ENDORIBONUCLEASES ASSOCIATED WITH RNAs IN CHICK EMBRYOS

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1. Introduction

It is well known that the ribosomal RNAs (rRNAs) can be degraded by ribonucleases (RNases) unless special care is taken during their extraction and purification. It is also known that the 28 S rRNA is a continuous single-stranded polynucleotide chain. However, hidden breaks which may be revealed by different denaturation procedures were reported in this rRNA obtained from several sources such as rat liver [1], *Bombyx mori* [2] and *Euglena gracilis* [3,4]. These fragments were described as products of a non-random degradation of the 28 S rRNA molecules probably produced during the extraction procedures.

Furthermore, in addition to the two major rRNA species (28 S, 18 S) several RNA fragments of mol. wt $0.88–1.25 \times 10^6$, were observed in the cytoplasm of rat liver cells [5]. These minor RNA components represent intermediates of the 'in vivo' breakdown of the 28 S rRNA and this degradation process could be enhanced 'in vitro' [6–8]. It has also been demonstrated that these minor RNA molecules are derived from the cleavage of the 28 S rRNA present in the 60 S ribosomal subunits which are part of the active polyribosomes [8,9]. The fragments can also be generated 'in vitro' by incubation of purified polyribosomes prior to rRNA extraction [4,10]. On the other hand, it has been reported that when a 28 S rRNA is located within the ribosome, the molecule is protected against the action of exogeneous nucleases such as lysosomal enzymes [6] or pancreatic RNase [9]. Hence, a close association between the 28 S rRNA and the endoribonuclease that generates the fragments might exist. It is noteworthy that a ribonuclease bound to the eukaryotic ribosomes has been described [11]. Moreover, some of the ribosomal proteins in hemopoietic rat cells were reported to have RNase activities [12]. Similarly, an endogeneous endoribonucleolytic activity was found in the 50 S ribosomal subunit of *Escherichia coli* [13]. This paper describes the existence of a RNA-bound cytoplasmic endoribonuclease in chick embryo. The enzyme can be obtained associated with the RNA (presumably as a ribonucleoprotein by extraction with phenol–chloroform. During the first steps of degradation of naked rRNA molecules this RNase produces fragments which are similar to those derived from the 28 S rRNA either 'in vivo' or 'in vitro' after the incubation of the polysomes (see above).

2. Materials and methods

2.1. *RNA extraction*

The RNAs were extracted from day 7–9 chick embryos by different methods.

Method A:

The embryos were removed from their shells and homogenized with ice-cold TKM buffer (Tris–HCl 0.050 M (pH 7.4); KCl 0.250 M; $MgCl_2$ 0.010 M) containing 0.05% Triton X-100 (Sigma), in a Potter Elvehjem apparatus using a Teflon pestle driven by hand. Buffer (1 ml) was used for each 0.16 g embryo tissue. A post-mitochondrial supernatant was obtained by centrifugation (Sorvall centrifuge HB 4 rotor, 10 000 rev./min, 10 min at 2°C). This supernatant was shaken with 0.020 M EDTA and with 1% (w/v) Sarkosyl (Sigma) for 5 min at room temperature. The mixture was extracted with phenol–chloroform [14], the resulting interphase was removed, mixed with 2 ml NET buffer (NaCl 0.1 M; EDTA 0.001 M; Tris–

HCl 0.050 M (pH 7.4)) and extracted with chloroform; then, the aqueous phases were combined and re-extracted again with phenol–chloroform. The RNAs were precipitated from the last aqueous phase with 2.5 vol. ethanol at −20°C.

Method B:

The embryos were removed from their shells and immediately immersed in cold (−70°C) ethanol. The frozen embryos were homogenized and the RNAs were extracted as described above (method A) but the temperature was maintained at ~0°C.

Method C:

The embryos were frozen, homogenized and the RNAs extracted as in method B, except that both the concentration and the time of exposure to Sarkosyl were lowered (0.05% for 30 s) during the extraction of the RNAs.

Method D:

RNase-free RNA was obtained as in method B but in this case the post-mitochondrial supernatant was extracted with hot (60°C) phenol–chloroform.

2.2. *RNase assay*

The RNase activity associated with the cytoplasmic RNAs was measured either by self-incubation of the RNAs in NET buffer at 37°C or by utilizing the ability of the enzyme to degrade a [^{3}H]uridine-labeled 28 S RNA (extracted by hot phenol) used as substrate. In every case, after incubation, the RNAs were heated for 5 min at 60°C and were characterized either on sucrose gradients or on polyacrylamide gels. Alternatively, the RNase activity was assayed using a [^{3}H]uridine-labeled RNA bound to Sepharose beads as substrate according to [15]. The Sepharose-bound RNA was a gift from Dr Mario Lebendiker. Contamination by spurious nucleases was avoided as in [16]. Perchloric acid (PCA)-soluble products were assayed as in [17].

3. Results

3.1. *Characterization of the cytoplasmic RNAs obtained by method A*

The analysis of the cytoplasmic RNAs extracted by method A, showed the presence of the three main components currently described (28 S, 18 S, 4 S). However, when these RNAs were heated for 5 min at 60°C prior to the analysis, a number of heterogeneous fragments were detected which indicated the presence of hidden breaks revealed by the treatment [18] (fig.1A). Moreover, as can be seen in fig.1b,c, when the 28 S and 18 S RNA molecules were isolated and submitted to a similar treatment, it was found that the hidden breaks were mostly located along the 28 S rRNA.

3.2. *Detection of RNases in cytoplasmic RNAs obtained by methods B and C*

Method B was designed to promote rapid inhibition of the nucleases during the extraction of the RNA. The RNAs obtained by this method were found to be unbroken even after the heat treatment (fig.2A). The lower sedimentation rate of the 28 S molecule in the heated RNA preparation (60°C) was an indication that these molecules had undergone a reversible denaturation [19,20]. These results also indicate that

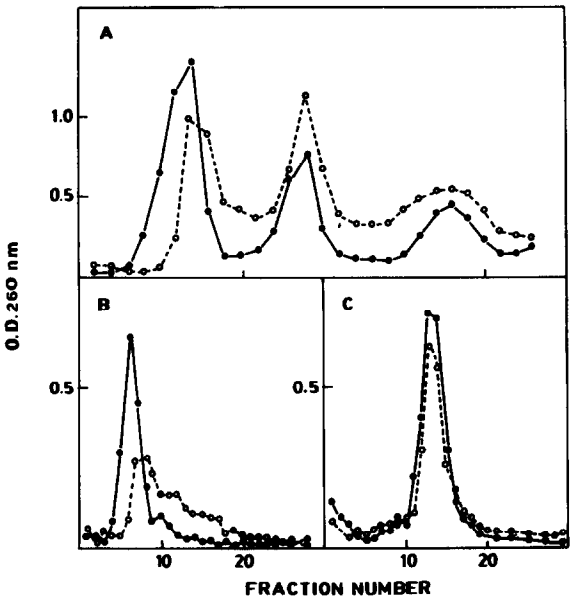

Fig.1. Detection of hidden breaks in the chick embryo cytoplasmic RNA obtained by method A. (A) The RNAs were suspended in NET buffer, divided into 2 aliquots. One of them was heated for 5 min at 60°C; both samples were then layered on top of 2 equal 10–30% sucrose gradients and run (SW25 rotor, 23 000 rev./min, 2°C, 21 h). 28 S (b) and 18 S (c) RNAs were isolated from a preparative gradient and analyzed as in (a). (•——•) control and (o– – –o) heated RNAs.

the hidden breaks detected in the 28 S RNA molecule obtained by method A were produced during the extraction procedures, probably by an endoribonuclease(s) present in the large ribosomal subunit. The stability at 37°C of the RNA extracted by method B was also tested. As seen in fig.2B, a considerable

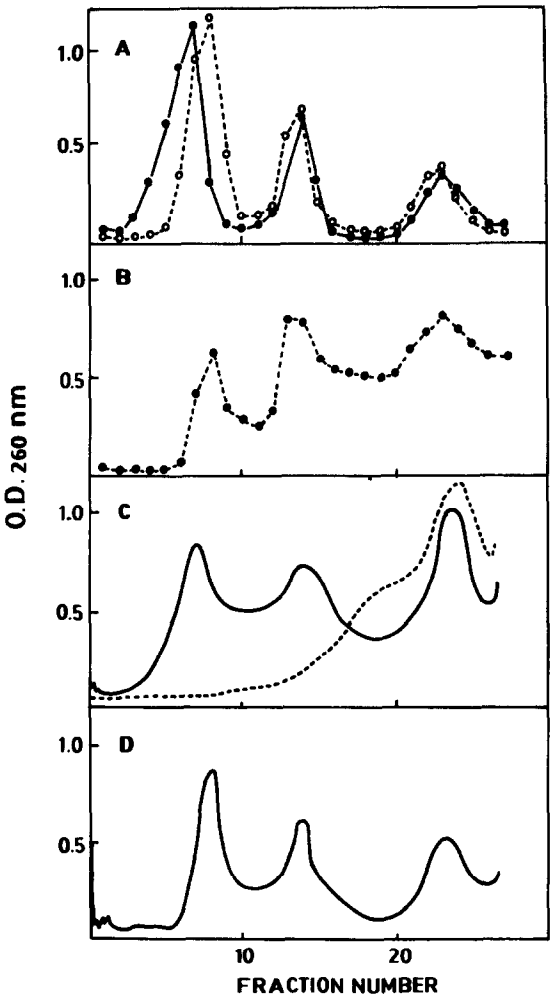

Fig.2. Analysis of the cytoplasmic RNAs obtained by method B. The RNAs were suspended in NET buffer; divided into 3 aliquots and treated as follows: (A) control (•——•) and heated RNAs (o– – –o); (B) incubated RNAs (1 h at 37°C and 5 min at 60°C) (•– – –•); (C) analysis of the cytoplasmic RNA extracted by method C. Control (——) and incubated RNAs (1 h, 37°C and 5 min at 60°C) (– – –); (D) Analysis of the RNA obtained by method D. The RNAs were suspended in NET buffer and incubated (20 h at 37°C and 5 min at 60°C). Conditions of analysis as in fig.1.

degradation of the incubated RNA was detected, indicating the presence of remnant nucleases that had not been removed by the extraction procedures. However, the amount of enzyme recovered by this method in different preparations was variable. Hence, with a view to studying these RNA-associated RNases more deeply a variant of method B (method C) was developed to assure the recovery of larger and more reproducible amounts of enzyme. Based on the findings in [21] both the concentration and the time of action of the detergent (Sarkosyl) during the extraction of the RNA were lowered, in order to disintegrate the ribosomes to a smaller extent prior to the addition of the phenol–chloroform mixture. Under these conditions, larger amounts of RNases were extracted with the RNA, and the RNA obtained by this method was rapidly degraded after brief incubation at 37°C (fig.2C). On the other hand, the enzymatic activity remained in the RNA preparation even after 2 or 3 additional extractions with ice cold phenol–chloroform. However, two similar extractions but performed at 60°C (method D) were enough to produce RNA preparations with no detectable RNase activity even after 20 h incubation (fig.2D).

3.3. *Analysis of the RNase products*

Two equal samples obtained by method C were incubated for 5 min and 20 h, respectively. The RNAs were analyzed on polyacrylamide gels. As can be appreciated from fig.3a, after 5 min incubation 4 RNA fragments of mol. wt 1.25×10^6, 1.00×10^6, 0.88×10^6, and 0.76×10^6 respectively, were detected. Fragments with analogous molecular weights derived from the 28 S RNA degraded 'in vivo' as well as upon storage or incubation of polyribosomes were also reported in other cells [5–10]. In addition, 5 of the RNA fragments, showing faster electrophoretic mobilities than the 18 S RNA, were similar to those found [10] during the incubation of polyribosomes. Complete degradation of the 28 S and 18 S RNA was detected after 20 h incubation (fig.3B). This pattern was similar to that found for the RNA incubated for only 1 h (fig.2C) indicating that these fragments were the final products of the degradation. Moreover, no appreciable ultraviolet-absorbing PCA-soluble products were detected even after 20 h of incubation when all the 28 S and 18 S RNAs were completely degraded as shown in fig.3B. Additionally, >97% of a ^{32}P-labeled

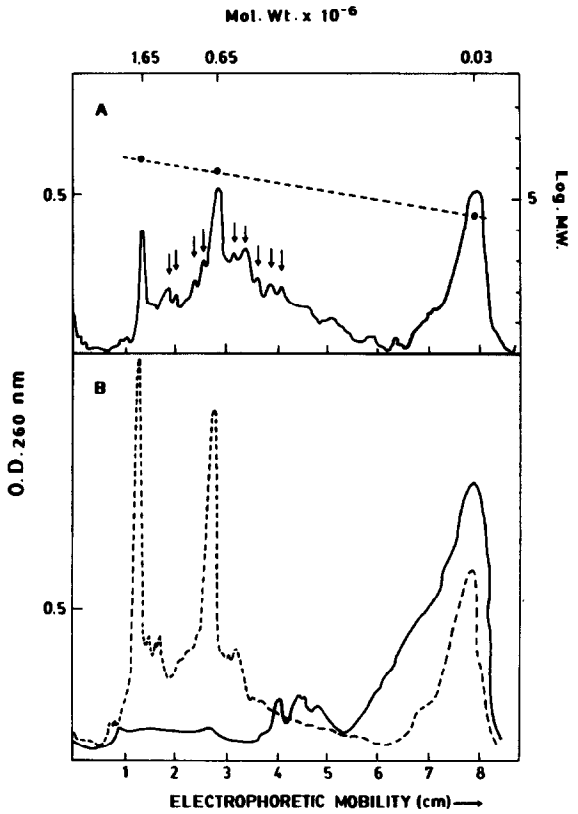

Fig.3. Characterization of the products generated by the RNase. The RNA was obtained as in fig.2c and incubated as follows: 5 min (a) or 20 h (b) at 37°C in NET buffer with (– – –) or without (——) 1% SDS. After incubation, the samples were ethanol-precipitated, re-suspended in buffer E [26], heated 5 min at 60°C and analyzed on 3% polyacrylamide gels (4 h, 5 mA/gel) [27]. Arrows indicate the principal fragments of the first steps of degradation.

RNA was trichloroacetic acid-insoluble after 20 h incubation with the RNase. The findings that the enzyme progressively degrades the RNA, producing characteristic fragments and that it does not produce acid-soluble products are indicative of the fact that this enzyme acts as an endoribonuclease.

3.4. *RNase activity associated with RNA*

The data presented above suggest that the enzyme is extracted together with the RNA. To gain further knowledge regarding the association of the enzyme with the RNA, the following experiment was performed. Chick embryo cytoplasmic RNA obtained by method C was analyzed on a gradient similar to that of fig.2C. Fractions from the gradient were pooled in 5 different zones and tested for RNase activity, as described in fig.4. As can be seen, an appreciable degradation of the substrate RNA was detected upon its incubation with material from zones 1,3 and 5 whereas no degradation was detected upon incubation with material from zones 2 and 4. The substrate RNA was also found to remain undegraded when incubated alone. The presence of enzymatic inhibitors or a substrate competition due to the large mass excess of the 28 S and 18 S RNA in zones 2 and 4, respectively, was tested as follows: *A* amounts of 28 S and 18 S RNAs, obtained from a preparative gradient (similar to that of fig.2C) were incubated alone or against aliquots from the other zones. In this case, the endogenous 28 S and 18 S RNA were not degraded when incubated alone, but they were, however, by the RNase present in zones 1,3 and 5, thus confirming the preceding results and ruling out the presence of nuclease inhibitors in those zones (not shown).

Figure 4 (central panel) shows a more precise location of the RNase along the sucrose gradient. In this case, the pellet and 16 different regions of the gradient were assayed for nuclease activity. As can be observed, the main peaks of enzymatic activity were located around the 36 S, 24 S and top fractions of the sucrose gradient. Similar results were obtained when the RNase activity was evaluated in each fraction using a Sepharose-bound [^{3}H]uridine-labeled RNA as substrate (not shown).

It is important to point out that the RNAse activity present at the 36 S and 24 S positions on the sucrose gradient varied with the state of preservation of the RNA preparation used; moreover, when the RNA–enzyme mixture was incubated at 37°C for different lengths of time, a breakdown of the RNA was observed with a concomitant shift of the enzymatic activity towards the lighter regions of the gradient. These results suggest that the enzyme could be associated with the RNA, probably as a ribonucleoprotein. Furthermore, when the RNA was found to be completely degraded, the enzyme was detected exclusively at the top of the gradient, presumably as a free polypeptide or associated with a shorter fragment of RNA. Similar results have been reported [22] in RNA preparations obtained from

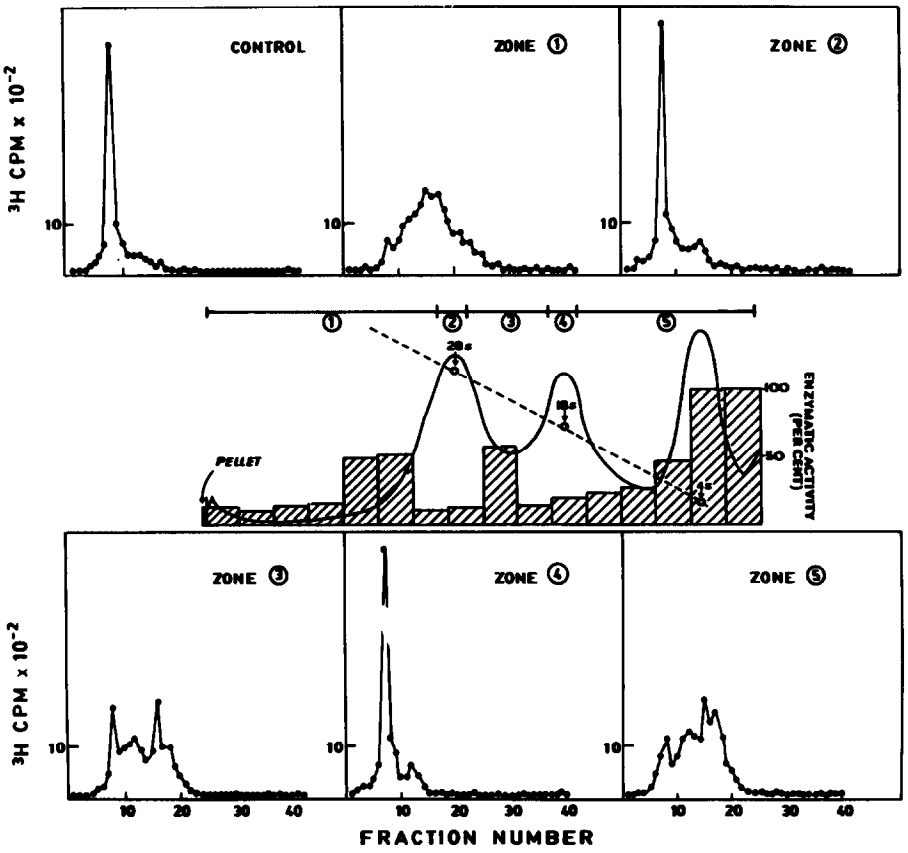

Fig.4. RNA-bound RNase activity in a sucrose gradient. A RNA preparation obtained as in fig.2c was analyzed on a sucrose gradient. Fractions from the gradient were pooled and 5 different zones were obtained as shown in the upper part of the central panel. Aliquots proportional to the volume of each pool were incubated in NET buffer together with a purified [^{3}H]uridine-labeled 28 S RNA (extracted with hot phenol–chloroform) for 1 at 37°C and 5 min at 60°C, and analyzed on 2.7% polyacrylamide gels (3 h, 5 mA/gel). The *A* profile in the central panel (——) corresponds to the RNA used as a source of the enzyme. In addition a duplicate gradient was divided into 16 different regions. The 16 pools and the pellet were analyzed for RNase activity as above. The shadowed columns indicate the enzymatic activity present in each fraction. The activity was evaluated as the percent diminution of the ^{3}H-counts under the 28 S RNA peak relative to a 28 S RNA incubated alone (control). The data were normalized in relation to the ^{3}H-counts recovered at the end of the analysis.

meristematic cells of *Vicia faba* roots. However, contrarily to what was reported by these authors, the enzyme described here did not produce acid-soluble nucleotides even after prolonged incubation at 37°C.

To test whether the RNase–RNA association arose artificially during the homogenization–extraction steps, the following experiment was performed. A day 9 chick embryo was labeled overnight with [^{35}S]methionine. The RNA was extracted by method C and analyzed on a sucrose gradient before and after incubation at 37°C (as in fig2C). In the control sample, it was found that the ^{35}S-label was associated with the RNA and showed a similar distribution to the *A* profile with the bulk of the counts located around the 4 S position of the gradient. In the incubated sample, both the RNA and the [^{35}S]methionine counts were only detected at the top of the gradient. The methionine-labeled material was found to be trichloroacetic acid-precipitable and RNase-resistant, but it was rendered trichloroacetic acid-soluble by treatment with pronase, suggesting that the ^{35}S-label was due to proteins extracted in association with the

RNA. The fractions containing these proteins were pooled and mixed with 20 A_{260} units of deproteinized cytoplasmic RNAs (obtained by method D (hot phenol)) in TKM buffer. The mixture was then analyzed on a sucrose gradient. The ^{35}S counts were again observed at the top of the gradient with no label associated with the macromolecular RNA (not shown), indicating that at least under these conditions there was no association between the RNA and the protein.

4. Discussion

Our results showed the existence of a RNA-bound endoribonuclease(s) that was extracted together with the RNA. The possibility that the association between both components might be produced during the extraction procedures cannot be completely ruled out. Nevertheless, the method described here allowed the isolation of a cytoplasmic ribonuclease probably at an advanced state of purification. Moreover, it is important to point out that Kwan [23] has described an exoribonuclease in HeLa cells located both in the soluble cytoplasm and in the ribosomes. However, the RNase described here did not show any exoribonucleolytic activity. On the other hand, it should be emphasized that the fragments produced by this RNase acting on naked RNAs, at the first steps of degradation, were similar to those reported to be present 'in vivo' as well as to those generated upon storage or incubation of polyribosomes [5–10], indicating that this enzyme might be the same as the one present in the large ribosomal subunits [8,9,13].

The afore-mentioned results are indicative of the fact that the disposition of the ribosomal proteins in the ribosomes may not be totally necessary for a non-random degradation of the rRNAs. Moreover, it was suggested that the secondary structure of the rRNA within the ribosome is similar to that of the free molecule [24]. Hence, it is therefore likely that the enzyme cleaves the same points on the polynucleotide chain regardless of whether the RNA is within the ribosome or is a free molecule in solution. Whether this RNA-bound enzyme(s) exists 'in vivo' or not; and what its functional role in the cell could be open questions. Nevertheless, a processing endoribonuclease attached to an essential RNA component has been recently observed in *E. coli* suggesting that the RNA component could provide the enzyme with specific recognition sites through RNA–RNA interactions [25].

Finally, preliminary experiments in our laboratory indicated that the chick embryo RNA-bound RNase described herein was able to process 'in vitro' the 28 S and 18 S rRNAs from the ribosomal RNA precursors.

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