THE IDENTIFICATION OF THE RNA BINDING SITE FOR A 50 S RIBOSOMAL PROTEIN BY A NEW TECHNIQUE

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1. Introduction

It is known that some of the proteins of *E. coli* ribosomes can bind individually and specifically to the ribosomal RNA's *in vitro* [1–5]. This presumably reflects fairly accurately the specific interactions between the RNA's and these proteins which exist *in vivo*. The identification and eventual sequence analysis of such binding sites on the RNA's will be necessary for any detailed description of these specific RNA–protein interactions which underlie both ribosomal assembly and function. Some progress has been made in this direction recently. The sites on the 16 S RNA to which certain proteins from the 30 S ribosomal particle become attached have been localised within the primary sequence of the RNA [6, 7]. This was done by binding the proteins to specific fragments of the RNA [6] or by isolating regions of the RNA protected against nuclease digestion by the presence of a protein [7]. Analogous studies of some components of the 50 S ribosomal particle have revealed that three proteins participate in binding the 5 S RNA to a region somewhere within the 3′-two-thirds of the 23 S RNA [8–10]. It was also shown that a portion of the 5 S RNA molecule within the complex is shielded against nuclease attack, and this area is therefore likely to be directly involved in forming the complex.

In this article we describe a new technique which we anticipate might prove generally useful in further studies of those regions of the RNA's involved in the specific binding of the ribosomal proteins. This method employs two gel electrophoresis steps: 1) in order to isolate firstly a specific ribonucleoprotein fragment resulting from nuclease digestion of the RNA–protein complex; 2) followed by the isolation and purification of the protected RNA component. It is particularly suitable for use with highly radioactive [^{32}P]RNA, which can be subsequently submitted to nucleotide sequence analysis.

We have applied this method in the first instance to obtain the binding site on the 23 S RNA for the 50 S protein L24. It has been previously reported that a protected area of the 23 S RNA can be recovered both from a nuclease-treated 23 S RNA–L24 complex and from an RNP fragment containing L24, isolated from nuclease-digested 70 S ribosomes [11]. In this paper we report the purification and characterization of this protected area, using ^{32}P-labelled material, and its position within the 23 S RNA molecule. This protected material consists of multiple fragments encompassing in all 350–370 nucleotides. A comparison of the products arising upon fingerprinting these fragments with the oligonucleotide maps which have been established during sequence analysis of the 23 S

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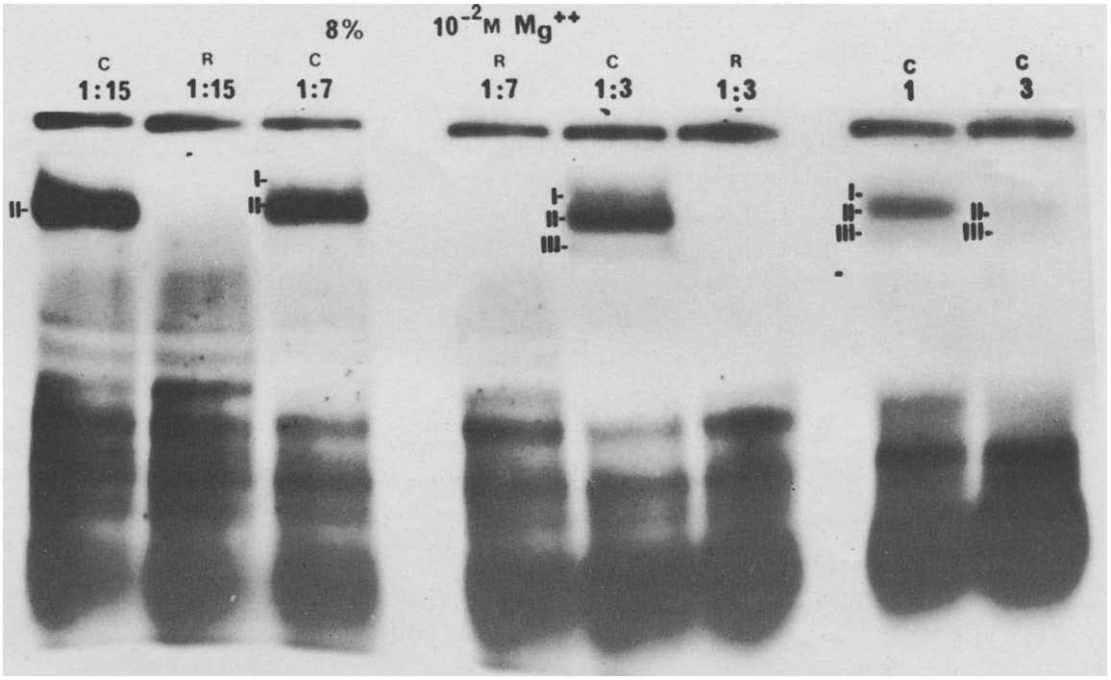

Fig. 1. Fractionation of the products arising from hydrolysis of the 23 S RNA–protein L24 complex (C) and the 23 S RNA alone (R) at different enzyme:substrate ratios, by electrophoresis on a polyacrylamide gel slab in the presence of 10^{-2} M magnesium (see Materials and methods section).

RNA has permitted us to localise the position of this protected area within the RNA molecule. It extends from 15 to about 550 nucleotides from the 5′ terminus of the 23 S RNA.

2. Materials and methods

2.1. *Preparation of [^{32}P] 23 S RNA*

Highly labelled 70 S ribosomes were prepared from *E. coli* (MRE 600) according to Fellner [12]. The ribosomes were suspended in a buffer (TM4) containing 10^{-4} M magnesium acetate, 10^{-2} M Tris–HCl, pH 7.5, at a concentration equivalent to about 0.8 mg RNA/ml. They were deproteinised by 3 successive extractions with equal volumes of buffer-saturated phenol. The 23 S RNA was fractionated from the other RNA components by sucrose density gradient centrifugation, according to [12].

2.2. *Complex formation*

Complexes of the 23 S RNA with the purified protein L24 (the purification was carried out in the way described in [11]) were prepared according to Stöffler et al. [5], in the reconstitution buffer of Nomura and Erdmann [13]. The 23 S RNA was dissolved in this buffer at a concentration of 2 mg/ml. We added 2 moles of protein/mole of RNA for complex formation. The total volume normally employed was kept small (not more than 0.5 ml), because of the subsequent gel electrophoresis step (see below).

2.3. *Enzymatic digestion of the complexes*

The RNA–protein complexes were digested with T_1 ribonuclease in the reconstitution buffer, for 30 min at 0°C. Enzyme:substrate ratios ranging from 1:15–3:1, on a weight basis, were used. The hydrolysates were directly subjected to electrophoresis.

2.4. *Polyacrylamide gel electrophoresis*

The products arising upon enzymatic digestion of the RNA–protein complexes were fractionated by 3

successive electrophoresis steps on polyacrylamide gel slabs. These procedures have been largely previously described by Ehresmann et al. [14], and are summarised here.

2.4.1 The digests were each mixed with an equal volume of a 50% (w/v) sucrose solution, containing bromophenol blue marker dye, and applied to an 8% gel made up in TMA buffer (10^{-2} M magnesium acetate, 5×10^{-3} M Tris–acetate, pH 8). Electrophoresis was continued for 16 hr at 4°C, with a potential difference of 10 V/cm. The products were detected by autoradiography. The bands containing RNP (see the Results section) were excised and dialysed against 8 M urea for 2 hr at room temperature, to remove the electrophoresis buffer, and facilitate the dissociation of the RNA and protein.

2.4.2. These bands were then subjected to electrophoresis on a second gel using a method based on that introduced by Vigne and Jordan [15]. The bands were placed on the top of a gel consisting of 2 layers, containing 12% and 15% polyacrylamide respectively. This is illustrated in fig. 2. These layers were made up in a buffer containing 8 M urea, 2.5×10^{-3} M EDTA, 0.1 M Tris–borate, pH 8. A further shallow layer of 12% gel, containing 0.1% SDS and 8 M urea, but no buffer, was polymerised around the bands. Electrophoresis was continued for 16 hr at 4°C, with a potential difference of 20 V/cm. This step served to dissociate the RNA fragments from the protein and from each other, and to fractionate them. The introduction of a gel layer lacking electrophoresis buffer caused a zone-sharpening effect, described by Vigne and Jordan [15] and Ehresmann et al. [14], which presumably contributed to the very good separation reproducibly obtained.

2.4.3. The RNA bands obtained in this way were subjected to a final repurification by electrophoresis on a further polyacrylamide gel slab. This was made up and run in the same way as the previous gel, except that no SDS was included in the upper layer and the lower layers of gel were more concentrated. Fragments contained in 12% gel after the second electrophoresis step were generally repurified by electrophoresis in 15% gels and material found in 15% gel layers was refractionated in 20% gels.

2.5. *Sequence analysis of RNA*

The sequence analysis of the radioactive RNA fragments was carried out by the methods of Sanger et al. [16, 17] and will be fully described elsewhere.

3. Results

3.1. *Isolation of RNP fragments*

The fractionation of the products arising from enzymatic digestion of the 23 S RNA–L24 complexes is shown in fig. 1. In the presence of the protein a part of the RNA is found in the upper part of the gel which is entirely absent in the control digestions carried out on the 23 S RNA incubated in the absence of protein L24. It would be expected that any resistant RNA fragments remain bound to the protein L24 under the electrophoresis and digestion conditions used, as ribonucleoprotein fragments. We confirmed this by isolating such RNP fragments using [^{3}H]*N*-methyl-labelled protein L24 at high specific activities, in the way described in [11]. This will be described more fully elsewhere.

The amount and nature of the RNP fragments obtained varies with the vigour of the enzymatic hydrolysis employed. It may be seen that the overall quantity of protected RNA decreases with increasing enzyme:substrate ratios, particularly at very high enzyme concentrations. It may also be seen that whereas a single rather broad band (II) is present under the mildest digestion conditions, a further band (III) appears with stronger digestion. Band I may represent a tailing of band II (see below) or may be slightly different.

3.2. *Characterization of the RNA present in the RNP fragments*

The fractionation of the RNA fragments released by dissociation of the RNP complexes is shown in fig. 2a. A large number of fragments of RNA are separated by gel electrophoresis. The primary RNP fragments I and II give similar patterns of products, and it is unclear whether there is any difference between them. The RNA fragments derived from the RNP band III also have similar mobilities, but their relative amounts clearly differ from the products arising from bands I and II. This is explicable in terms of the results described below.

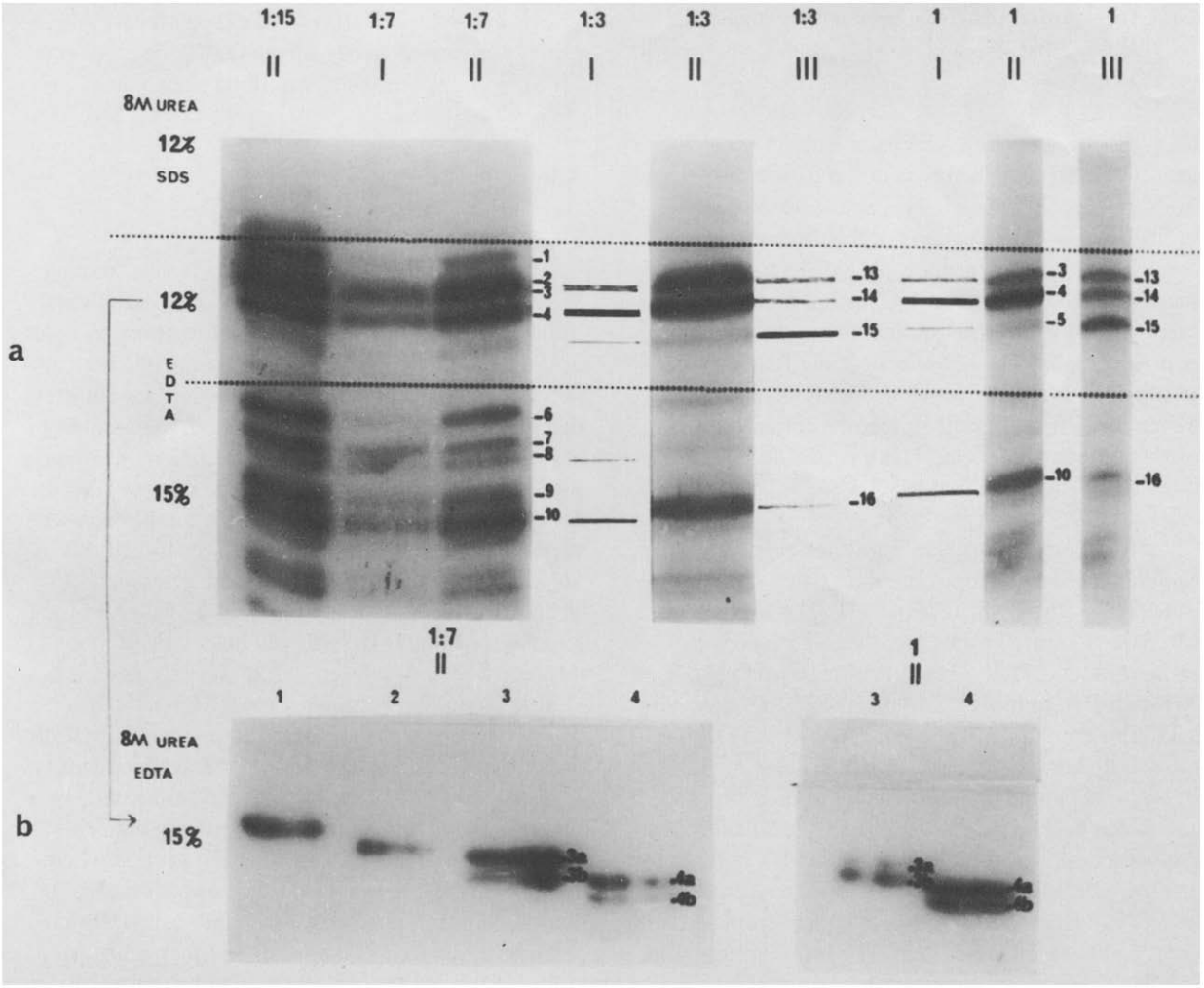

Fig. 2: a) Fractionation of the products resulting from dissociation of the RNP fragments shown in fig. 1. by electrophoresis in a polyacrylamide gel slab containing SDS and 8 M urea; b) repurification of these products by electrophoresis on a polyacrylamide gel slab of higher concentration (see Materials and methods section).

These RNA fragments were further characterized by fingerprinting methods, after refractionation by electrophoresis on 15% or 20% polyacrylamide gel slabs (see Materials and methods). The complete nucleotide sequences of all of the products arising from T_1 ribonuclease or pancreatic ribonuclease digestion of all of these fragments have been determined in connection with studies on the primary structure of the 23 S RNA which are being carried out (C. Branlant et al., in preparation). Quantitative studies of the frequencies of occurrence of the oligonucleotides in individual fragments, and overlapping fragments derived from other types of partial enzymatic hydrolysis carried out on the 23 S RNA provide us with both estimates of the lengths of the various fragments and their positions relative to each other and in the 23 S RNA. This work will be fully described elsewhere, but is used here to interpret the results presented concerning the nature of the RNA fragments from the protein binding site.

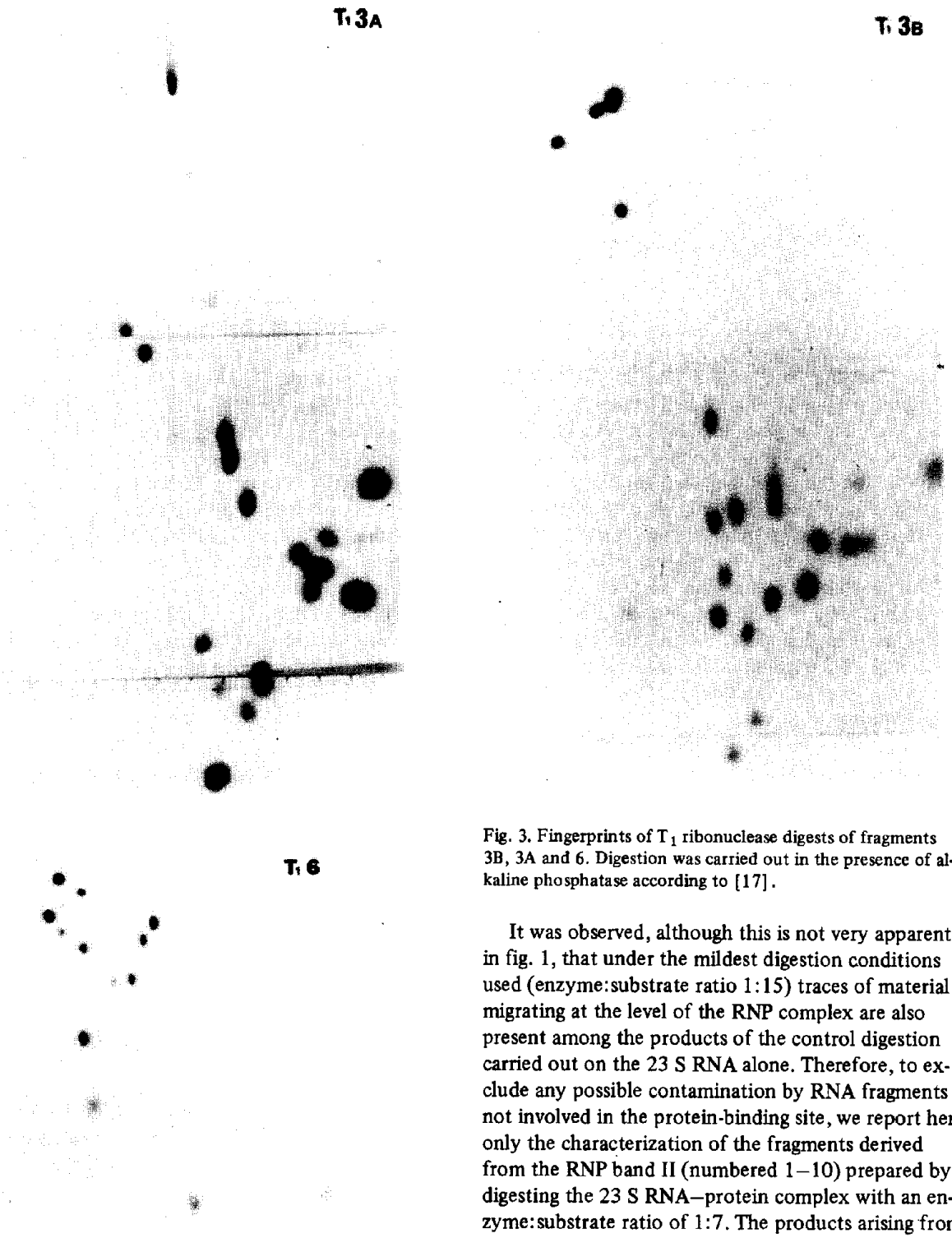

Fig. 3. Fingerprints of T_1 ribonuclease digests of fragments 3B, 3A and 6. Digestion was carried out in the presence of alkaline phosphatase according to [17].

It was observed, although this is not very apparent in fig. 1, that under the mildest digestion conditions used (enzyme:substrate ratio 1:15) traces of material migrating at the level of the RNP complex are also present among the products of the control digestion carried out on the 23 S RNA alone. Therefore, to exclude any possible contamination by RNA fragments not involved in the protein-binding site, we report here only the characterization of the fragments derived from the RNP band II (numbered 1–10) prepared by digesting the 23 S RNA–protein complex with an enzyme:substrate ratio of 1:7. The products arising from RNP complex III (numbered 13–16), obtained with very high enzyme:substrate ratios, are also discussed.

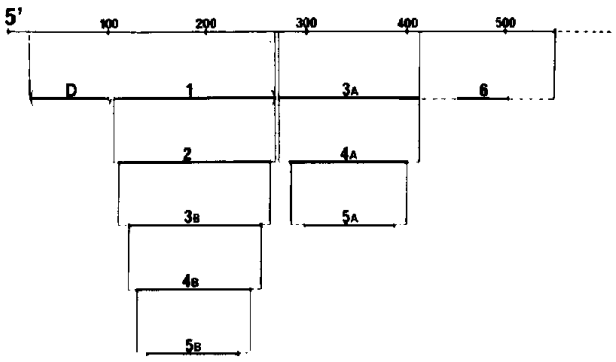

Fig. 4. A plan of the positions of the fragments isolated from the RNA–protein complex in the 23 S RNA molecule.

Fragments 3, 4 and 5 each yielded two components on refractionation (numbered 3A, 3B, 4A, 4B, 5A, 5B) as shown in fig. 2b. However, sequencing studies showed that all of these fragments are subsections of fragment 1 and 3A. The products 4A and 5A are related to fragment 3A, and the products 2, 3B, 4B and 5B to fragment 1. The fragments 15–17 from the RNP band III, only contained single components, identical with 3B, 4B and 5A respectively. Presumably the absence of fragments 3A, 4A and 5B from the RNP complex III is related to its greater electrophoretic mobility, and its appearance at very high enzyme substrate ratios.

The nucleotide sequence studies carried out on fragments 1 and 3A reveal that they contain about 160 and 140 nucleotides respectively, the fingerprints of fragments 3A and 3B are shown in fig. 3. The sequence analysis operations carried out on this region of the 23 S RNA also indicate that the fragment 1 and 3A are either contiguous or separated by a fragment D of about 80 nucleotides depending on whether the fragment D is placed before or after the fragment 1. The fragments 1 and D encompass a region extending from about 15–270 nucleotides from the 5′ terminus of the 23 S RNA while the fragment 3A occupied the region from about 270–410 nucleotides from the 5′ terminus of the 23 S RNA. This information is summarised diagrammatically in fig. 4. Fragment 6, migrating into the 15% gel, contains about 50 nucleotides, The fingerprint of this fragment is shown in fig. 3. Overlapping partial enzymatic digestion products from the 23 S RNA indicate that this fragment is derived from an area to the right of fragments 1 and 3A, but adjacent, lying 410–550 nucleotides from the 5′ terminus of the molecule. The remaining smaller products arising from the RNP band II have also been analysed, and contain sub-fragments of 1, 3A and 6 and some further small pieces believed to be derived from the neighbourhood of fragment 6.

4. Discussion

We have described above a new method of isolating the specific RNA binding site of a ribosomal protein which we believe may be generally applicable. However, it is clear that some limitations in the use of the technique exist. Firstly, the RNA–protein complex must remain stable under the electrophoresis conditions used. Secondly, the enzymatic digestion conditions must be sufficiently vigorous to digest the unprotected RNA to fragments with a size-range smaller than those contained in the complex. This might also damage the RNA in the complex sufficiently that it no longer remains attached to the protein, and therefore the size of the binding area may be subsequently underestimated. Equally (as discussed below) some RNA adjacent to the binding areas, but not in direct contact with the protein, may also be specifically protected. Finally, this method affords no formal proof that the specific binding site isolated with this experimental protocol completely corresponds to the site occupied by the protein within the ribosomal particle, although this is clearly very likely to be the case.

The nature of the protection of the RNA afforded by the presence of the protein is unclear. The protein (mol. wt. 11 000) is capable of directly shielding probably only a small part of the RNA (mol. wt. about 115 000) which is associated with it. Two explanations of the extensive protection which is observed occur to us: a) In the presence of the protein the RNA assumes a compact, specific tertiary structure, which renders it highly resistant to T_1 ribonuclease digestion despite being not directly in contact with the protein in the main; b) This region of the molecule has been found during sequence analysis to be relatively resistant to T_1 ribonuclease digestion even in the absence of the protein (C. Branlant et al., unpublished observations). The 23 S RNA is known to contain a similar amount of secondary structure to the 16 S RNA [18] involving as much as 70% of

the nucleotides at 25°C under physiological conditions. The 16 S RNA, on the basis of sequence analysis, is thought to consist of a series of hairpins of various sizes, linked by very short single-stranded regions ([19] and C. Ehresmann et al., unpublished results). If the 23 S RNA has a similar secondary structure, the observed protection could be due to the protein* directly protecting a small number of fragile points between a series of hairpins. In the control the RNA would be cut at these points, and the resistant hairpins would be found undigested amongst the products in the lower half of the gel (fig. 1). These products range up to a maximum of about 40 nucleotides in size. It is not possible to distinguish between these possibilities, and it is quite likely that both mechanisms contribute to the observed resistance. In this connection the considerable resistance of the protein L24 to digestion by trypsin and other proteases [11], both in the ribosomal particle and in the isolated protein 23 S RNA complex, is interesting. It suggests that the protein is surrounded by RNA, which occludes it from the enzyme. It might be imagined that the protein lies between several hairpins.

It is possible to exclude at least partially the argument that the observed resistance is more apparent than real, and is due to the retention of pieces of RNA in an RNA–protein complex, which would otherwise migrate in the lower part of the first gel (fig. 1). The larger components derived from the complex, when fractionated on the second and third gels (fig. 2) in the presence of urea, are shown by their mobilities and their fingerprints to contain over 100 nucleotides each. They cannot therefore be present in the digestion products of the control sample, which only range up to about 40 nucleotides in size.

It is interesting that in the 30 S particle the protein S 4 binds to an area close to the 5′-terminus of the 16 S RNA. This region extends over several hundred nucleotides [6, 7], and it has been suggested that multiple distinct binding sites on the RNA are in contact with the protein, separated by non-binding areas which are nevertheless protected to some extent against nuclease action in the presence of the protein. The results presented above suggest that the binding site for L24, at the 5′-terminus of the 23 S RNA, might possess rather similar structural characteristics to the S 4 binding site. Complete nucleotide sequence analysis of the 5′-terminal 500 nucleotides in the 23 S RNA has been undertaken (C. Branlant et al., unpublished work) and the amino acid sequence of the protein L24 is far advanced (R. Crichton, et al., unpublished work). It is clear that when this information is complete it may be possible to understand in much greater detail the specific nucleic acid–protein interactions responsible for the binding of this protein to the 23 S RNA.

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