A STUDY OF THE ARRANGEMENT OF 18 S rRNA WITHIN 40 S SUBPARTICLES OF RABBIT RIBOSOMES

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1. Introduction

Knowledge of the disposition of both rRNA and protein moieties within the active ribosome should contribute to the unravelling of the mechanisms of ribosome function. Although nucleotide sequences of 18 S rRNA (S-rRNA) are becoming available [1,2] much remains to be discovered about the arrangement of the RNA chain within functional ribosomes and ribosomal subparticles. One method of studying rRNA conformation in situ is to treat ribosomes with nucleases to hydrolyse internucleotide bonds in regions of the RNA moiety at the ribosome surface and then to examine the digestion products. This approach, however, yields a complex mixture of products that is difficult to analyse. For example, it was shown that rRNA of rabbit ribosomes is cleaved at intervals of ~100 nucleotides [3]. The problem can be simplified by scrutinising only the population of fragments carrying the 3′-end of the rRNA species under investigation. This report describes the molecular size distribution of radioactive RNA fragments obtained after nuclease treatment of the smaller subparticle (S-subparticle) of rabbit ribosomes labelled with [^{32}P]pCp at the 3′-end of 18 S rRNA. The results confirm the wide distribution of cleavage sites along the polynucleotide chain and also reveal their positions measured from the 3′-end.

2. The principle of the method

The position of a cleavage site and its relative sensitivity to hydrolysis can be derived from a knowledge of the size distribution of terminally-labelled fragments and the relative radioactivity of the fragments. The principle is applicable to any polymer, but for simplicity it is illustrated by reference to radioactive S-rRNA, labelled at the 3′-end.

A radioactive S-rRNA fragment that is a single polynucleotide chain free of hidden breaks is generated by a single cleavage. The position of this cleavage measured from the 3′-end of S-rRNA is revealed by the size of the fragment. The radioactivity of a particular labelled fragment is proportional to its molar concentration because there is one radioactive residue/molecule. Thus, a plot of the radioactivity of the fragments *vs* fragment size reveals not only the positions of cleavage points but also the relative molar proportions of the fragments.

The distribution of radioactivity among fragments separated according to size can be analysed quantitatively. A fragment of n residues is generated when the constituent $n-1$ bonds remain intact and the n-th bond is cleaved. The probability of the fragment being produced is measured by the mole fraction of the product and this is related to the radioactivity (A_n) of this fragment. Under the conditions of the experiment the probability of bonds $1 \ldots n$ being cleaved is $p_1 \ldots p_n$ and the probability of bonds $1 \ldots n$ remaining intact is $(1-p_1)(1-p_2)\ldots(1-p_n)$. The probability of generating a radioactive product of n residues is given by eq. (1) where K is a proportionality constant:

$$K A_n = (1-p_1)(1-p_2)\ldots(1-p_{n-1})p_n \qquad (1)$$

Similarly, the probability of producing a radioactive fragment of $n+1$ residues is given by eq. (2):

$$K A_{n+1} = (1-p_1)(1-p_2)\ldots(1-p_n)p_{n+1} \qquad (2)$$

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The ratio of the radioactivities of fragments of n and $n + 1$ residues reflects the relative sensitivities of the n-th and $(n + 1)$-th bonds to hydrolysis [see eq. (3)]:

$$A_{n+1}/A_n = (1 - p_n)p_{n+1}/p_n \quad (3)$$

It is evident that the probability of n bonds remaining intact diminishes as n increases. Very low levels of hydrolysis are needed to reveal sensitive sites distant from the 3′-end and a complete spectrum of more sensitive sites is obtained when intact molecules can be detected after partial hydrolysis. If the intact molecule comprises Z bonds and Z + 1 residues, then p_z may be evaluated by applying eq. (1–3) [see eq. (4)]:

$$A_{z+1}/A_z = (1 - p_z)/p_z \quad (4)$$

Once p_z is known $p_1 \ldots p_z$ may be evaluated. Cleavage sites close to the 3′-end may be more closely scrutinised afer more extensive hydrolysis.

3. Materials and methods

The enzymes used were bought from the sources listed below: T_4 RNA ligase (Miles Biochem.), S_1 nuclease (Bethesda Res. Lab.), T_1 RNase (Calbiochem.), bovine pancreatic RNase (Boehringer). Amersham supplied [5′-^{32}P]pCp (2000–3000 Ci/mmol) and Fuji Photofilm provided X-ray film. Phenol was freshly distilled before use. Acrylamide and bisacrylamide (N,N^1-methylene bisacrylamide) were purchased from BDH and all other chemicals were of Analar quality.

Polyribosomes, isolated from rabbit reticulocytes [4], were dissociated into subparticles [5]. The S-subparticles were labelled with [^{32}P]pCp by means of T_4 RNA ligase as in [6] and kept at −80°C in storage buffer (0.1 M Tris–NH_4Cl/2 mM $MgCl_2$/10 mM Tris–HCl (pH 7.6)). Before nuclease treatment, S-subparticles in storage buffer were incubated at 37°C for 10 min to promote the native conformation (cf. [7]). A sample was quickly cooled on ice and kept at 0°C during the course of the experiment to provide a control which was otherwise treated in the same way as the nuclease-treated samples.

For treatment with pancreatic RNase and T_1 RNase, the reaction mixture contained 60 μg ^{32}P-labelled S-subparticle/10 μl storage buffer and either 0.05 ng pancreatic RNase, or 0.001 units of T_1 RNase. Treatment with pancreatic RNase was for 15 min at 0°C, whereas treatment with T_1 RNase was for 20 min at 20°C. Treatment with S_1 nuclease (2 or 20 units/assay) was for 5 min at 37°C and the reaction mixture comprised 60 μg ^{32}P-labelled S-subparticle/10 μl S_1-buffer [10 mM NH_4Cl/50 mM NaCl/0.2 mM $MgCl_2$/1 mM $ZnSO_4$/5% (v/v) glycerol/30 mM sodium acetate (pH 4.6)]. In each case the reaction was stopped by homogenising the reaction mixture immediately after the addition of 0.5 ml SDS buffer [0.2% (w/v) SDS/0.25 M sucrose/25 mM KCl/1 mM $MgCl_2$/50 mM Tris–HCl (pH 7.6)] and 0.5 ml water-saturated phenol. After centrifugation the aqueous phase was removed and the RNA precipitated by the addition of 1/10th vol. 1 M NaCl and 2.5 vol. ethanol and storage at −20°C. The precipitate was dissolved in sterile ion-free water and freeze dried.

Samples were dissolved in 10 μl 8 M urea/20 mM Tris–HCl (pH 7.4)/1 mM EDTA/0.05% (w/v) xylene cyanol/0.05% (w/v) bromophenol blue, heated to 90°C for 30 s, then cooled quickly on ice and layered on a 12 cm polyacrylamide gel [2.5% (w/v) acrylamide/0.19% (w/v) bisacrylamide/7 M urea/1 mM EDTA/50 mM Tris–HCl (pH 8.3)]. The products of partial digestion were separated by electrophoresis at 200 V for 3 h. The molecular size of the S-rRNA fragments were calculated from their mobility using as markers 5 S rRNA (120 nucleotides), 5.8 S rRNA (160 nucleotides) and rabbit S-rRNA (1825 nucleotides), by analogy with *Xenopus laevis* S-rRNA [1]. The plot of the logarithm of molecular size *vs* mobility was linear.

After electrophoresis, the gels were dried and exposed with X-ray film for 1–5 days. Several autoradiographs differing in exposure time were obtained to establish the linear dose–response range of the photographic film and to visualise minor components. After autoradiography the optical density of the photographic film was measured using a microdensitometer (Joyce-Loebl, Newcastle-upon-Tyne, model E15, mark IIB).

4. Results

S-Subparticles in which the 18 S rRNA component had been labelled with [^{32}P]pCp at the 3′-terminus were treated with nucleases as above. These procedures were designed to produce ^{32}P-labelled fragments free of hidden breaks and so allow the position of the cleavage site generating the fragment to be measured

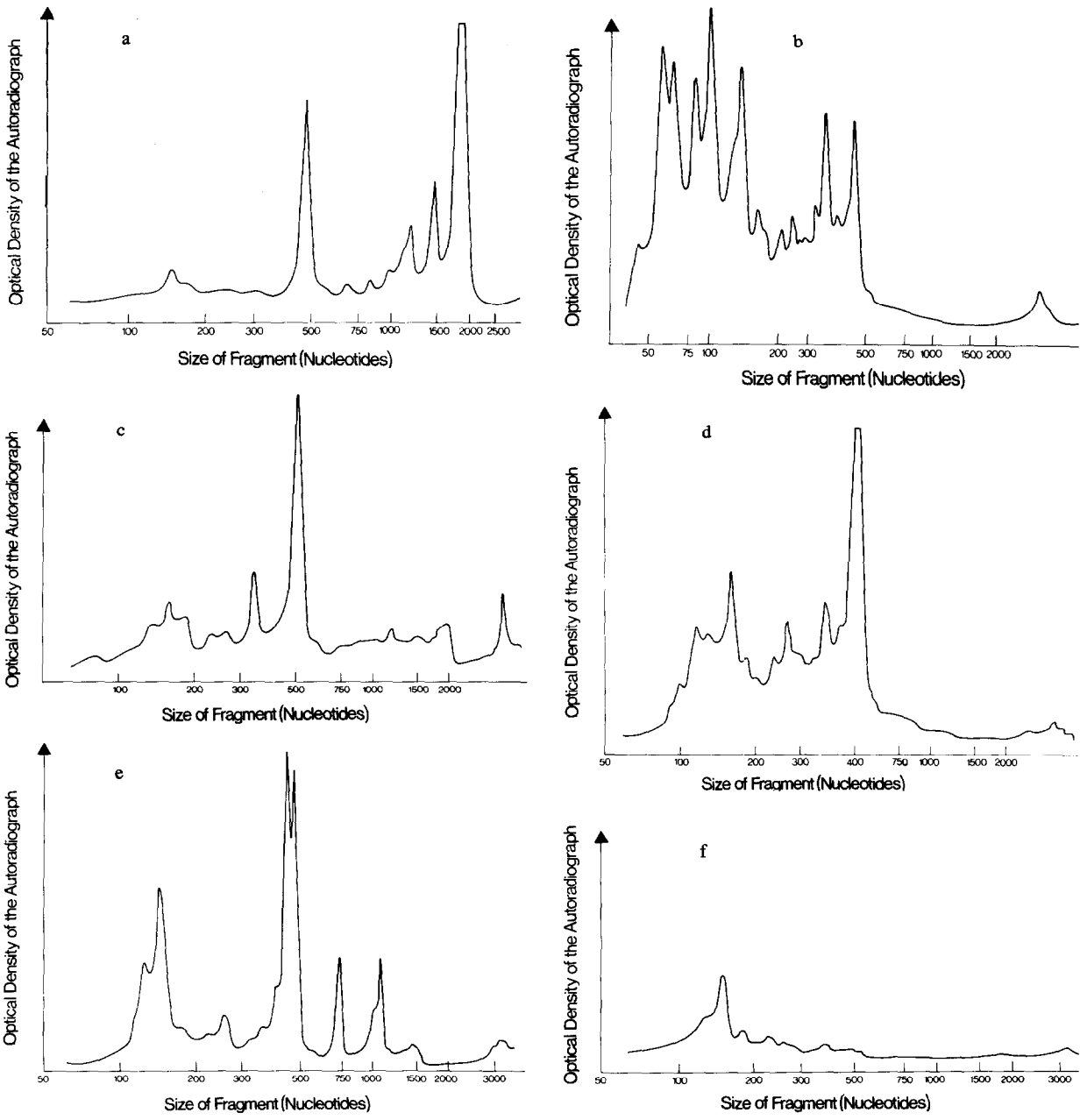

Fig.1. Separation of ^{32}P-labelled S-rRNA fragments by electrophoresis through 2.5% (w/v)-polyacrylamide gels under denaturing conditions. The results are presented as the profile of the absorbance of the photographic film versus the molecular size of the radioactive fragments. S-Subparticles were treated with nuclease, RNA was isolated, the fragments separated by gel electrophoresis, and mobility was related to fragment size as in section 2. The ordinate is a measure of the radioactivity of the ^{32}P-labelled fragments and hence of their abundance. Indeed, the ordinate is directly proportional to the mole fraction of the radioactive components. (a) S-rRNA from control S-subparticles which were partly hydrolysed by endogenous nuclease; (b) S-rRNA isolated after S-subparticles were treated with pancreatic RNase (0.05 ng enzyme/60 μg S-subparticle) at 0°C for 15 min; (c) S-rRNA isolated after S-subparticles were treated with T_1 RNase (0.001 units of enzyme/60 μg S-subparticle) at 20°C for 20 min; (d) S-rRNA isolated after S-subparticles were treated with T_1 RNase (0.01 units of enzyme/60 μg S-subparticle) at 20°C for 20 min; (e) S-rRNA isolated after S-subparticles were treated with S_1 nuclease (2 units of enzyme/60 μg S-subparticle) at 37°C for 5 min; (f) S-rRNA isolated after S-subparticles were treated with S_1 nuclease (20 units of enzyme/60 μg S-subparticle) at 37°C for 5 min.

from the 3′-end of S-rRNA. The radioactivity of the fragments is directly proportional to their abundance (see above). Plotting the absorbance of bands on the photographic film *vs* mobility is equivalent to a plot of the mole fraction of the radioactive fragment *vs* molecular size (see fig.2).

Interesting results were provided by S-rRNA from control S-subparticles exposed only to endogenous nucleases (fig.1a). More than 65% of the radioactivity was associated with intact S-rRNA showing that a low level of a partial hydrolysis had taken place. This revealed the widest spectrum of the more sensitive sites. At least 28 clear bands, distributed over 100–1780 nucleotides were identified on inspection of the autoradiograph. The most sensitive regions were found at 450–500 nucleotides, 1000–1200 nucleotides and 1350–1470 nucleotides measured from the 3′-end. From the distribution of fragments in the region 100–150 nucleotides from the 3′-end, it appears that sensitive sites are distributed at intervals of up to 35 nucleotides. Equations (3) and (4) were used to calculate values of p_n and the following examples serve to show the relative sensitivity to cleavage of different bands: $p_{150} = 0.02$; $p_{480} = 0.16$; $p_{1200} = 0.07$ and $p_{1470} = 0.12$. Thus, the bands in the region of 480 and 1470 residues from the 3′-end are most susceptible to cleavage.

Although each of the enzymes, pancreatic RNase, T_1 RNase and S_1 nuclease gave rise to its own distinctive pattern of radioactive fragments of S-rRNA, several features are common to all 3 profiles of radioactivity *vs* fragment size (see fig.1b,c,e). The degree of partial hydrolysis was not the same in each case and this is sufficient to account for the different proportions of the larger fragments in the partial hydrolysates. More extensive treatment diminished the yield of the larger fragments still further and more vividly reveal the presence of cleavage sites nearer to the 3′-end of S-rRNA (fig.1b,d,f). The profiles of mole fractions of these radioactive fragments *vs* size (see fig.2) show that the region of S-rRNA most readily cleaved by endogenous nucleases were also readily

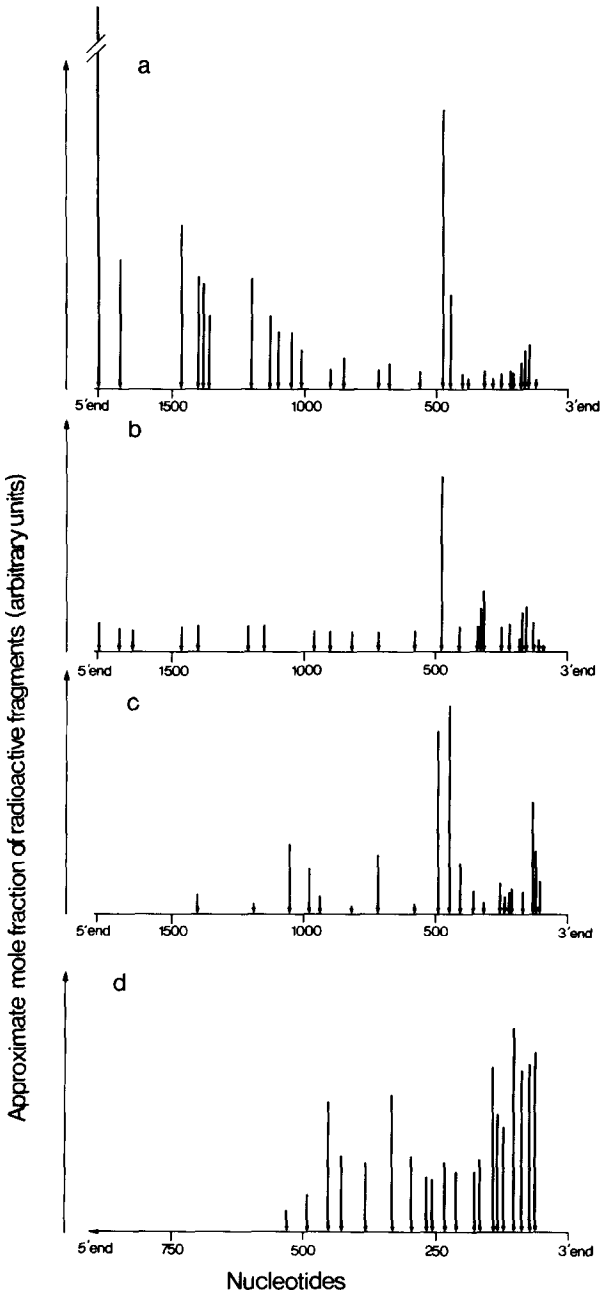

Fig.2. Summary of the location, measured from the 3′-end of S-rRNA, of internucleotide bonds cleaved by nuclease treatment of S-subparticles: (a) endogenous nuclease (see fig.1a); (b) T_1 RNase (see fig.1c); (c) S_1 nuclease (see fig.1e); (d) pancreatic RNase (see fig.1b). The length of the shaft of each arrow is proportional to the mole fraction of radioactive fragments produced as a result of the treatments in fig.1, and the width of the arrow-head indicates the possible error in locating the cleavage site. Only those fragments that gave rise to distinct bands in the autoradiographs are included in the figure. The ordinate is directly proportional to the probability (p_n) of hydrolysing the n-th bond during the course of the experiment (see above), e.g., in the experiment summarised in fig.2a, $P_{150} = 0.02$, $P_{485} = 0.16$ and $P_{1470} = 0.12$. Inevitably, the method highlights cleavage sites nearer to the 3′-end of S-rRNA (e.g., fig.2d).

attacked by the other enzymes. Regions of S-rRNA which were unaffected by nuclease extended over a region of ⩽150 nucleotides, e.g., nucleotides 580–630, nucleotides 720–820, nucleotides 1200–1300 and nucleotides 1470–1560 measured from the 3′-end.

5. Discussion

The S-subparticles used in these experiments were well characterised [8,9] and were shown to retain their capacity to function in protein synthesis after treatment to label the 18 S rRNA 3′-terminus [6]. Furthermore, S-subparticles treated with RNase under conditions similar to those used here retained activity in poly(U)-directed polyphenylalanine synthesis [9]. We infer that the S-rRNA radioactive fragments obtained after treatment of S-subparticles with either pancreatic RNase or T_1 RNase reflect the disposition of S-rRNA in the functional subparticle. The same conclusion is also likely to apply to the results obtained by S_2 nuclease treatment, even though this enzyme functions in acidic solutions and requires Zn^{2+}. Exposure to pH 5 does not diminish ribosome activity [10] and the secondary structure of S-rRNA is scarcely altered at pH 4.5 [11].

The results summarised in fig.2 confirm the earlier conclusion that S-rRNA is packaged in the S-subparticle in such a way that regions are accessible to RNase at intervals of ⩽100 nucleotides on average [3]. This report reveals the approximate location of sensitive sites and it also provides an indication of their relative sensitivity to enzymic hydrolysis. The sensitive sites of S-rRNA must be accessible to nucleases for cleavage to occur and so comprise regions that are exposed on the surface of the S-subparticle. Therefore, a substantial part of S-rRNA lies on the surface of the S-subparticles. A few regions were identified where up to ~150 nucleotides were found to be inaccessible to nucleases.

The above approach is capable of refinement. For example, the cleavage sites within 200 nucleotides of the 3′-end of S-rRNA can be identified with precision using long 8% polyacrylamide gels [12]. It is now possible to identify regions of S-rRNA that are sensitive to cleavage in the isolated S-subparticle but which are protected in the 80 S ribosome because of the interaction of the S-subparticle with the larger L-subparticle. The 3′-end of the S-rRNA component of S-subparticles reacts 17-times more readily with T_4 RNA ligase than the S-rRNA component of ribosomes [6]. The 3′-end of L-rRNA can also be labelled in functional 60 S subparticles and the same procedures used to investigate the conformation in situ. It may also be possible to investigate RNase-sensitive sites close to the 5′-end of S-rRNA or L-rRNA in situ if a method can be developed for attaching a label to the 5′-termini.

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