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Affinities of ribosomal protein S20 and C-terminal deletion mutants for 16S rRNA and S20 mRNA

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ABSTRACT

We have measured the binding of E. coli ribosomal protein S20 and a number of C-terminal deletion mutants to 16S rRNA and in vitro transcribed S20 mRNA. Mutant S20s of interest were synthesized in vitro from the appropriate plasmid templates by coupled transcription and translation. The affinity of S20 produced in vitro for 16S rRNA is 1.2×10^7 (M^{-1}) in a gel filtration assay. Removal of as few as 6 residues from the C terminus of S20 results in a sharp loss of binding activity, suggesting the presence of critical residues in this region. Analysis of the amino acid sequence of S20 indicates that these residues may constitute part of a segment of α helix. Although S20 is known to autoregulate its own synthesis, we were unable to demonstrate any measurable affinity of S20 for its own mRNA.

INTRODUCTION

The architecture of the E. coli ribosome is based largely upon non-covalent interactions of protein and RNA. So too it would appear, are the processes ribosomal proteins utilize in regulating their expression - in particular, that of autogenous translational regulation (1,2). Elucidation of the nature of these interactions depends upon techniques capable of identifying the features which provide for their selectivity. Much attention has been devoted to defining the locations of protein binding on ribosomal RNA's (reviewed in 3) and more recently, to delimiting the protein domains responsible for this binding and various other functions. The principal means utilized in defining these domains has involved proteolytic digestion followed by assay of the ability of the resulting peptides to bind or perform other activities. This approach has been useful in identifying functional domains in a number of ribosomal proteins, particularly in the case of S4 (4-9). However, these methods suffer from the

limited range of chemical and enzymatic cleavages available.

Two separate experimental approaches have implied that the amino terminus of ribosomal protein S20 contains residues essential for its binding to 16S rRNA. In the first, Paterakis et al found that two proteolytic fragments of E. coli ribosomal protein S20 lacking 14 or 35 amino acids from its N-terminus exhibited reduced or no 16S rRNA binding activity, respectively (10). In a second, an amino terminal fragment of S20 up to 50 residues in length cosedimented with 16S rRNA after the latter had been incubated with crude extracts of heat shocked cells (11). However, in neither of the above cases were quantitative measurements obtained. Accordingly, we have employed here a comprehensive means of obtaining an assortment of deletions at the C-terminus of the S20 protein, without proteolysis. Mutation is accomplished at the DNA level, and altered peptide products are generated by coupled in vitro transcription/translation of the new sequences. In this case, groups of deletions were made for internal as well as C-terminal sequences and a quantitative analysis of their 16S rRNA binding properties applied.

Ribosomal protein S20 is also of interest in that it is an autogenous regulator of its own synthesis (12,13) and therefore is presumed to be capable of binding either to 16S rRNA for ribosome assembly or its own messenger RNA to effect translational repression. In general, very little is known about the latter ability among ribosomal constituents (14,15), or the relationship between the two activities (ie. do they use the same site on the protein? What are their relative strengths? etc.). Hence we have measured the binding of S20 synthesized in vitro to both 16S rRNA and to S20 mRNA transcribed in vitro. Unlike the case of S4 (15), which has been found to bind either to 16S rRNA or its own mRNA with equal affinities, we find that S20 shows at least 100 fold greater affinity for 16S rRNA than for its mRNA.

MATERIALS AND METHODS

Strains and Plasmids:

The source plasmid (pGM24) from which all mutants were derived has been previously described (16). Vectors used for cloning and expressing mutant sequences were pKK223-3 (17) from Pharmacia and pCQV2 (18) which was obtained from Dr. D.T.

Denhardt. Host strains were JM103 (19) and MM294 (endA1, thi-1, hsdR17, supE44 ; from M. Meselson) respectively. Strains used for cell-free synthesis extracts were RD100 (rns$^-$, pnp$^-$, met$^-$, trpD9778, relA, lacZ ; from M. L. Pearson) and BU8049 (F$^-$ Δ[lac pro] strA, recA, lon$^-$) from the Bukhari collection (obtained through Dr. G. Chaconas).

Mutagenesis and Sequencing:

A. C-terminal Deletions: The source plasmid pGM24 was digested at a unique HindIII site just distal to the S20 coding sequence and then treated with Bal 31 exonuclease (from BRL Inc.) under conditions previously established to effect the removal of up to a maximum of 200 base pairs (bp). After addition of HindIII linkers and digestion with EcoRI and HindIII, fragments containing the deleted S20 sequences were purified from polyacrylamide gels. These were ligated into M13mp8 and sequenced by the modified dideoxy technique of Biggin et al (20). Selected sequences were then sub-cloned from M13 RFs into expression vectors (above).

B. Internal Deletions: An EcoRI/HindIII fragment containing the S20 coding region was isolated and partially digested with the enzyme Sau3A. The mixture was randomly ligated into M13mp8 which had been previously digested with EcoRI and HindIII and calf intestinal alkaline phosphatase. Inserts of appropriate sizes for deletions (and rearrangements) were sequenced. Selected EcoRI-HindIII fragments were sub-cloned into expression vectors as above.

Coupled Transcription/Translation in vitro:

The procedure of Zubay (21) was followed as modified by Mackie (22) and with these further changes. Plasmid concentrations were limited to approximately 5 μg per ml in incubations performed at 34°C for 30 min. [^{35}S]-Methionine was included for labelling and aliquots were immediately frozen in liquid nitrogen. Yields of S20 were assessed as follows. A fraction of each incubation was precipitated with 5 volumes of acetone and applied to a 16.25% polyacrylamide slab gel (0.75 mm thick) based on the SDS/urea system developed by Swank and Munkres (23). Wet gels were processed for fluorography and then dried and exposed. Slices containing S20 or its derivatives were excised and rehydrated in 50 μl ddH_2O. After 1 hr, 10 mls of a

mixture of 3% Protosol in Econofluor (NEN) was added and the vials incubated at 37°C overnight before counting. This method was suggested by New England Nuclear. Another sample from each incubation was counted directly to obtain the specific activity of methionine, from which yields of S20 were derived.

Isolation of 16S rRNA:

RNA was isolated from ribosomes of strain MM294 by centrifuging crude 70S ribosomes through sucrose gradients containing 0.1 M LiCl, 0.5% SDS, 10 mM Tris (pH 7.5), and 1 mM EDTA (24). Where required, cultures were labelled with [^{3}H]-uridine (100 μCi / 200 mls of culture). Dilutions of radioactive and unlabelled RNA yielded approximately 660 DPM per μg of 16S rRNA.

RNA-Protein Association:

Samples of [^{3}H]-16S rRNA (11 pmoles containing 4000 DPM) were incubated at 37°C in 40 μl of TMK buffer (50 mM Tris [7.6], 20 mM $MgCl_2$, and 350 mM KCl) containing 6 mM β-mercaptoethanol for 30 minutes, and then chilled. Aliquots of the in vitro translations were thawed immediately before use and centrifuged for 30 sec. at 10,000 xg to remove precipitates. Protein, RNA, and 2 μg bovine serum albumin (BSA) were incubated for 30 minutes on ice in a total volume of 50 μl. Incubations were immediately loaded onto Bio-Gel A-0.5m columns (25) and eluted with TMK buffer containing 6 mM β-mercaptoethanol and 10 μg per ml BSA. Bio-Gel A-0.5m was found to give the best separation between 16S rRNA and unbound proteins and unincorporated [^{35}S]-methionine from the in vitro translation. Twelve fractions of 7 drops each were collected and counted directly in 2.5 mls PCS (Amersham). In each case a blank reaction (minus RNA) was run to quantify the radioactivity coeluting with 16S rRNA.

S20 Messenger RNA:

Fragments containing the S20 coding and proximal promoter regions were cloned into the vector pSP65 (26) and used as templates for transcription of S20 mRNA essentially as suggested by Promega Biotech. Each 50 μl transcription reaction included 1 μCi of [^{3}H]-UTP. Transcripts were denatured with glyoxal in 50% DMSO and verified to be intact by electrophoresis in a 1.5% agarose gel followed by staining with ethidium bromide (27).

RNA-protein association was assayed essentially as above with the following changes. Reactions contained a total of

approximately 6 pmoles of transcript containing 60,000-80,000 DPM of [^{3}H] in a total volume of 25 μl. S20 translations were made 2 mM in DTT and supplemented with 1-2 U/μl of RNasin before being added to RNA.

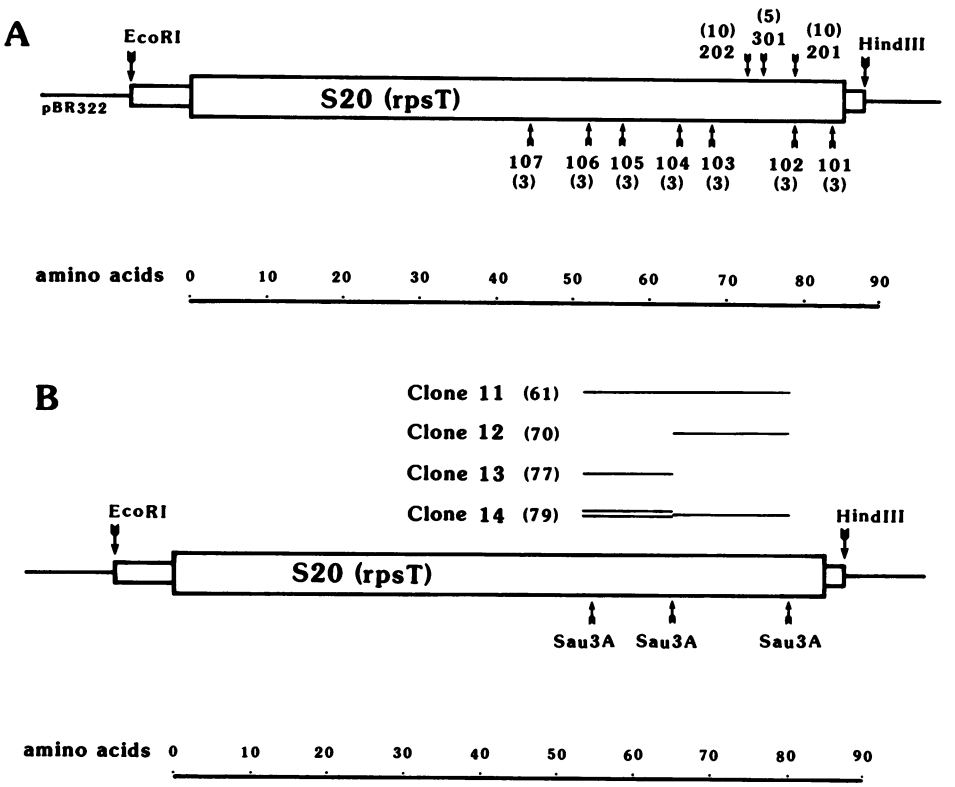

Fig. 1.(a) Map of C-terminal deletions of ribosomal protein S20. The plasmid pGM24 contains an EcoRI-HindIII fragment which includes the S20 coding region and leader sequence in the vector pBR322. The end point of each of the characterized Bal 31 generated deletions is marked. Numbers in brackets indicate the number of additional amino acids added to the C terminus by run-on. The plasmid carrying a full length EcoRI-HindIII insert in pKK223-3 is designated pCD6.

(b) Map of internal deletions of ribosomal protein S20. The locations of the three Sau3A restriction sites used in constructing these deletions are indicated. Deleted sequences are represented by single lines (——), while a duplicated sequence is shown as a double line (═══). Numbers in brackets indicate the number of amino acid residues remaining in each mutant.

RESULTS

S20 Deletions:

The diagrams in Figure 1 illustrate the structure of the S20 sequence used for all constructions (pGM24), as well as the end points of the characterized deletions. Since Bal 31 digestion of the EcoRI-HindIII fragment coding for S20 removes the natural S20 stop codon, C-terminal deletions were cloned into expression vectors directly adjacent to a pBR322 fragment (residues 29 to 651) which carries closely spaced stop codons in all three reading frames. As a result, C-terminal deletions carried amino acid run-ons of 3, 5 or 10 residues depending on reading frame. The three possible "tails" are shown in Table 1, and their potential effects on the proteins are considered below. Each of the numbered deletions was sequenced to predict the actual C-terminal sequences of the S20 derivatives accurately.

Coupled Transcription/Translation:

Coupled transcription/translation produced the different S20 products with varying efficiencies. In general, the shorter the coding sequence remaining, the lower the final yield of peptide. Actual yields varied from as high as 425 pmoles of S20 per pmole of template with the full length S20 sequence, to as low as 60 pmoles of mutant peptide per pmole of template with shorter clones such as 106 and 107. Attempts to increase these yields by including a number of inhibitors of proteolytic enzymes in the incubations were unsuccessful, and it remains unclear if this reduction in yield is a result of lower translational efficiency or increased proteolytic breakdown. Yields were also found to vary appreciably with the strain and growth state of cultures

Table 1. Sequences of possible S20 C-terminal run-ons.

Run-on length	Sequence
3 residues	-(x) lys leu stop
5 residues	-gln ala leu met arg stop
10 residues	-ala ser phe asn ala val val tyr his ser stop

x = gly, ser, cys, or arg.

used for preparing crude extracts, with the best extracts being obtained from cells harvested early in log phase.

Binding of in vitro synthesized S20 to 16S rRNA:

Protein-RNA complexes between S20 or its derivatives and 16S rRNA were formed and isolated as described in Materials and Methods. A binding curve obtained using complete S20 synthesized in vitro from pCD6 is shown in Fig 2(a). Panel (b) of Fig 2 represents a double reciprocal analysis of the same experiment. By this method, we estimate an apparent binding constant for S20 with 16S rRNA of approximately 1.2×10^7 (M^{-1}), with the number of binding sites calculated as 1.1. This compares favourably with an equilibrium value of 1.4×10^7 (M^{-1}) obtained by Craven and Schwarzbauer using the nitrocellulose binding method for measuring complex formation (28).

All of the points illustrated in Fig 2 represent incubations utilizing protein derived from a single translation. Although some variations in binding activity among the products of individual translations were found to occur, numerous different in vitro translations of normal S20 yielded no products with measured K_a values less than 1.0×10^7 (M^{-1}). Also, denaturing agarose gel analysis of 16S rRNA after separation of complexes indicated that it remained fully intact.

Specificity for S20 binding to 16S rRNA was established by several means. First, authentic unlabelled S20 competed for binding with labelled S20 that was produced in vitro: 30 pmoles of unlabelled S20 reduced the binding of 2 pmoles of [^{35}S]-S20 to 12 pmoles 16S rRNA in the standard assay by 50%. Secondly, analysis of proteins coeluting with 16S rRNA showed S20 to be the only labelled protein present. Finally, S20 synthesized in vitro was shown to have a low affinity for an unrelated RNA (clover yellow mosaic virus RNA). These methods were employed since the yields of S20 from in vitro translation are inadequate for demonstrating saturation of the 16S rRNA.

Binding of S20 Derivatives to 16S rRNA:

The ability of mutant S20 proteins synthesized in vitro to bind to 16S rRNA was assessed in the standard assay. The data in Fig 3 show that the removal of any residues from the C-terminus of S20 results in an immediate drop in its ability to bind to 16S RNA. The loss of only 6 C-terminal residues (clone 102)

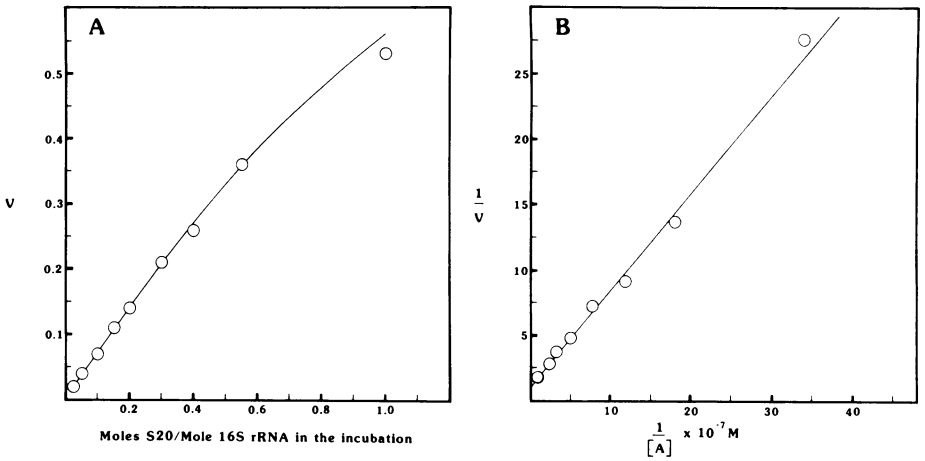

Fig. 2.(a) Binding curve for ribosomal protein S20 with 16S rRNA. Increasing amounts of a [35S]-labelled, pCD6 directed, in vitro translation were reconstituted with purified [3H]-labelled 16S rRNA as described in Materials and Methods. v = moles S20 bound / mole RNA.
Fig. 2.(b) Double reciprocal plot of S20 binding to 16S rRNA (from Fig. 2a). [A] is the molar concentration of unbound S20 ; the 1/v intercept is 1/n ; the slope is 1/nK where n is the number of binding sites and K is the apparent association constant.

eliminated most of the normal activity, and the loss of another 14 (clone 104) completely removed it. A third clone, (202), which carries the longest amino acid run-on, is in fact of the same size as normal S20 and differs from it only in the identity of the last 10 residues. This derivative displayed less than 4% of the binding of normal S20. Examination of the activity of a second similarly sized deletion carrying the alternative 5 residue run-on (clone 301), shows that this "tail" can confer an intermediate level of binding activity relative to the longer, but less active 10 amino acid version. The signifigance of these "tail" effects will be discussed below.

Several internal deletions and a rearrangement of internal sequences were also produced, by making use of 3 in-frame Sau3A sites in the coding region of S20 (Fig 1b). Removal of the entire region bounded by these sites or just the downstream half resulted in a total loss of measurable binding. However, after removal of only the upstream fragment (clone 13), an intermediate

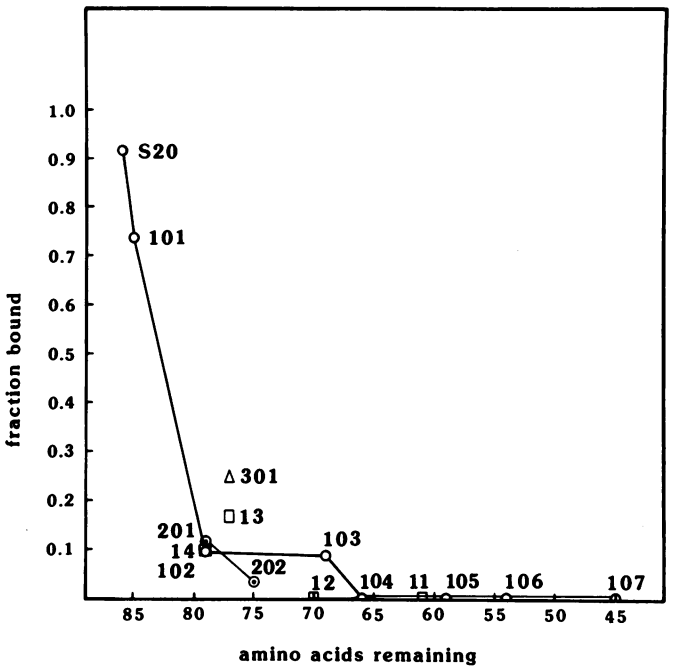

Fig. 3. Binding levels of mutant S20 polypeptides to 16S rRNA as a function of deletion size. The graph shows the fraction bound of each peptide to 11 pmoles of 16S rRNA in the standard assay. Input of protein is constant at 1 pmole and the levels are plotted against the number of amino acid residues remaining in the peptide. Each family of amino acid run-ons (Table 1), is represented by a different symbol: ○ = 3 amino acids, △ = 5 amino acids, ⊙ = 10 amino acids, □ = internal deletion.

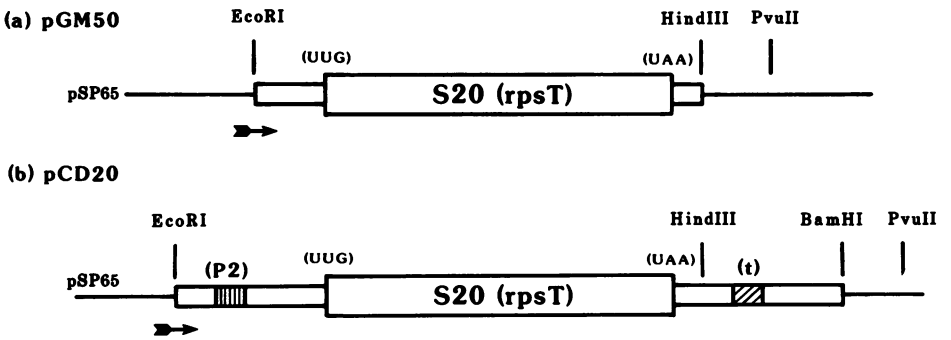

Fig. 4. Structure of plasmids used to generate S20 mRNAs in vitro. Two S20 encoding fragments were cloned into the expression vector pSP65 using the EcoRI and HindIII or EcoRI and BamHI sites in the vector polylinker to create the plasmids pGM50 and pCD20. (⟶) = transcription start site. (P2) = second S20 promoter. (t) = S20 terminator.

level of activity remained. Again, the extreme C-terminal region was critical. Replacement of the deleted downstream fragment with a duplication of the upstream Sau3A fragment (clone 14) generated a mutant S20 slightly less active than that in clone 13.

S20 Binding to S20 mRNA:

Fragments containing the S20 leader and coding region, or second promoter, leader, coding region and terminator, were cloned in pSP65 as illustrated in Fig 4. The constructs were cut at the HindIII or PvuII sites for (a), and HindIII or BamHI sites for (b) before transcription. The expected transcript sizes were 335 and 518 bases, and 403 and 545 bases respectively. A single reaction for each was visualized on a denaturing agarose gel (see Materials and Methods), and the appropriate bands are marked on Fig 5. The SP6 polymerase also showed some tendency to terminate on encountering the natural S20 terminator (lane b of Fig 5).

Samples representing 6 pmoles of transcript were tested for binding with 3 to 4 pmoles of full length S20 in a total volume of 25 μl by essentially the same method as that used for 16S rRNA

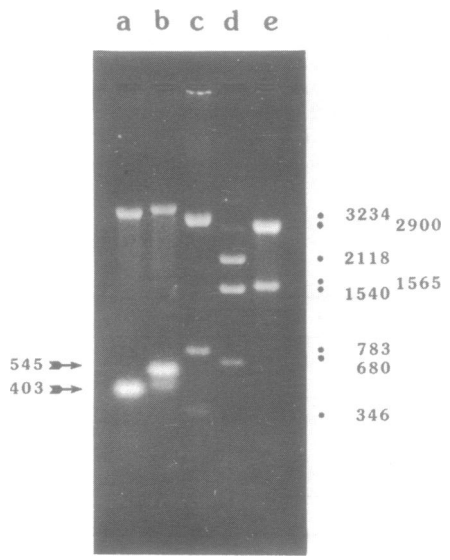

Fig. 5. Denaturing agarose gel of pCD20 transcription products. Lane a, pCD20 (HindIII); lane b, pCD20 (BamHI); lanes c to e, size markers. Transcript sizes are as marked in the left hand margin. Marker sizes are indicated in the right hand margin. Lane b also shows a band (436 residues) corresponding to transcripts terminated at the natural S20 terminator.

(see Materials and Methods). No binding was detected. We also tested for binding using the "medium salt" (MS) buffer used by Deckman and Draper (15) in measuring the affinity of S4 for α operon mRNA. No measurable binding was found with any transcript under these conditions. Examination of the RNA on a denaturing gel after assay showed that it was still intact. In addition, further attempts to detect association using a gel retardation assay also failed to show any binding of S20 to its own mRNA.

DISCUSSION

The method presented here for studying protein structure-function relationships is significant in its utilization of mutant polypeptides derived by *in vitro* synthesis for binding studies. The main advantages realized by this approach are: (1) changes in protein structure can be more readily implemented through manipulation of DNA than by alteration of protein structure via proteases, (2) potentially labile protein conformations can be obtained without the requirement of purification and any harsh treatments this usually entails, (3) the ability to study partially active mutants whose properties make them impossible to generate or purify *in vivo*, and (4) the ability to synthesize labelled proteins of sufficient specific activity to be detected in very small quantities without purification.

The presence of other labelled proteins and unincorporated [^{35}S] in our standard assay precludes the use of filter binding methods for measuring protein-RNA association. A limitation of the alternative gel filtration method is the inability to measure equilibrium values. This is probably of minor significance in the case of a strong interaction with a large electrostatic component such as that of 16S rRNA binding a very basic ribosomal protein like S20, but weaker mutant interactions might be more strongly affected. Competition experiments performed with the most active S20 mutants (data not shown), suggest that their equilibrium binding activities could in fact be somewhat larger than our measurements derived using gel filtration might indicate.

An analysis of the amino acid sequence of S20 by the method of Garnier *et al* (29) predicts that S20 may be composed of 3 major helical segments, with the C-terminus representing a region of high probability for α helix. A similar analysis of the mutant

sequences indicates that while the shortest 3 residue run-on is effectively neutral, the 5 and 10 residue "tails" favour helical and extended conformations respectively. The loss of virtually any residues from the C-terminus (as represented by clones 101-107), results in an immediate deterioration of 16S rRNA binding, leading to its complete elimination after the loss of more than 14 amino acids. In addition, substitution of the 10 C-terminal residues of S20 with a random sequence favouring an extended configuration (clone 202), also led to the loss of binding activity. These results suggest a helical configuration is crucial in this region. This conclusion is further supported by the intermediate level of binding activity produced by substituting the 5 residue helical "tail" in another deletion missing a similar number of normal S20 residues (clone 301 compared to 202).

Manipulations of internal S20 sequences are also consistent with this conclusion. The two segments delimited by Sau3A restriction sites in the S20 coding region each represent essentially one half of the third helical region of the protein. Removal of this whole region or the downstream half (clones 11 and 12) resulted in total loss of measurable binding, while the loss of just the upstream fragment (clone 13) left an intermediate amount of activity. Again, the extreme C-terminal region was critical. Replacement of the deleted downstream fragment with a duplication of the upstream one (clone 14) failed to restore more than a low level of activity.

These data suggest that C-terminally deleted mutants of S20 are not significantly capable of binding 16S rRNA due to the absence of a critical region of α helix proximal to the normal C-terminus. This region could be essential for the integrity of the secondary and tertiary structure of S20. In view of the earlier data of Paterakis et al (10), it seems likely that N and C terminal segments of S20 interact with each other to generate a conformation of the protein capable of binding to 16S rRNA.

We were surprised to find that the apparent affinity of S20 for its mRNA was so much lower than for 16S rRNA. We estimate that the K_a for S20-S20 mRNA binding would be no greater than at most 10^5 (M^{-1}), or essentially non-specific. Alternatively, if

S20 does indeed bind specifically to its own mRNA, it must also dissociate very quickly. However, rapid association-dissociation of protein and nucleic acid does not appear to be characteristic of interactions of known regulatory signifigance, as in for example, the lac operon (30) or phage lambda (31). It has previously been thought that to be competitive, both assembly and translational repression would utilize a common site on the protein, and therefore recognize similar sites on RNA. The finding that S4 binds to both 16S rRNA and to transcripts containing its presumptive "translational operator" supports this hypothesis, at least for the α operon. In view of the convincing evidence that S20 exhibits regulation at a post transcriptional step (12,13), the fact that we cannot detect an interaction by S20 with its mRNA suggests the possibility of a different mechanism, perhaps involving recognition by S20 of an intermediate in the initiation of its own synthesis (G.D. Parsons, B.C. Donly, and G.A. Mackie; manuscript submitted). However, the full implications of this result with S20 will have to await the emergence of quantitative data from other ribosomal operons.

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