Polynucleotide fragments from the 28S ribosomal RNA of insects

Hajime Ishikawa

Department of Biology, College of General Education, University of Tokyo,
Meguro-ku, Tokyo 153, Japan

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ABSTRACT

If RNA is extracted from the ribosomes which had been isolated from frozen-thawed tissue of *Galleria mellonella*, the 28 S rRNA, when heated or treated with urea, dissociates into seven different species of polynucleotide fragments. They were designated as R1, R2, R3, R4, R5, R6, and R7, whose molecular weights were estimated to be 1.15×10^6 , 0.75×10^6 , 0.55×10^6 , 0.40×10^6 , 0.30×10^6 , 0.25×10^6 , 0.20×10^6 daltons, respectively. It is likely that R1 and R5 arise from a single nick in original 28 S rRNA. Experiments with isolated R1 suggest that it is made up of a hydrogen-bonded complex of R2 and R4. R5 is a complex of R6 and an unidentified species, X. It is suggested that these fragments result from nicks which are introduced, secondarily, in the phosphodiester bonds by an endogenous endonuclease(s). Since the secondary nicks are limited in number and located in specific points of the molecule, it appears that the reaction is quite specific.

It was also shown that the 28 S aphid RNA, which apparently lacks the primary nick, is susceptible to nicking.

INTRODUCTION

The 28 S ribosomal RNA from the larger ribosomal subunit of mammalian cells¹, Deuterostomian animals², and the 23 S rRNA from bacteria³ consist of single, uninterrupted polynucleotide chain. In contrast to this, a distinguishing feature of insect 28 S rRNA and other Protostomian and Protozoa 28 S rRNA's^{2,4} is their dissociation into 18 S products on treatment with heat, urea or dimethyl sulfoxide^{5,6,7}. This conversion results from a dissociation of two chains in 28 S rRNA held together by non-covalent bonding⁷. The monodispersity of the dissociation product suggested that the nick (primary nick) lies near or in the middle of the chain⁷.

In an earlier paper, the author demonstrated that other nicks (secondary nicks) can be introduced at specific points of the 28 S rRNA from insect tissue depending on the experimental conditions used during the isolation⁸. In this paper, polynucleotide products due to these secondary nicks are further described, and a mapping of these fragments is suggested permitting a further defining of the structure of insect 28 S rRNA and its topography in the ribosomes.

MATERIALS AND METHODS

(a) Materials

The greater wax moth, Galleria mellonella (L.) was cultured by the methods reported previously^{9,10}. Last instar larvae which had just started spinning were used throughout the present study.

A species of aphid, Aphis laburni (order Hemiptera; suborder Homoptera) was collected from sprouts of acacia trees in the Tokyo University campus, and reared on broad-bean plants in the laboratory. Asexual females of the aphid were used exclusively in the present study.

(b) Methods

The whole tissue of Galleria larvae was frozen and kept at -20°C for a week prior to homogenization. The thawed tissue was homogenized in 3 volumes of 0.02 M-Tris-HCl (pH 7.6) containing 0.4 M-sucrose, 0.02 M-KCl, 5 mM-MgCl₂ and 5 mM-phenylthiourea in a Waring blender at the maximum speed for 1 min at 4°C . The homogenate were filtered through a double fold of gauze and filtrate centrifuged at 10,000xg for 15 min. The resulting supernatant was again centrifuged at 105,000xg for 180 min. The microsomal pellet was kept overnight at -20°C , and then suspended in 0.05 M-Tris-HCl (pH 7.6) containing 1 mM-MgCl₂, 2 mM-phenylthiourea and 0.5 % sodium deoxycholate, and centrifuged at 105,000xg for 120 min. The crude ribosomes were rinsed twice in the buffer containing deoxycholate, once in the buffer without deoxycholate, and frozen^{11,12,13,14}. The ribosomes were homogenized in a glass homogenizer and the RNA isolated by published procedures¹⁵. The homogenizing solution consisted of equal volumes of acetate buffer (0.01 M-sodium acetate, 4 ug/ml potassium polyvinyl sulfate, 0.8 mg/ml Macaloid¹⁶, 0.5 % sodium dodecyl sulfate, pH 5.0) and water-saturated, neutralized phenol^{17,18}. The RNA solution was layered over a linear sucrose gradient and the 28 S RNA isolated^{7,19}. The 28 S rRNA of aphid tissue was isolated by the same procedures as above. The method of Bishop *et al.*²⁰ for polyacrylamide gel electrophoresis was followed with slight modification¹⁰. After electrophoresis, the gels were scanned for ultraviolet absorbancy with a Gilford recording spectrophotometer adapted for this purpose.

RESULTS

(a) Differential dissociation of 28 S RNA from Galleria ribosomes

The 28 S rRNA was extracted from the Galleria ribosomes, isolated by sucrose density gradients, and dissolved in E buffer (0.04 M-Tris, 0.02 M-sodium acetate, 1 mM-EDTA, pH 7.2). The solution of 28 S rRNA was divided

into more than fifteen equal fractions, which were then heated at various temperatures. After heating samples were rapidly cooled in ice, and subjected to gel electrophoresis. The results of nine different experiments are summarized in Fig. 1. It was evident that thermal treatments of the 28 S rRNA from 42°C to 75°C for 1 min gave rise to seven major products. They are designated, in accordance with their electrophoretic mobilities, as R1, R2, ---, and R7, while the 28 S RNA was represented by R0. Of interest is the parallelism observed in the rise and fall of different products. At lower temperatures, R1 arises in parallel with R5 (Fig. 1b, c), and as R1 decreases R4 rises (Fig. 1d, e, f). At higher temperatures, a decrease of R5 occurs in parallel with a rise of R6 (Fig. 1f, g, h, i).

(b) Precursor-product relationships

To further clarify these relationships, the thermal products were isolated and re-electrophoresed. For this purpose a large amount of 28 S rRNA was heated at 45°C for 1 min, and subjected to preparative gel electrophoreses. During the scanning the gel by U. V. absorbancy, the linear transport apparatus was switched off halfway, and the gel portions containing the product R1, R2 and R5 were cut out (see the inset of Fig. 2). This procedure permits the isolation of R1 from R0, and R5 from R4 without appreciable contamination. The gel containing the desired RNA was homogenized in E buffer, and stood overnight at 4°C to extract the RNA. The extracted RNA fragment was heated at various temperatures, and again subjected to gel electrophoreses in parallel with a standard 28 S rRNA which had been heated at 50°C for 1 min. The results with R1 are shown in Fig. 2. It is evident that R1 dissociates on further heating into two products, R2 and R4. This suggests that R3 and R5 are structurally independent of R1.

In the next experiments, R1, R2 and R5 which had been isolated as described previously were heated at 70°C for 1 min, and resolved on gel electrophoreses in parallel with the products from 28 S rRNA obtained by heating at the same temperature (Fig. 3). Again, the isolated R1 dissociated into R2 and R4 (Fig. 3a) while the isolated product R2 was stable and underwent no further dissociation (Fig. 3b). Further heating of the product R5 gave rise to the smaller product R6 (Fig. 3c).

(c) Effect of urea

The dissociation of 28 S RNA from fresh tissue of insects into the 18 S product is observed at room temperature provided that urea or dimethyl sulfoxide is present in the preparation^{5,7}. In order to further compare the

Figure 1. Differential dissociation of 28 S RNA from *Galleria* ribosomes. 0.1 ml aliquots of preparation were heated for 1 min at various temperatures as follow: (a) 0°C; (b) 42°C; (c) 45°C; (d) 47°C; (e) 50°C; (f) 55°C; (g) 60°C; (h) 70°C; (i) 75°C.

Figure 2. Further dissociation of product R1.

The gel portion containing the fragment R1 (see the inset) was gently homogenized in E buffer, and kept overnight at 4°C. The RNA, thus extracted, was again subjected to gel electrophoreses, after heated for 1 min at various temperatures as follow: (a) 0°C; (b) 45°C; (c) 47°C; (d) 50°C. What was electrophoresed in parallel with these re-extracted products is the 28 S RNA of the same origin which was heated at 50°C for 1 min (-----).

Figure 3. Thermal stability of isolated products.

The dissociation products R1, R2, and R5 were isolated (see the inset of Figure 2), and heated in a small amount of E buffer at 70°C for 1 min. What was electrophoresed in parallel with these is the 28 S RNA which was heated at 70°C for 1 min (----). For convenience, four separate electropherograms were redrawn on a single figure. (a) R1; (b) R2; (c) R5.

secondary nicks with the primary one, effect of urea on the 28 S rRNA from the frozen-thawed ribosomes was studied. The 28 S rRNA, which had been isolated on sucrose gradients was dissolved in E buffer, and the solution adjusted to various concentrations with respect to urea. The solutions were then incubated at 25°C for 60 min, cooled, and directly submitted to gel electrophoresis. As shown in Fig. 4a to d, the 28 S rRNA isolated from fresh tissue was dissociated only into the 18 S product in response to 4 or 8 M-urea. As is the case with thermal treatments of this RNA, no product other than 18 S RNA was observed. On the other hand, urea treatments of the 28 S RNA isolated such that secondary nicks are present gave rise to the same seven dissociation products (Fig. 4e, f, g, h).

(d) Thermal dissociation of 28 S RNA from Aphis ribosomes

It was of interest to investigate whether the 28 S aphid RNA is susceptible to nicking when extracted from the frozen-thawed ribosomes, since the RNA lacks the primary nick²¹. As shown in Fig. 5a and b, if the 28 S RNA was extracted directly from the fresh tissue of Aphis, no dissociation occurred even after heating at 70°C for 10 min, which confirms the results of Shine and Dalgarno²¹. However, if the 28 S rRNA is extracted from frozen-thawed ribosomes, the 28 S aphid RNA was readily dissociated into a number of products (Fig. 5c, d, e, f). The peaks found are not the same as those from Galleria RNA. Further, since the peaks on the gel electropherograms are for the most part not discrete, it is likely that the number of secondary nicks in Aphis 28 S rRNA are more than found in Galleria.

(e) Molecular weight estimations of dissociation products

In order to permit a tentative mapping of the fragments from the Galleria 28 S rRNA, the molecular weights of the various products from heat or urea treatment were estimated by comparing their mobilities with those of E. coli and mouse liver rRNA's on the gel electrophoresis^{20,22,23}. The results from these experiments are summarized in Fig. 6. The molecular weights of mouse liver RNA were assumed to be 1.71×10^6 and 0.70×10^6 daltons, and that of E. coli RNA, 1.07×10^6 and 0.56×10^6 daltons^{16,23,24}. A straight line relationship was obtained between mobility and the logarithm of the above assumed molecular weights. The molecular weights estimated for the eight species of RNA fragments are: R0-- 1.45×10^6 , R1-- 1.15×10^6 , R2-- 0.75×10^6 , R3-- 0.55×10^6 , R4-- 0.40×10^6 , R5-- 0.30×10^6 , R6-- 0.25×10^6 , R7-- 0.20×10^6 daltons. These results are consistent with the precursor-product relationships suggested, i.e. R0=R1+R5, and R1=R2+R4.

Figure 4. Dissociation caused by urea treatment. The 28 S RNA without the secondary nicks was prepared from the fresh tissue of *Galleria*, and treated as follow: (a) 0 M; (b) 2 M; (c) 4 M; (d) 8 M. The RNA with the secondary nicks was isolated as described, and treated as follow: (e) 0 M; (f) 2 M; (g) 4 M; (h) 8 M.

Figure 5. Thermal dissociation of the aphid RNA.

The 28 S RNA was heated in E buffer at 0°C (a) or 70°C (b) for 10 min. the 28 S RNA used on the experiments (c) to (f) was prepared from the ribosomes which had been isolated from the frozen-thawed tissue. The RNA was heated in E buffer at various temperatures for 1 min. (c) 0°C; (d) 50°C; (e) 60°C; (f) 75°C.

DISCUSSION

The present study further demonstrates the existence of secondary nicks in addition to the primary one^{5,7,21} in the 28 S rRNA from insects.

Unlike the primary nick, the secondary ones are not pre-existing, but are introduced at specific points in the polynucleotide chain depending on the experimental procedure used⁸. As is the case with the primary nick, the secondary ones are unmasked by a brief heat treatment or treatment with urea. The results suggest that 'nicked' covalent chain are held together by hydrogen bonds involved in the secondary structure of the molecule^{7,25,26,27,28}. It should be noted that the secondary nicks are not unmasked simultaneously when the 28 S rRNA is treated with heat or urea. For example, one (R6) is not unmasked until heated at or above 60°C, while another one (R4) is unmasked at or below 50°C (Fig. 1). It is likely that such differential unmasking may be due to the difference in the number of hydrogen bonds involved in masking each nick.

In order to simplify the discussion, a tentative mapping of the seven products due to the nicks is provided (Fig. 7). In this map, P is considered to be the primary nick in the 28 S rRNA and I, II, and III are secondary nicks. The primary nick occurs in vivo and the result is the conversion of 28 S rRNA to two 18 S products⁷. Among the secondary nicks, I and II are expected to be the most unstable, since they are readily unmasked by heating and the products R1, R5 and R7 arise (Fig. 1). The primary nick, P is assumed to be more stable than the secondary nicks, I and II, thus accounting for a later appearance of the products R2, R3 and R4.

It is to be expected that the secondary nicks I and II, are not in every molecule of 28 S rRNA²⁹. One molecule may have the primary nick (P) only; another, P and secondary nick I; and the third, P, I and II. It is likely that nick II occurs seldom since the products R3 and R7 are small in amount when compared to R2, R4 or R5, and become relatively greater when the R2 is less in amount. The nicking at the point I is also likely not complete⁸. Therefore, the fragment R2 will be a mixture of R2_A and R2_B in less amount.

The nick III seems to be unique in that it is not unmasked until heated at or above 60°C. Since the fragment R6 is smaller in size than R5 by approximately 0.05×10^6 daltons, another dissociation product from R5 is assumed and designated as X. This product was not observed in these studies, likely due to its small size. The fragment X was located at a terminus of the molecule, as shown in Fig. 7. It is proposed that this fragment X may be

Figure 6. Estimation of molecular weights of the products.
 (A) mouse liver 28 S RNA; (B) *E. coli* 23 S RNA; (C) mouse liver 13 S RNA; (D) *E. coli* 16 S RNA; (18 S) *Galleria* 18 S RNA.

Figure 7. A tentative mapping of the RNA fragments from the 28 S RNA of *Galleria*.
 P: primary nick; I, II, III: secondary nicks. The 3'- and 5'-termini are so called here just for convenience, and are interchangeable on the figure.

identical to the lRNA which is non-covalently attached to the 28 S rRNA. The lRNA, a small RNA about 150 nucleotides in length, was first demonstrated in the mammalian ribosome by Pene *et al.*³⁰, and a similar molecule from yeast ribosomes was recently sequenced by Rubin³¹. The mammalian lRNA can be released not only from isolated 28 S rRNA but also from ribosomes¹⁴. The existence of a similar molecule has been suggested for the insect 28 S rRNA by Shine and Dalgarno²¹. In addition, end-labelling of the 28 S rRNA of insects showed no more than two pre-existing nicks²¹. Consequently, it is likely that the lRNA is attached to one of the two termini of the 28 S rRNA. Also, the fragment X is not released from R5 to give R6 before the primary nick is unmasked (Fig. 1), and the lRNA can be readily reassociated with the 28 S RNA¹⁴, though the dissociation product due to the primary nick is not reassociated with each other under any conditions examined so far^{7,21}. Therefore, in addition to the similarity of the assumed molecular sizes, the fragment X is also like lRNA in that the hydrogen bonds involved in attaching these molecules to the 28 S rRNA are considerably more in amount than those in the primary nick of the insect RNA. If the fragment X is identical to the lRNA, it may follow that the nick III is not really "secondary" but pre-existing²¹.

The proposed map can also account for the existence of small shoulder of the peaks in the electropherogram. A small transient shoulder at the light side of R1 (Fig. 1) may be explained by assuming an unstable product, R3+R4. Likewise, a persistent shoulder at the light side of R2 may represent the product R4+R6, which does not have a nick at the site I.

It was shown that the 28 S aphid RNA also is susceptible to secondary nicking. However, the resulting fragments from the Aphid RNA are different from those from the *Galleria* RNA. Yet, since the dissociated profile is highly reproducible, the secondary nicks are likely introduced at the specific points as is the case with the *Galleria* RNA. Perhaps, the points at which the secondary nicks are introduced to the RNA molecule are species-specific.

These high-molecular-weight fragments which were isolated from a eukaryotic ribosomal RNA may provide an excellent system for the study of the structure of 28 S rRNA. It will not be very difficult, nowadays, to analyze the base sequence of the fragment R5 and R6, the molecular size of which is much smaller than that of the *E. coli* 16 S rRNA^{32,33}. Furthermore, each of the RNA fragments can be studied with respect to an interaction with the

ribosomal proteins³⁴, which will facilitate study of the topography³⁵ of larger subunit of the ribosome from eukaryotic cells.

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