IN VITRO PROCESSING OF HeLa CELL PRERIBOSOMES BY A NUCLEOLAR ENDORIBONUCLEASE

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1. Introduction

The biosynthesis of ribosomes in eukaryotic cells involves the sequential processing of a common nucleolar ribosomal precursor particle (pre-rRNP) into the small and large ribosomal subunits. The nascent 45 S pre-rRNA [1] of the mammalian pre-ribosome [2–4] is cleaved into discrete intermediate pre-rRNA molecules (41 S, 32 S; 20 S) which in turn are shaped finally into mature 18 S and 28 S rRNAs (for references, cf. reviews by Maden, 1970 [5] and Grierson et al. 1970 [6]). The mechanism of this processing is not yet known, but it is very probable that an endoribonuclease is involved.

We report the isolation of a nucleolar ribonuclease obtained from HeLa cells. This enzyme is shown to convert *in vitro* the isolated preribosomes containing the 45 S pre-rRNA into particles containing RNA molecules which correspond in size to the nucleolar RNAs normally found *in vivo*. Virtually no alcohol-soluble reaction products are found in this reaction, hence we conclude the enzyme is an endoribonuclease.

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Abbreviations:

rRNA : ribosomal RNA
pre-rRNA: precursor RNA to ribosomal RNA
A.S. : ammonium sulfate

2. Material and methods

2.1. *Chemicals*

Sucrose, RNase free from Mann, USA. The other chemicals were reagent grade. DEAE-cellulose SH from Serva, Germany. 5-^{3}H-uridine with more than 20 Ci/mM from the Radiochemical Centre, Amersham, England. Deoxyribonuclease RNase free from Worthington, EC 3.1.4.5. sRNA, of *E. coli*, from General Biochemicals, USA.

2.2. *Solutions*

Preribosome extraction buffer: 0.01 M triethanolamine, 0.01 M KCl, 0.1 mM $MgCl_2$, 0.01 M dithiothreitol, 0.1% Brij 35, pH 7.4. Na-Mg buffer: 0.01 M triethanolamine, 0.01 M NaCl, 0.001 M $MgCl_2$, pH 7.4. NH_4Cl-EDTA (2 X stock solution): 2 M NH_4Cl, 0.02 M EDTA, pH 8.0. Dialysis buffer: 0.05 M triethanolamine, 0.05 M KCl, 0.001 M β-mercaptoethanol, pH 8.0. Incubation buffer (10 X stock solution): 0.5 M triethanolamine, 0.5 M NaCl, 0.5 M KCl, 0.05 M $MgCl_2$, 0.01 M β-mercaptoethanol, pH 7.4. SDU solution (5 X stock solution): 5% SDS, 2.5% DOC and 2.5 M urea.

2.3. *Preribosome isolation*

Preribosomes were extracted from nucleoli as described elsewhere [4]. Detergent-washed nuclei were lysed with 0.1% heparin and 0.2% DOC, the chromatin digested with DNase (1 μg/ml) and the nucleoli sedimented through a 2.2 M sucrose cushion. The nucleolar pellet was resuspended in extraction buffer, and homogenized 10 min at 25° to extract

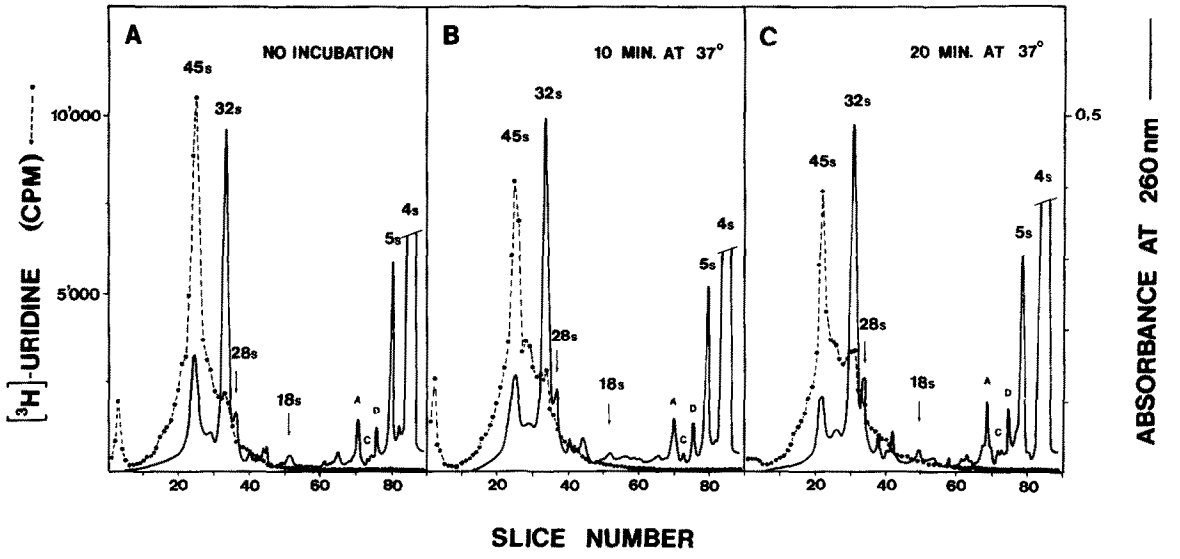

Fig. 1. Preribosome stability. Preribosomes (0.2 A_{260} units, 125 μl) isolated from HeLa cells labelled 15 min with 5-^{3}H-uridine (100 ml at 4 × 10^6 cells/ml, 20 μM uridine, 10 μCi/ml) were incubated under the assay conditions for the following times: A) 20 min at 0°, B) 10 min at 37° and C) 20 min at 37°. The reaction was stopped by adjusting to 1% SDS–0.5% DOC and 0.5 M urea (SDU treatment); the released RNA was precipitated by 3 vol of ethanol in presence of 150 μg sRNA as a carrier and analysed by electrophoresis on polyacrylamide exponential gels (L = 12 cm; C_1 = 15%; C_2 = 2%; V_j = 1.6 cm^3; r = 0.3 cm; n = 2; 8 hr at 100 V; 1.5 mm slices, cf. [4]). (———) Absorbance at 260 nm; (- - - - -) ^{3}H-radioactivity.

the preribosomes, which remain in the supernatant after sedimentation of the nucleoli (20 min, 20,000 *g*). The preribosome concentration was determined by measuring absorbance at 260 nm and the samples were stored at –70°.

2.4. *Isolation of the nucleolar endoribonuclease*

2.4.1. Preparation of nucleoli

All steps were carried out at 0–4°. Exponentially growing HeLa cells were collected and washed with Earle's solution (Ca^{2+} free). 5.4 ml packed cells were resuspended in 20 ml hypotonic Na-Mg buffer for 15 min of swelling, and then isotonicity was restored to 0.25 M sucrose. The cells were opened with a precision bored (0.035 mm clearance) stainless steel mechanical Dounce homogenizer and the nuclei were sedimented (5 min, 280 *g*). The nuclear pellet was resuspended in 10 ml isotonic (0.25 M sucrose), Na-Mg buffer, stirred on a Vortex and sedimented. (This step removes residual lysosomes and mitochondria which would be lysed by the detergent purification.) The pellet was resuspended as before, Triton X-100 added to 0.5%, the mixture stirred 1 min on a Vortex, and the nuclei were sedimented. The pellet was finally resuspended by stirring 1 min with 1% Tween–0.33% DOC (pH 7.4) and then resedimented. The washed nuclei of this last pellet (2–2.5 ml) appeared under the microscope to be free from any cytoplasmic contamination [4]. After this detergent treatment the structure of the nuclei was partially disrupted but the further isolation of nucleoli was not impaired.

Nucleoli were obtained by lysing the suspended nuclear pellet in 10 ml 0.5 M sucrose and Na-Mg buffer (2 mM $MgCl_2$) by the addition of DOC to 0.5% and 2 μg/ml DNase, vigorous mixing (Vortex and pipetting) and a 15 min digestion on ice. The lysate was centrifuged 45 min at 27,000 rpm (SW 27 rotor) on a double layer sucrose cushion (1 M and 2.2 M sucrose in Na-Mg buffer) in order to sediment and purify the nucleoli. The processing endoribonuclease was extracted from this nucleolar pellet.

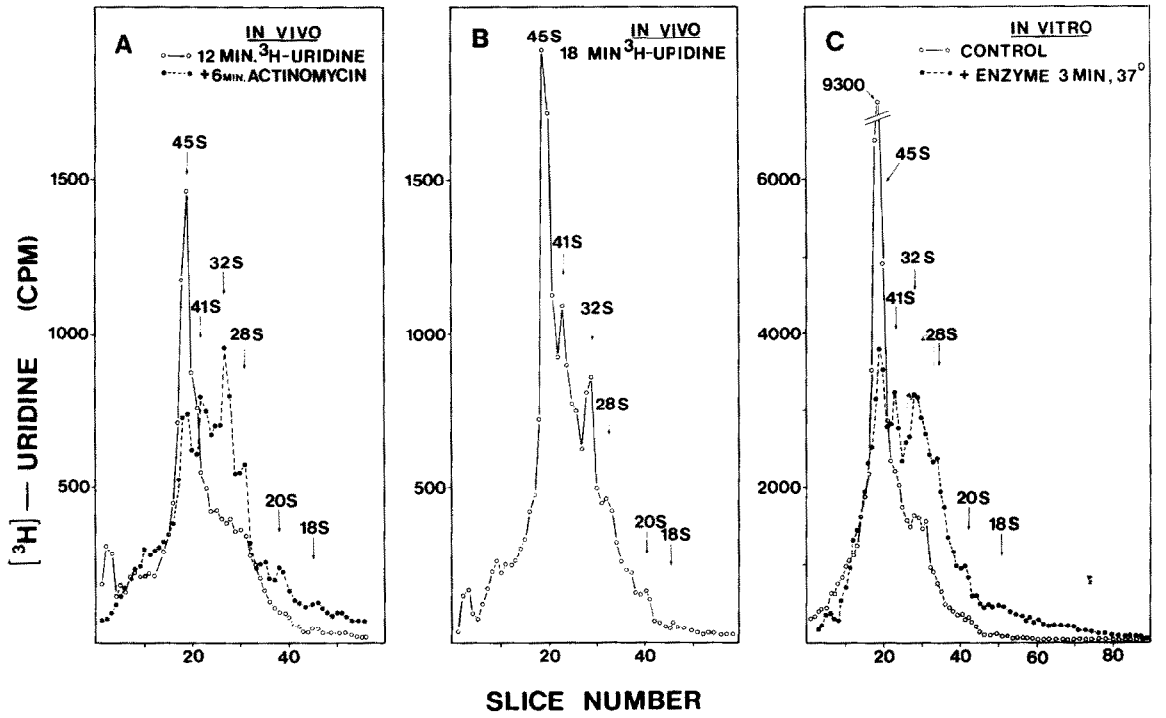

Fig. 2. *In vivo* and *in vitro* processing of preribosomes. A, B (*in vivo*): Exponentially growing HeLa cells were labelled 12 min with 5-^{3}H-uridine (1.2 l at 4 X 10^5 cells/ml, 10 μM uridine, 0.5 μCi/ml. Thereafter, the culture was divided in 3 parts of 400 ml; 1 batch was chilled on ice, to the second actinomycin D was added to 1 μg/ml and the third was left growing. The 2 latter batches were chilled on ice 6 min later. Nucleolar preribosomes were isolated and their RNA analysed by electrophoresis on exponential gels. (L = 9 cm; C_1: 5.5%; C_2 = 2%; V_i = 1.6 cm^3; r = 0.3 cm; n = 2; 14 hr at 90 V; 1.5 mm slices) A) 12 min, ^{3}H-uridine (——); 12 min ^{3}H-uridine and 6 min actinomycin (- - - -). B) 18 min ^{3}H-uridine (——). C) (*in vitro*): 0.2 A_{260} units of preribosomes labelled 15 min (same batch as in fig. 1) were incubated 6 min at 37° under assay conditions without enzyme (——), or 3 min at 37° with 120 μl "crude enzyme" (1.75 μg protein) (- - - -). The RNA of both samples was analysed by electrophoresis on exponential gels (L = 13 cm; C_1 = 20%; C_2 = 2%; V_i = 1.6 cm^3; r = 0.3; n = 2; 10 hr at 130 V; 1.5 mm slices). ^{14}C-labelled ribosomal RNA was used as internal radioactive markers; the position of 18 S and 28 S rRNA is displayed by arrows.

2.4.2. Extraction of the enzyme

The nucleoli were resuspended in 5 ml preribosome extraction buffer and homogenized 10 min at 25° in a glass Dounce homogenizer. After addition of 1 ml triethanolamine (1 M) at pH 8 and 6 ml of NH_4Cl-EDTA (2 X stock solution), this mixture was homogenized once more at 25° for 5 min and then centrifuged for 20 min at 20,000 *g* at 0°. To the 12 ml crude nucleolar supernatant was added 4.3 g solid ammonium sulfate (A.S.). After 30 min stirring (at 0°) the precipitate was removed by sedimentation (20 min at 20,000 *g*) and the supernatant was dialysed extensively against "dialysis buffer" at 4°. The precipitate was removed by sedimentation (20 min at 20,000 *g*) and the supernatant, containing the endoribonucleolytic activity and designated "crude enzyme" was kept frozen at −20°.

2.5. *Processing enzyme assay*

The assay is defined by testing the ability of an enzyme fraction to convert preribosomes *in vitro* into specific products containing RNA corresponding in size to the nucleolar RNA types found *in vivo*. Since a complex multistep reaction is involved, such an assay has only a qualitative significance but can hardly be used for the quantitation of the nucleolar processing activity.

The *in vitro* processing incubation mixture con-

tained: 0.125 ml pulse-labelled preribosomes (15 min ^{3}H-uridine) at 1.6 A_{260} units per ml, 0.050 ml stock incubation buffer (10 X), up to 0.25 ml enzyme solution and water to 0.5 ml. The complete mixture was transferred from ice into a waterbath at 37° for short time incubations (3–10 min). The reaction was stopped by adding first 0.125 ml of stock SDU solution (5 X) with rapid Vortex mixing followed immediately by 2 ml of cold absolute ethanol. After 2 hr at -20° the mixture was centrifuged for 30 min at 7,000 *g*. The supernatant was kept for further analysis. The RNA pellet dissolved in 50 μl of RNA electrophoresis buffer containing 10% glycerol was analysed by electrophoresis. Aliquots of the alcohol supernatant were dried on GF/B glassfiber filters and counted for measuring the amount of alcohol-soluble radioactive RNA products.

2.6. *RNA electrophoresis on polyacrylamide exponential gels*

This analysis was carried out according to the new technique we described recently [4].

2.7. *Protein determination*

Protein contents were estimated by the micromethod of Heil and Zillig [8].

3. Results

3.1. *Preribosome stability*

The relative stability of preribosomes during incubation is a prerequisite for detecting an enzyme-dependent processing reaction under the conditions of our assay. We reported previously a method to prepare stable preribosomes [4]. Fig. 1 shows the pattern of the RNA contained in preribosomes (from cells labelled for 15 min with ^{3}H-uridine) of a control and of those incubated *in vitro* at 37° for 10 and 20 min. Since no degradation occurs, we conclude that our nucleolar particles are stable enough over short incubation times (less than 10 min) to serve in our standard assay. Nevertheless their stability is not absolute: upon prolonged incubation, some processing does occur similar to that observed during *in vivo* maturation (cf. fig. 1C, 2A and B).

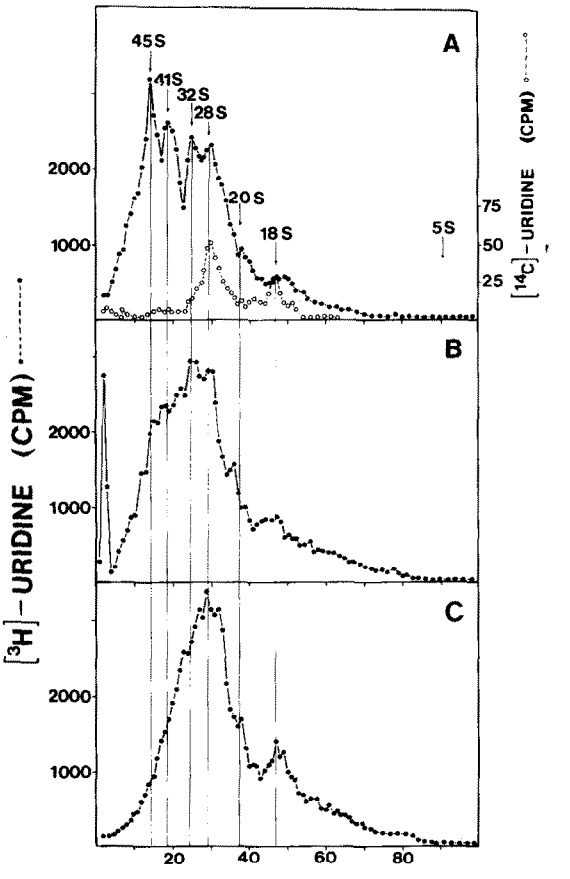

Fig. 3. Enzyme-dependance of processing. 0.2 A_{260} units of preribosomes (same batch as in fig. 1) was incubated at 37° under assay conditions: A) 3 min with 60 μl DEAE-enzyme (cf. text) equivalent to 0.5 μg protein. B) 3 min with 120 μl DEAE-enzyme. C) 6 min with 240 μl DEAE-enzyme. The RNA released by SDU treatment and precipitated by ethanol was dissolved in E-RNA buffer–8 M urea at room temp and analysed by electrophoresis on exponential gels (L = 13 cm; C_1 = 20%; C_2 = 2%; V_i = 1.6 cm^3; r = 0.3; n = 2; 10 hr at 130 V; 1.5 mm slices). ^{14}C-uridine labelled HeLa cell ribosomal RNA was used as internal marker. (——) ^{3}H-radioactivity; (- - - -) ^{14}C-radioactivity.

3.2. *In vivo and in vitro processing of preribosomes*

Any observed *in vitro* processing must be compared with the corresponding step of preribosome maturation *in vivo*. In order to make such a comparison a cell culture was divided into 3 equal batches, and these were labelled with ^{3}H-uridine for 12 min, 18 min and 12 min, respectively, followed by a 6 min chase in the presence of actinomycin D (1 μg/ml).

Nucleoli and preribosomes were isolated and RNA was released by SDU treatment. Fig. 2A shows the superimposed RNA patterns of the 12 min pulsed preribosomes and of the actinomycin chased preribosomes. The predominantly labelled 45 S pre-rRNA peak disappears during the short chase, giving rise to intermediate 41 S, 32 S and 20 S pre-rRNAs as well as to some 28 S and 18 S rRNA. This result is essentially in agreement with previous published experiments of the same kind [9]. The radioactive RNA pattern of preribosomes labelled 18 min without any actinomycin D is shown in fig. 2B. In this case where normal RNA synthesis and processing were concomitant, the radioactivity pattern is qualitatively similar to that found in the former case where RNA synthesis was blocked by actinomycin D, indicating that the drug does not give rise to anomalous early processing. Fig. 2C demonstrates the *in vitro* processing in the presence of the nucleolar enzyme (crude enzyme). The RNA pattern of labelled preribosomes (15 min ^{3}H-uridine *in vivo*) incubated alone as a control is superimposed on that of preribosomes incubated with the nucleolar enzyme. There is no doubt that the *in vitro* pattern (fig. 2C). is strikingly similar to those observed *in vivo* (fig. 2A and B).

3.3. *Enzyme purification and reaction course*

Most of the detectable nucleolar processing activity was found in the supernatant after ammonium sulfate precipitation which removed about one third of the protein and much of the nucleic acids found in the nucleolar supernatant after the salt extraction of nucleoli. Only 1–2% of the activity was found associated with the A.S. precipitate. For a further purification step, we took advantage of a procedure developed by Lazarus and Sporn [10] allowing separation of endoribonuclease from exoribonuclease activity. This latter enzyme has been shown to possess the major ribonucleolytic activity in the nuclei of various mammalian cells [11].

To 2.5 ml of the "crude enzyme" (30–40 μg protein) was added 0.3 ml DEAE cellulose equilibrated with dialysis buffer (low salt). After this treatment the non-adsorbed fraction (called "DEAE-enzyme") contained 2 or 3 times more endoribonuclease activity and less protein (about one half). Thus, by this purification step and "unmasking" of more activity, the specific activity could be raised by a factor of 3 to 5. The absorbance ratio A_{280}/A_{260} of 1.05 indicated a nucleic acid contamination of probably less than 3%.

Fig. 3 shows the RNA patterns resulting in a typical processing assay in which increasing amounts of the DEAE-enzyme were incubated with preribosomes; the same patterns were obtained in kinetic experiments with fixed amounts of enzyme. These results confirm the involvement of an endonucleolytic activity in processing: the originally labelled 45 S RNA of the preribosomes (fig. 2C, solid line) was converted sequentially into 41 S, 32 S, 28 S and 20 S, and 18 S RNA. However, in addition to the predominant 28 S and 18 S reaction end products found in the more advanced stages of processing (fig. 3C), other by-products could be detected, such as an RNA species ($\sim 1.4 \times 10^6$ M.W.) migrating between the 28 S and 20 S RNA's. It remains to be determined if this cleavage product is identical to the "24 S" RNA described by Weinberg and Penman [12] and proposed to arise from 41 S cleavage (41 S → "24 S" + 28 S), or if it arises from the first cleavage of the 45 S pre-rRNA (45 S → 41 S + "24 S"). Since the RNA was loaded in 8 M urea for electrophoresis on exponential gels (without urea), a treatment which dissociates completely the 28 S associated 7 S RNA from the 28 S rRNA [13], hidden breaks are very unlikely to invalidate this analysis. However, in order to prove the *in vitro* reaction products to be identical to those found *in vivo*, a finger print analysis would provide better evidence than gel electrophoresis for this identity.

Incubation of preribosomes with increasing amounts of either crude (A.S.) or DEAE-enzyme never resulted in more than 2–3% alcohol-soluble reaction products, while incubation without added enzyme gave up to 2% soluble products. From this we conclude that our nucleolar processing activity is an endonuclease, and that the early maturation of preribosomes does not seem to require an intensive exonucleolytic trimming. The shift in RNA size distribution and the discrete intermediates observed as the *in vitro* enzymatic reaction proceeds (fig. 3) supports this conclusion. Work is in progress to purify and characterize further this nucleolar endoribonuclease, and to find out what directs the specificity of the preribosome processing.

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