ISOLATION OF FOUR RIBONUCLEOPROTEIN FRAGMENTS FROM THE 30 S SUBUNIT OF *E. COLI* RIBOSOMES*

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1. Introduction

One approach to obtain more insight into the structure of ribosomes is the partial fragmentation of the ribosomal subunits. Digestion of the 30 S subunit with ribonucleases leads to 30 S fragments containing proteins and pieces of rRNA. The analysis of protein contents of such fragments established groups of proteins, which were interpreted as being close neighbours in the 30 S subunit [1–3].

In this paper we will describe the isolation of four fragments of the 30 S subunit obtained by mild ribonuclease T_1 digestion and the identification of the proteins of the fragments by the two-dimensional gel electrophoresis [4]. Our results agree with a rearranged assembly map, i.e. the assembly map reflects the topological arrangement of the proteins within the 30 S particle. Furthermore the ability of the isolated fragments to bind streptomycin was studied.

2. Materials and methods

2.1. *Materials*

Ribonuclease T_1 (EC 2.7.7.26) was purchased from Boehringer, Mannheim, Germany; Bentonite-SF from Serva, Heidelberg, and activated charcoal, amidoblack and toluidineblue from Merck, Darmstadt. 30 S subunits were prepared as described previously [5].

2.2. *Isolation of the ribonucleoprotein fragments*

The conditions of hydrolysis were similar to those of Brimacombe et al. [6]. 3000 A_{260} units of 30 S subunits in 3 ml TMNSH buffer (0.01 M Tris-HCl, pH 7.5, 0.01 M $MgCl_2$, 0.06 M NH_4Cl, 0.006 M mercaptoethanol) were digested with 1000 units/ml T_1-ribonuclease for 20 hr at 5°. The reaction mixture was centrifuged for 17 hr at 25,000 rpm in the B XV zonal rotor which contained a hyperbolic gradient from 5–34% sucrose (w/v) with ionic conditions used for ribosomal reconstitution [7]: 0.03 M Tris-HCl, pH 7.5, 0.02 M $MgCl_2$, 0.3 M KCl, 0.006 M mercaptoethanol. Before use, the sucrose was washed with bentonite and activated charcoal as described previously [8]. The fractions containing the ribonucleoprotein fragments (RNP) were collected and the RNP's precipitated with 0.7 vol of ethanol [9]. RNP II and RNP III were purified with the Spinco SW 40 rotor in a sucrose gradient from 17–34% (w/v) and with ionic conditions as described above. 10 A_{260} units of the RNP were applied per tube. The particles were centrifuged for 10 hr at 36,000 rpm.

2.3. *Characterization of the RNP's*

The proteins in the fragments were identified by two-dimensional gel electrophoresis [4]. The gel electrophoresis of the fragments or of the RNA was performed as described by Wrede and Erdmann [10]. The fragments (0.5 A_{260} units per tube with 3.1% polyacrylamide gel) were run in two gels in a buffer with low ionic strength (0.01 M Tris-HCl, pH 7.8, 0.01 M $MgCl_2$, 0.03 M NH_4Cl, 0.006 M mercaptoethanol). One gel was stained with toluidineblue, the other with amidoblack.

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To demonstrate the RNA content, the RNA was extracted with the phenol method as previously described [8]. 0.5 A_{260} units were applied per tube of a 3.1% polyacrylamide gel. The buffer with high ionic strength contained 0.09 M Tris-boric acid, pH 8.3, 0.0025 M NA_2EDTA. The current was adjusted to 2 mA per gel and the electrophoresis was stopped when the dye marker bromophenolblue had run 7.5–10 cm.

Streptomycin binding assays were carried out by the method of equilibrium dialysis as described by Schreiner and Nierhaus [11]. Analytical centrifugation was performed in a Spinco Model "E" ultracentrifuge equipped with UV light source, multiplexer and photoelectric scanner. 5-Cell runs were made at 19° using an An-G Titanium rotor.

3. Results and discussion

3.1. *Characterization of fragments*

The ribonuclease T_1 digest from the 30 S subunits resulted in four peaks after zonal centrifugation (fig. 1). Peaks II and III containing the ribonucleoprotein fragments RNP II and RNP III were purified in a SW 40 rotor, since peaks I and IV showed very little or no contamination with the adjacent peaks. The RNP's

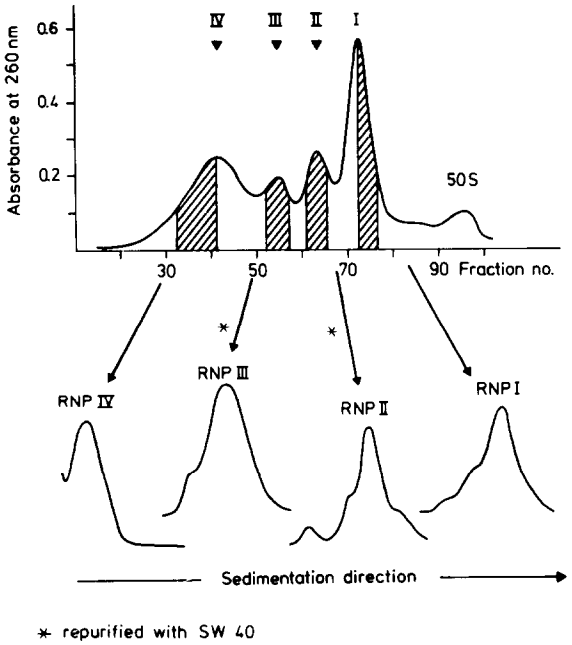

Fig. 1. Isolation of RNP fragments. The upper half shows the sedimentation profile after zonal centrifugation. RNP II and RNP III were repurified with the Spinco SW 40 rotor. The lower half shows the SW 40 profiles of the fragments used for the experiments.

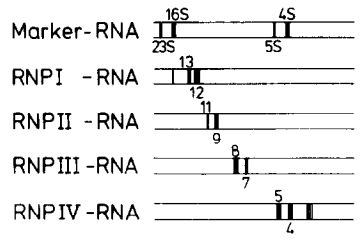

Fig. 2. RNA patterns of the RNP fragments. Electrophoresis in 3.1% polyacrylamide. As marker we used a RNA mixture extracted from 30 S subunits mixed with about 10% 50 S subunits. Soluble 4 S RNA was added.

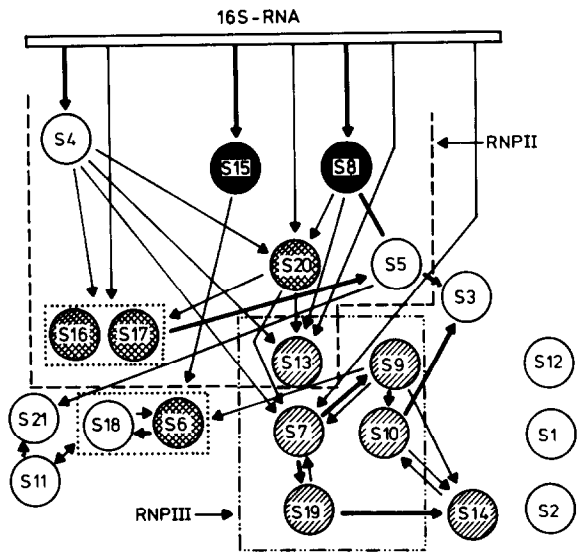

Fig. 3. Assembly map [13], taking in account the rearrangement by Morgan and Brimacombe [2] and the data of table 1. Different cross-hatchings identify the proteins from Morgan and Brimacombe's fragments Nos. 4, 7, and 8. Protein S21 is not in RNP fragment No. 8 (Brimacombe, personal communication). Dotted lines: Proteins of RNP II (- - -), RNP III (·--). S16 and S17 are separated by the two-dimensional electrophoresis, in contrast to the methods used by Nomura and Brimacombe. The placement of (S18, S6) is according to Mizushima and Nomura [13].

Table 1
Protein content of the RNP particles.

	S1	S2	S3	S4	S5	S6	S7	S8	S9	S10	S11	S12	S13	S14	S15	S16	S17	S18	S19	S20	S21
RNP I	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
RNP II	–	–	–	+	+	(±)	(–)	+	(–)	(∓)	–	–	(+)	–	+	+	(+)	–	–	+	–
RNP III	–	–	–	(±)	(±)	–	(+)	–	+	(+)	–	–	+	(±)	(±)	–	–	(±)	+	(±)	–

+ Normal; (+) reduced; (±) in traces; – absent.

had a purity of at least 90%. This was demonstrated by recentrifugation of an aliquot of the material on a sucrose density gradient in the SW 40 rotor and in gel electrophoresis (fig. 1). RNP I, II and III gave one band in a gel electrophoresis at low ionic strength (see sect. 2.3). RNP IV showed 3 bands in the 4 S–5 S RNA region; but only the band with the lowest mobility was stainable with the dye amidoblack specific for proteins. From the results obtained with the gel electrophoresis we concluded that each peak contains only a single fragment. The S-values of RNP I, II, III and IV were "32 S", "16 S", "11 S" and "3 S", respectively.

3.2. *RNA and protein content of the fragments*

After phenol extraction of each fragment, the RNA was applied to gel electrophoresis at high ionic strength. A schematic representation of the RNP-RNA's is shown in fig. 2. Each fragment contains two major RNA pieces. The smaller the fragment, the smaller were its RNA pieces.

The protein content was determined with the two-dimensional gel electrophoresis [4]. The data are summarized in table 1.

Table 2
Streptomycin binding to RNP particles and their RNA's.

	(% Streptomycin bound by) Particle	RNA
30 S	30.92 ± 2.35	6.53 ± 1.1
RNP I	31.44 ± 1.9	6.18 ± 0.51
RNP II	2.14 ± 1.13	6.78 ± 0.51
RNP III	5.78 ± 0.78	5.93 ± 0.74

Values are % of streptomycin input. Equilibrium dialysis was performed in cells with two chambers separated by a dialysis membrane, details are described elsewhere [11]. Each chamber had a volume of 50 μl; one was filled with 30 μl containing 1 A_{260} unit of RNP's or RNA's, the other with 30 μl streptomycin solution.

RNP I contained all of the 30 S proteins, although the RNA was cut in at least two long pieces. RNP I had no activity in the poly U assay (supplemented with the 50 S subunit) and the activity could not be restored after incubation under the reconstruction conditions of Traub and Nomura [12]. This loss of activity is most probably due to the conformational change indicated by the S-value (32 S) and induced by the hidden break(s).

The RNP II contained six proteins in normal amounts (S4, S5, S8, S15, S16, S20) and two proteins in reduced amounts (S13, S17). This RNP particle fits nicely in the assembly map [13] rearranged by Morgan and Brimacombe [2], see fig. 3. This particle resembles the 7 S particle described by Schendel et al. [3]. Both particles contain 8 proteins. However, these authors described S1 and S6 which we did not find in RNP II. Also we have found S8 and S13 in RNP II which were not present in their particle. The RNA of RNP II must be similar to the 12 S RNA piece [14], to which proteins, S4, S8, (S13), S15 and S20 bind. These binding proteins, together with S5, S16 and S17 are present in our RNP II.

In RNP III five proteins are contained in normal or reduced amounts: S7, S9, S10, S13 and S19. This particle is identical to Morgan and Brimacombe's RNA–protein fragment number 4 [2]. These authors could not distinguish between S14 and S19, but it is evident from our data that S19 is present in the fragment and S14 is not. All the proteins of RNP III except S13 belong to the second assembly step in 30 S biosynthesis (precursor p30 S → mature subunit) [8, 15, 16]. In contrast to the other particles it is very difficult to isolate RNP IV with a reproducible protein composition. Therefore we omit this particle from further discussion in this paper.

3.3. *Streptomycin binding to the fragments*

We tested RNP I, II and III with respect to the streptomycin binding ability (table 2). The binding behaviour of RNP II and RNP III is of particular interest: The RNA's of both particles bind about 6% of the input streptomycin whereas the RNP's show a different binding. RNP II binds significantly less and RNP III about the same amount of streptomycin as their corresponding RNA's.

Biswas and Gorini [17] recently reported evidence that RNA is the streptomycin binding element in the ribosome. However, Schreiner and Nierhaus [11] could demonstrate two binding types on ribosomes for streptomycin, an unspecific RNA type and a specific protein type. The unspecificity of the RNA type is demonstrated in table 2: an equal amount of the different RNA pieces bound the same streptomycin amount as 16 S RNA. Concerning the specific binding type we assume that the binding protein(s) is (are) not present in RNP II, because RNA from RNP II exerted a three fold higher streptomycin binding than the corresponding particle. The equivalence of streptomycin binding to RNP III and to its RNA indicates that the proteins of RNP III do not bury the streptomycin binding region of RNA, or, alternatively, the binding protein or one of the binding proteins is present in the RNP III. Thus, the four RNP III proteins S7, S9, S10, and S19 are candidates for the streptomycin binding sites. As S12 was shown to be involved in streptomycin binding [18] and this protein is not present in RNP III, the binding site for streptomycin should be composed of more than one protein. A further investigation into the binding properties of these proteins has been carried out by Schreiner and Nierhaus [11].

Acknowledgements

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