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RE-JOINING OF THE 18S FRAGMENTS DISSOCIATED FROM THE 28S RIBOSOMAL RNA OF INSECT: A STRUCTURAL ROLE OF 5.8S RNA

Hajime Ishikawa

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

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SUMMARY

The 18S RNA fragments, A and B, and 5.8S rRNA were dissociated from 28S rRNA of cultured insect cells, NIAS-Px 58. When a mixture of fragments, A + B, was incubated with ^{3}H-labeled 5.8S RNA under appropriate conditions, a considerable amount of radioactivity was observed with 28S rRNA. It is highly likely that the 5.8S rRNA induced a re-joining of fragment A and B to form the 28S RNA molecule.

INTRODUCTION

The 28S rRNA from insects except aphids, and most of other protostomes is characterized by occurrence of a hidden break at or near the mid-point of the molecule (1). Due to the hidden break, the 28S rRNA is dissociated into two 18S fragments, tentatively named A and B, on treatment with agents which disrupt hydrogen bonding. This conversion results from a dissociation of two polynucleotide chains in the 28S rRNA molecule (2, 3). Though the hidden break was studied from several aspects (4-11), its biological significances have been totally unknown. It will be partly because no attempt to re-join the two RNA fragments, A and B, due to the break has been made successfully. Concerning this, what should be considered is that the 5.8S RNA, along with the two fragments, is released from 28S rRNA upon heated. It will be reasonable to assume, therefore, that the 5.8S rRNA plays an important role in a reconstruction of 28S RNA molecule from the fragment A and B. This communication describes the first attempt at re-joining the two fragments by means of 5.8S rRNA.

MATERIALS AND METHODS

NIAS-Px 58, a cell line from the pupal ovaries of the swallow tail, Papilio xuthum, Linne (12) was used throughout. The insect cells were cultured in synthetic medium MGM-431, containing fetal bovine serum (Gibco.) at 25°C (13). The population doubling time under the conditions employed was 45 hr. To label the rRNAs, the culture medium was supplemented with [5-^{3}H] uridine (Radiochemical Centre, 28 Ci/mmole) at 16 uCi/ml. [U-^{14}C] Uridine (Radiochemical Centre, 513 mCi/mmole) was used at 1 uCi/ml. The labeling was performed at 25°C for 48 hr. Extraction of total RNA from the

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whole cell and treatment with DNase were done by published procedures (9). Isolation of the 28S and 18S rRNAs in sucrose density gradient was as before (9). The 28S rRNA was dissolved in 0.01 M Tris-HCl (pH 7.4) containing 1 mM EDTA and 0.05 % sodium lauryl sulfate, and heated at 65°C for 5 min (14). The treated sample was chilled immediately in an ice bath and then centrifuged on 50 ml linear 10-20 % sucrose gradient in the same buffer at 23,000 rev/min for 25 hr at 4°C. The gradient was monitored through the flow cell of recording spectrophotometer (Fig. 1). A mixture of RNA fragments (A + B) and the 5.8S rRNA were pooled separately as indicated. The 5.8S rRNA was further purifed through sucrose density centrifugation under the same conditions as above. The fragments (A + B) and 5.8S rRNA were mixed in a molar ration as specified and subjected to annealing.

Annealing was performed in a small volume of 0.4 M NaCl, 3 mM EDTA, pH 7.3 in a sealed capillary pipette at specified temperatures for 30 or 60 min (15). The sample was slowly cooled down to room temperature, and was diluted fourfold with 3 mM EDTA (pH 7.3) prior to application to polyacrylamide gel (15). Electrophoresis was performed at 5 mA/column for 4 hr at room temperature. As internal markers, the Px cell total RNAs which had been labeled with [^{14}C] uridine for 48 hr were used. In an experiment shown in Fig. 2, electrophoresis was on 6 % polyacrylamide (7 cm long) for 2 hr. The gel was sliced and counted for radioactivity (16) in a Beckman LS 9000 liquid-scintillation spectrophotometer. When necessary, the gels were scanned for ultraviolet absorbance with a Gilford 250 recording spectrophotometer equipped with a linear transport. During the scanning, the linear transport was switched off halfway, and the gel portions containing the 28S and 18S rRNA marked and cut out later (9). To extract the RNA species, the gel disk was homogenized in 0.1 M Tris-HCl (pH 9.1) containing 0.5 M NaCl, 10 mM EDTA and 50 ug/ml yeast RNA. The RNAs were precipitated in cold trichloroacetic acid (10 %), dried on glass filter paper and counted for radioactivity.

RESULTS

(a) Separation of 5.8S rRNA

Prerequisite to the present experiments was to obtain a mixture of RNA fragments, A + B, which had been completely stripped of resident 5.8S rRNA. This was achieved by centrifuging the heated sample through sucrose gradient at low salt concentrations at 4°C (Fig. 1) (14). When the RNA fragments, thus isolated, were heated and centrifuged again under the same conditions, no further release of low molecular weight RNAs was observed (data not shown).

In order to test the purity and identity, the isolated 5.8S [^{3}H] rRNA was analyzed on polyacrylamide gel. The low molecular weight RNAs from Px cells, which had been labeled with [^{14}C] uridine and isolated by sucrose gradient, were used as internal standards. By comparing the mobility with those of the 5S and transfer RNAs on the gel, the ^{3}H-labeled species was identified as the 5.8S rRNA. Also, it was evident that the two cycles of sucrose gradient centrifugations were effective enough to isolated the 5.8S molecule free from the fragments, A + B (Fig. 2).

(b) Annealing of 5.8S rRNA with RNA fragment A and B

The 5.8S rRNA, which had been labeled with [^{3}H] uridine, was precipitated in cold ethanol together with unlabeled RNA fragments, A + B from the 28S

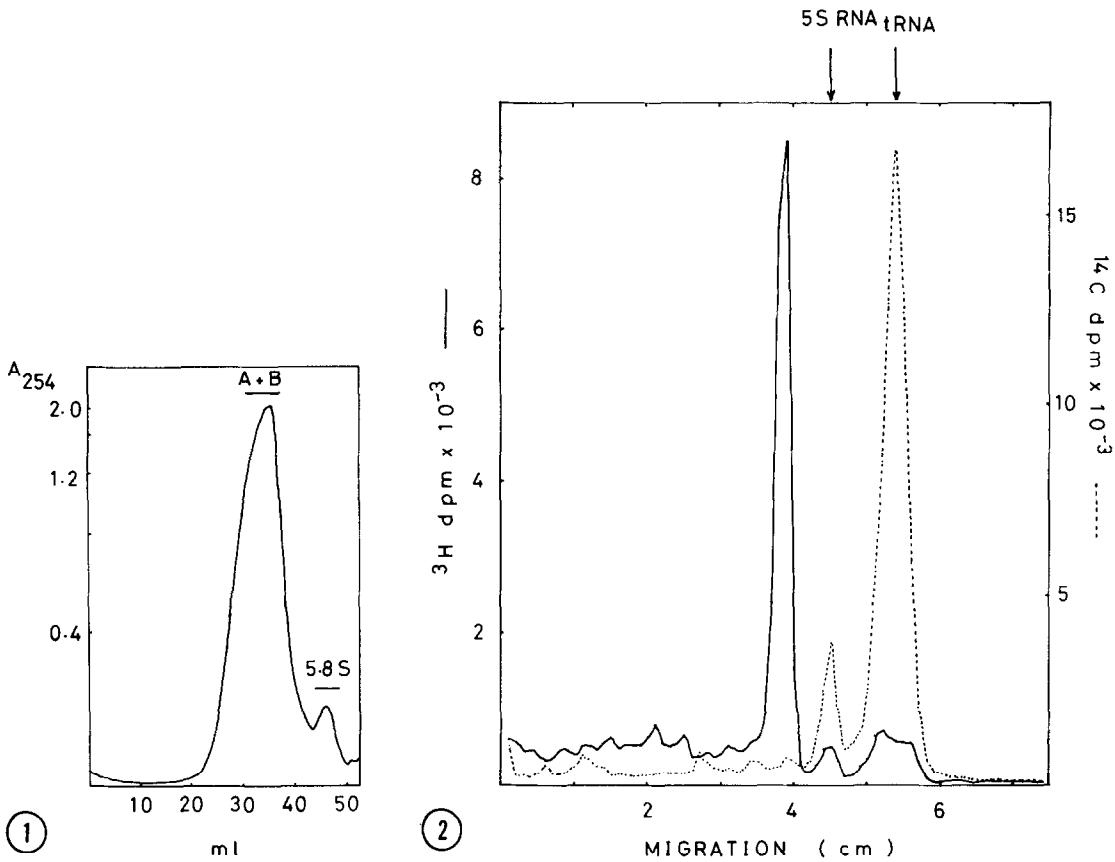

Fig. 1. Separation of 5.8S rRNA from the 18S RNA fragments (A + B) in sucrose density gradient. The 28S rRNA was dissolved in 0.01 M Tris-HCl (pH 7.4) containing 1 mM EDTA and 0.05 % sodium lauryl sulfate, and heated at 65°C for 5 min. The treated sample was centrifuged in 10-20 % sucrose gradient in the same buffer at 23,000 rev/min for 25 hr at 4°C. The gradient was monitored, and the fragments (A + B) and 5.8S rRNA pooled, as indicated.

Fig. 2. Gel electrophoresis of [^{3}H] 5.8S rRNA isolated by sucrose density gradient. The 5.8S rRNA, isolated as in Fig. 1, was purified again through the same sucrose density gradient. The RNA, thus purified, was mixed with ^{14}C-labeled low molecular weight RNAs from Px cells, and subjected to polyacrylamide (6 %, ethylene diacrylate-cross linked) gel electrophoresis. Electrophoresis was performed at 5 mA/column for 2 hr at room temperature. The gel was sliced and counted for radioactivity.

rRNA. The molar ratio of A + B to 5.8S RNA was approximately 10 in this experiment. The RNA mixture was treated as described under Methods and electrophoresed together with ^{14}C-labeled markers. As shown in Fig. 3, about 25 % of radioactivity applied on gel was found in the region of 28S rRNA while 60 % found in the region of 18S rRNA. Not much radioactivity was observed in the 5.8S region (about 6 cm from the top). It will be reasonable to presume that by means of 5.8S RNA the fragment A and B were joined together

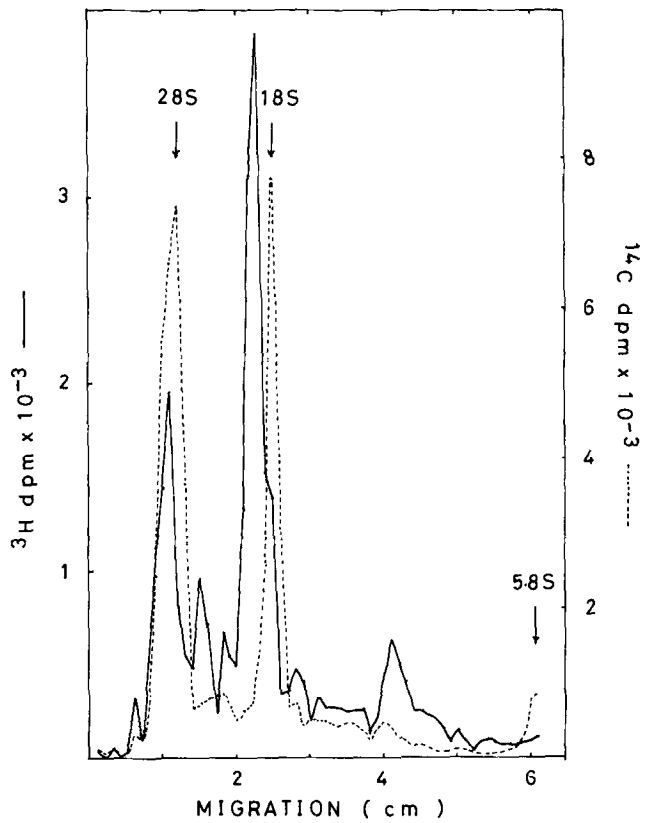

Fig. 3. Gel electrophoresis of a mixture of 5.8S rRNA and the 18S RNA fragments. The 5.8S rRNA labeled with [^{3}H] uridine, was incubated with about ten-fold molar amount of the 18S unlabeled fragments (A + B) in a small volume of 0.4 M NaCl, 3 mM EDTA, pH 7.3 at 55°C for 60 min. The sample was diluted four-fold with 3 mM EDTA (pH 7.3), and subjected to polyacrylamide gel electrophoresis. The upper 4 cm of the gel column consisted of 3 %, and the lower 2 cm of 6 % polyacrylamide. Electrophoresis was performed under undenaturing conditions at 5 mA/column for 4 hr at room temperature. The Px cell total RNAs were labeled with [^{14}C] uridine for 48 hr, and employed as internal standards.

to form 28S RNA complex. A minor peak, which was observed at about 4 cm from the top, was not further characterized for the present. The possibility that the radioactivity observed with 28S rRNA was due to unspecific attachment of the 5.8S rRNA to high molecular weight RNAs was excluded by an experiment shown in Fig. 4. In the latter, an annealing experiment was performed employing 18S rRNA in place of the mixture, A + B. It was apparent that [^{3}H] 5.8S rRNA was not associated with the 18S rRNA. In Fig. 5, the ^{3}H-labeled RNA fragments were incubated with non-radioactivie low molecular weight RNAs, which did not contain the 5.8S molecule but tRNA species and 5S rRNA, under the same conditions as in Fig. 3. It is evident that without 5.8S rRNA the fragment A and B were not joined.

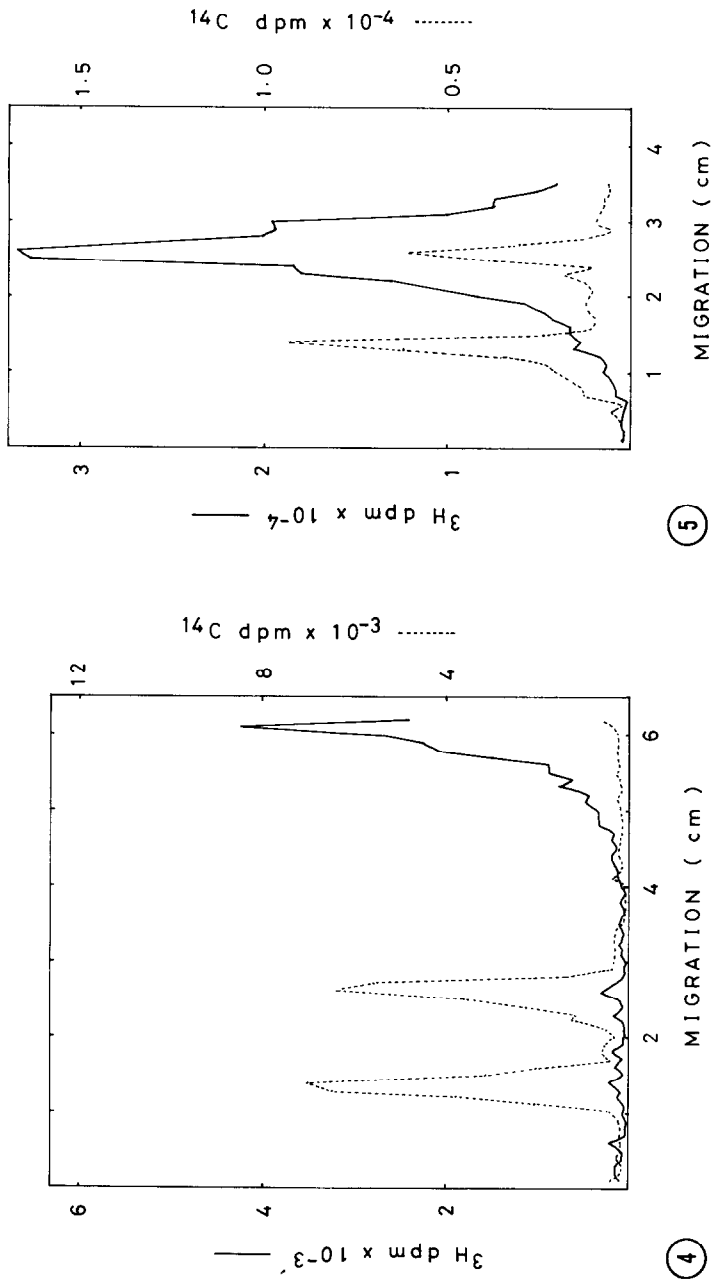

Fig. 4. Gel electrophoresis of a mixture of 5.8S rRNA and 18S rRNA. The 5.8S rRNA, labeled with [^{3}H] uridine, was incubated in the presence of about ten-fold molar amount of the 18S unlabeled rRNA under the same annealing conditions as in Fig. 3.

Fig. 5. Gel electrophoresis of a mixture of low molecular weight RNAs and the RNA fragments. Low molecular weight RNAs, which had been isolated in sucrose density gradient, were incubated in the presence of one-tenth molar amount of 18S RNA fragments labeled with [^{3}H] uridine.

(c) Optimum temperature for the formation of 28S RNA

The temperature dependence of joining reaction was evaluated by holding mixtures of ^{3}H-labeled 5.8S rRNA and non-radioactive RNA fragments, A + B, in 0.4 M NaCl, 3 mM EDTA (pH 7.3), at various temperatures for 30 min. Reaction products were chilled and diluted with 3 mM EDTA (pH 7.3) containing ^{14}C-labeled Px total RNAs and unlabeled total RNAs from posterior silkglands of Bombyx mori. The diluted products were resolved in polyacrylamide gel electrophoresis as before and the gels scanned for ultraviolet absorbance. The gel portions containing the 28S and 18S RNA were sliced out, and the RNAs extracted and counted for radioactivity as described under Methods. Since in the present procedure the recovery of ^{3}H-labeled RNA is apt to be changed due to several artifactual factors, an amount of ^{3}H-radioactivity was corrected in terms of the amount of ^{14}C-radioactivity observed with the same fraction. The results were summerized in Table 1. It will be noteworthy that the rather high temperatures were required for 28S RNA complex formation.

DISCUSSION

In the present studies, the 5.8S rRNA was isolated by passing twice through sucrose density gradient (Fig. 1). The 5.8S rRNA, thus isolated, was sufficiently pure (Fig. 2) and was not contaminated by the RNA fragment A and B (Figs. 2 and 4). When the ^{3}H-labeled 5.8S rRNA was incubated with a mixture of unlabled RNA fragments (A + B), the radioactivity was observed with molecular species a little larger than the 18S rRNA (Fig. 3). This will indicate that the 5.8S molecule tends to bind to either or both of the RNA fragment A and B under the annealing conditions used. The result is not surprising since in mammalian systems the 5.8S-28S rRNA association was restored by the same annealing procedure as in the present (15). Of important will be that the radioactivity due to 5.8S rRNA was found also with the 28S rRNA (Fig. 3). The most straightforward interpretation of this result will be that the fragments, A and B, from the 28S rRNA were joined together, and that the radioactive 5.8S RNA was associated with the molecule. At present, dimerization of the same species of fragments should be equally considered since in insects the two RNA fragments, A and B, are not separable from each other (2). This point is being extensively studied. In the present stage, for simplicity, let me assume that the two different fragments were joined together. What can be emphasized for the present is that in the absence of 5.8S rRNA, the fragment A and B were not joined (Fig. 5).

As shown in Table 1, a complex of 5.8S rRNA and fragment A (and/or B) is readily formed at as low as 50^{o}C. The rather high temperature is required for

Table 1. Effect of temperature on re-joining.

Temperature	28S dpm[a]	28S P[b]	18S dpm[a]	18S Q[b]	P/(P+Q) x 100
50°C	2623 16347	0.1605	3308 8957	0.3693	30.3
55°C	2937 14397	0.2040	3407 8436	0.4039	33.6
60°C	2230 13582	0.1642	2291 7507	0.3052	35.0
65°C	3115 12893	0.2416	2012 8137	0.2473	49.4
70°C	3083 15634	0.1972	2956 8166	0.3619	35.3

^{3}H-Labeled 5.8S rRNA was incubated with about ten-fold molar amount of the unlabeled fragments (A + B) at various temperatures for 30 min, diluted, and then electrophoresed on 3 % polyacrylamide gel together with ^{14}C-labeled total RNAs of Px cells. The RNA species were extracted from the gel portions containing the 28S and 18S rRNA, dried on filter paper, and counted for radioactivity.

a: upper, ^{3}H dpm; lower, ^{14}C dpm
b: ratio of ^{3}H dpm to ^{14}C dpm

formation of 28S RNA complex. This observation will suggest that the joining process is a specific one, involving a relatively extensive region of hydrogen bonding. Even at the optimum temperature (65°C), no association of 5.8S molecule with 18S rRNA was observed (data not shown, but see Fig. 4).

As for the structural role of 5.8S rRNA in the joining process, two possibilities will be conceivable. One presupposes that 5.8S rRNA is associated with either fragment A or B. The other assumes the molecule's association with the both. In the former, upon being associated with 5.8S rRNA, one fragment assumes a conformation suitable for association with the other. In the latter, simply, the 5.8S rRNA joins one fragment with the other. No conclusive evidences, so far, have supported either of the two assumptions. And yet, a few circumstancial evidences seem favorable for the latter, which should be further substantiated. This line of studies will provide an interesting model for RNA-RNA interaction in the three-component system.

It remains unclear whether the re-joining reaction seen here represents true restoration of 28S rRNA. If it is the case, several new approaches on the structure of 28S rRNA complex will be feasible. These will lead to not

only an unmasking of biological significances of the hidden break in 28S rRNA but an understanding of structural roles pertained to the 5.8S rRNA, a stigma to the eukaryotic cell.

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