible spur formation. This result shows that S1-F4 has the intact NH_2-terminal region of S1. Lack of spur formation in Figure 3A would indicate [as previously noted with m1-S1 in Subramanian & Mizushima (1979)] that the antigenic determinants of S1 in its C-terminal region are mostly silent.

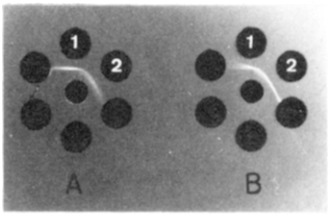

FIGURE 3: Double immunodiffusion of protein S1 and fragment S1-F4 against (A) anti-S1 serum and (B) anti-F2a serum. Wells numbered 1 and 2 contained 2 μg of S1 and S1-F4, respectively. Center wells contained 30 μL of antiserum.

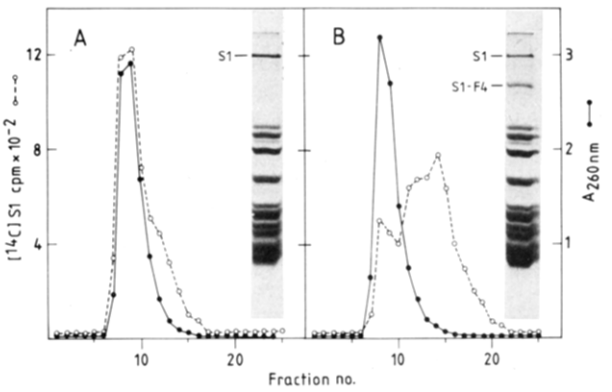

FIGURE 4: Competition between S1-F4 and S1 for the binding site on ribosomes. 30S subunits lacking S1 (138 pmol) were incubated with either (A) *N*-[1-^{14}C]ethylmaleimide labeled S1 or (B) the same amount of labeled S1 *plus* a 5-fold molar excess of S1-F4. The samples were then subjected to gel filtration as described under Materials and Methods. Radioactivity and UV absorption of the fractions are given. The inset in each panel shows the Coomassie blue stained pattern of the 30S subunit proteins (from the UV peak) after sodium dodecyl sulfate gel electrophoresis.

That S1-F4 still carries one free –SH group (i.e., on Cys-292) was confirmed by two different experiments: S1-F4 could react, in the presence of 8 M urea, with *N*-[1-^{14}C]ethylmaleimide, and the incorporated radioactivity corresponded to 1 mol of label/mol of protein. It could also be further cleaved by NTCB reaction in the presence of urea, and a smaller fragment migrating faster than S1-F4 was produced under these conditions (data not shown).

Binding of S1-F4 to 30S Subunits. S1-F4 was incubated with S1-lacking 30S subunits, and the latter was separated from unbound protein on a sucrose gradient. Analysis of the 30S subunit protein by sodium dodecyl sulfate gel showed the presence of S1-F4 (data not shown). The results from a competition experiment between radiolabeled S1 and unlabeled, 5-fold molar excess of S1-F4 for the S1-binding site on 30S subunits is shown in Figure 4. S1-F4 depressed the level of S1 binding, and the 30S subunits eluting from the column contained both S1 and S1-F4 as determined by gel electrophoresis (Figure 4B). This experiment qualitatively shows the retention of ribosome binding affinity by S1-F4, while more quantitative experiments would reveal whether the S1·30S binding constant has been affected by the cleavage.

Binding of mRNA by S1-F4. S1 and its fragments that contain the RNA binding domain bind free poly(U) (Subramanian, 1983); Figure 5A shows such binding by S1-F4. There is evident binding between S1-F4 and poly(U), although to a lesser extent than with intact S1. The binding between poly(U) and the RNA binding fragments S1-F1 and F3 is also weaker than it is with S1.

We tested the ability of S1-F4 to confer to 30S subunits the capacity to bind phage MS2 RNA. Szer & Leffler (1974)

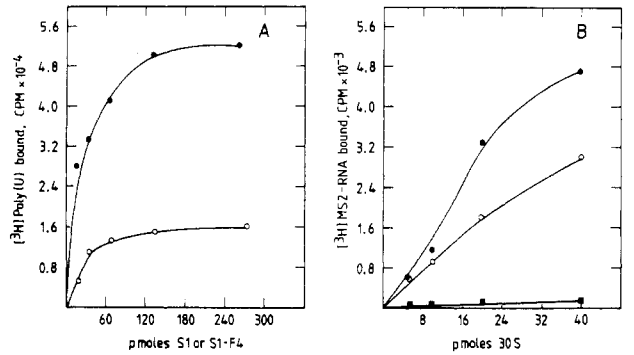

FIGURE 5: RNA binding properties of free S1-F4 and 30S subunits reconstituted with S1-F4. (Panel A) Binding of radiolabeled poly(U) to S1 (●) and S1-F4 (○), as determined by the Millipore assay. (Panel B) Binding of [^{3}H]MS2 RNA to 30S subunits lacking S1 (■), to 30S subunits containing S1 (●), and to 30S subunits with S1-F4 in place of S1 (○).

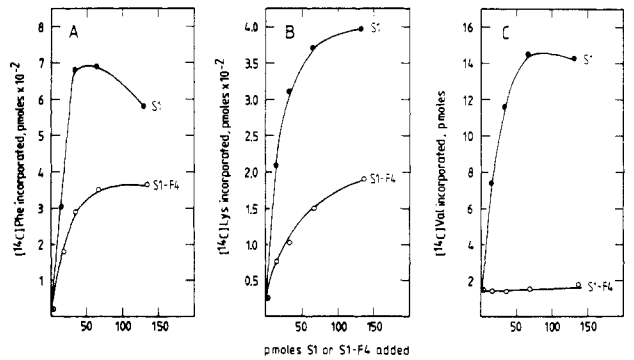

FIGURE 6: Protein synthesis activity of S1-lacking ribosomes in the presence of increasing amounts of either S1 or S1-F4. Panels A–C show the translation of poly(U), poly(A), and phage MS2 RNA, respectively. One picomole of [^{14}C]Phe, [^{14}C]Lys, and [^{14}C]Val corresponded, respectively, to 55, 53, and 232 cpm. Blank values (i.e., incorporation without added mRNA), which were 21, 5, and 1.1 pmol in panels A–C, have been subtracted.

have previously shown that 30S subunits lacking S1 are virtually unable to bind MS2 RNA (see Figure 5B, bottom line). S1-F4 confers a relatively high degree of MS2 RNA binding capacity to 30S subunits (Figure 5B). We may thus infer that a truncated S1 containing its ribosome binding domain and two of the repeating homologous sequences is able to function as a ribosomal mRNA binding protein.

Is S1-F4 Active in Protein Biosynthesis in Vitro? The answer to this question in the case of three messenger RNA species is given in Figure 6. Ribosomes containing S1-F4 instead of S1 can translate poly(U) and poly(A) at levels that, although lower than that with intact S1, are considerable, i.e., about 50%.

The most interesting results were obtained with phage MS2 RNA. Ribosomes reconstituted with S1-F4 were practically totally inactive in translating this messenger RNA (Figure 6C). In further experiments, a mixture of S1-F4 and purified C-terminal fragment (residues 349–557) was also found inactive in translation (data not shown). The striking contrast seen in the functional capability of S1-F4 vis-ă-vis synthetic mRNAs vs. a natural mRNA allows (as discussed below) some insights into the probable function of the homologous repeats of S1.

Discussion

The decoding of messenger ribonucleic acid with biologically acceptable speed and accuracy, which takes place on the ribosome in vivo, is evidently a precisely ordered process. Since the primary structures of all the components of *E. coli* ribosome have been determined [see Wittmann (1982)], it is now possible to probe the role in this process of individual ribosomal components at molecular and intramolecular levels. The finding that rRNA and r proteins of higher plant chloroplast ribosomes are close structural homologues of *E. coli* ribosome [e.g., Subramanian et al. (1983)] adds more generality to the results from such studies. We should point out here an interesting and probably highly significant feature of the ribosome as revealed from structure and stoichiometry studies. The *E. coli* ribosome actually contains two different, significantly long, repeat sequences. They are the four intramolecular repeats of S1 (Figure 1) in the small subunit and the four intermolecular repeats of protein L7/L12 [generated by the presence of four copies of this protein; see Subramanian (1975)] in the large subunit. Both S1 and L7/L12 are functionally essential ribosomal proteins.

We previously described (Subramanian & Mizushima, 1979) a short form of S1 obtained from a mutant *E. coli*. Structural work has shown this form (m1-S1) to have the same NH_2-terminal region as S1 but terminating at Ala-437; i.e., it is 120 amino acid residues shorter than S1 (K. Foulaki, M. Kimura, B. Wittmann-Liebold, and A. R. Subramanian, unpublished results). It contains only three of the repeating stretches of S1 (Figure 1). m1-S1 can replace S1 in the translation of poly(U), poly(A), and phage MS2 RNA or in the transcription of Qβ RNA; the activity levels are 70–90% that of S1 (Subramanian & Mizushima, 1979; Guerrier-Takada et al., 1983; A. R. Subramanian, unpublished results). Thus, the S1 molecule can tolerate the loss of one homologous stretch (R4) with only minor impairment in function. The loss of R3 *and* R4 leads to the novel result described in this paper (Figure 5): the ability to translate synthetic homopolynucleotides is only impaired while the ability to translate a natural mRNA is virtually abolished.

Homopolynucleotide messengers do not contain the initiation codon, and therefore, their translation bypasses the initiation steps required for the translation of natural mRNA. One of these steps is the in-phase placement of mRNA on the ribosomal decoding site, i.e., the precise fitting of the AUG codon at the P site. It is likely that this in-phase placement is achieved by a movement of mRNA subsequent to its initial binding onto the ribosome. Our results with S1-F4 suggest that all four (or at least three) of the repeating stretches of S1 are necessary to accomplish this movement of ribosome-bound mRNA.

Many nucleic acid binding proteins contain two oligonucleotide binding sites in their quaternary structure, e.g., gene 5 protein of bacteriophage fd (McPherson et al., 1980) or adenosine 3′,5′-phosphate receptor protein (McKay & Steitz, 1981). The repeating segments of S1 are each about as long as the gene 5 protein monomer. Had the repeating segments of S1 been evolved only to bind mRNA, two segments (i.e., R1 and R2) would have been sufficient. The presence of four segments points to an additional function, and we suggest (from our results on S1-F4 and other S1 fragments) that this additional function is concerned with the movement of ribosome-bound mRNA. In recent publications, we have described a model for the function of S1 that can accommodate this additional role (Subramanian, 1983; Suryanarayana & Subramanian, 1983).

Registry No. 2-Nitro-5-thiocyanobenzoic acid, 30211-77-9; poly(U) (homopolymer), 27416-86-0; poly(A) (homopolymer), 24937-83-5.

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Potentiometric Analysis of the Purified Cytochrome *d* Terminal Oxidase Complex from *Escherichia coli*†

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ABSTRACT: The cytochrome *d* terminal oxidase complex is a principal component of the aerobic respiratory chain of *Escherichia coli*. This purified complex contains two polypeptides as analyzed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis, and the reduced minus oxidized spectrum indicates that cytochromes *b*-558, a_1, and *d* are all components of the complex. Changes in the absorption spectrum of the complex between 500 and 700 nm were analyzed as a function of the solution electrochemical potential. A spectral resolution algorithm was used to extract the reduced minus oxidized spectrum of each electrochemically active species from the set of spectra of the complex at different solution potentials. The procedure yielded reduced minus oxidized difference spectra for cytochromes *b*-558, a_1, and *d*, along with the midpoint potentials and Nernst *n* values for each cytochrome. The midpoint potentials (E_m) and *n* values for the solubilized complex were as follows: cytochrome *b*-558, E_m = 61 mV and n = 0.8; cytochrome a_1, E_m = 113 mV and n = 1; cytochrome *d*, E_m = 232 mV and n = 1. The spectrum of cytochrome *b*-558 was typical of *b*-type cytochromes, and that of cytochrome *d* was dominated by a band centered at 628 nm. The difference spectrum of cytochrome a_1 indicated an α band at 594 nm, a strong β band near 560 nm, and a trough near 645 nm. The spectrum is similar to the spectra of high-spin heme *a* model compounds.

The cytochrome *d* terminal oxidase complex is a principal component of the aerobic respiratory chain of *Escherichia coli* (Miller & Gennis, 1983; Bragg, 1979; Haddock & Jones, 1977). The respiratory chain is branched and contains two terminal oxidases, cytochrome *d* and cytochrome *o* [see Bragg (1979) and Haddock & Jones (1977)]. The cytochrome *d* terminal oxidase is induced when the cells are grown under conditions of oxygen limitation (Kranz & Gennis, 1983), and previous work has shown that the affinity for molecular oxygen of cytochrome *d* is higher than that of cytochrome *o* (Rice & Hempfling, 1978). Both terminal oxidases have been purified to homogeneity (Matsushita et al., 1983; Miller & Gennis, 1983; Kita et al., 1982). The reduced minus oxidized difference spectrum of the cytochrome *d* complex is complicated

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