THE PRODUCT OF RIBOSOME-ASSOCIATED RNA-DEPENDENT RNA POLYMERASE IN IMMATURE CHICKEN ERYTHROCYTES

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SUMMARY

The radioactively labelled product obtained after incubation of chicken erythrocyte ribosomes with [^{3}H]-UTP was shown by sucrose density gradient centrifugation to have a sedimentation coefficient of 4-5S; the ratio of UMP to uridine incorporated was 6.09. The product synthesized and isolated after incubation of intact cells in the presence of [^{3}H]-uridine and actinomycin D was shown to be very similar with respect to sedimentation coefficient and the ratio of UMP/uridine incorporated, providing for the first time evidence of a ribosome-associated terminal ribonucleotidyl-transferase activity in intact cells.

1. INTRODUCTION

Wilkie and Smellie (1) were amongst the first to describe the presence of ribosome-associated terminal ribonucleotidyl transferases. These authors observed that the enzyme activities ".... may only be artefacts brought about by the conditions of assay or of isolation of the free ribosome fraction...." Although a number of workers have since described the presence of similar ribonucleotidyl transferase activities in different species (2,3,4,5,6) the question of whether a cytoplasmic enzyme activity (or its products), incorporating UTP into oligonucleotide material, can be detected in intact cells, still remains unanswered.

In continuation of our previous studies on the ribosome associated ribonucleotidyl transferase activities from immature chicken erythrocytes *in vitro* (4,7), the existence of the product(s) of such enzyme activities *in intact cells* is considered in this communication.

2. MATERIALS AND METHODS

Precoated TLC-plates (Cellulose F) were obtained from Merck Chemicals and actinomycin D from P.L. Biochemicals Incs. The sources of other chemicals used in this study have been mentioned previously (4,7).

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Preparation of anaemic chicken blood, ribosomes and ribosomal RNA* were as described before (4,7) except that incubations of ribosomes with [^{3}H]-UTP (250 µCi/µmole) were carried out for 30 min.

For the study of RNA synthesis in intact cells aliquots of whole anaemic blood (1.89 x 10^9 cells/ml) were mixed in a ratio of 3:2 with a solution containing 2% (w/v) glucose, 2% (w/v) sodium citrate, and incubated at 37°C for 2 h with [^{3}H]-uridine (100 µCi/ml, 42 Ci/mmol) in the presence or absence of actinomycin D (80 µg/ml). After incubation the cells were sedimented by centrifugation at 4 500 g for 10 min at 20°C. The pellet was washed, cells were lysed and ribosomes and ribosomal RNA prepared as previously described (4,7). The "nuclear" pellet (sedimented at 6 000 g, 10 min) was washed with a buffer containing 0.1 M Tris-HCl, pH 9.0, 0.1 M NaCl, 1 mM EDTA, and RNA extracted using the method of Aviv and Leder (8).

Analysis of hydrolysis products of RNA was carried out as follows : Washed RNA precipitates were dissolved in 0.05 M KOH and hydrolysed at 97°C for 40 min. The solutions were then neutralized with HCl and applied onto TLC-cellulose plates, with the appropriate standards. Electrophoresis in pyridine:acetic acid:water (1:10:292) at pH 3.6 was performed for 1 h at 2000 V (25 mA). Sample migration was towards the cathode. The plates were dried, and analysed under ultraviolet light. For determination of radioactivity, the appropriate sections of the plate were divided into rectangles of 1 cm x 0.5 cm. The cellulose was scraped off, collected in a scintillation vial and counted.

3. RESULTS AND DISCUSSION

The RNA-dependent synthesis of RNA in the presence of actinomycin D is well known (9). We have shown that the incorporation of UTP into oligonucleotides (or RNA) by a ribosome-associated terminal ribonucleotidyl transferase from immature chicken erythrocytes was insensitive to the presence of this antibiotic (10). Therefore if this enzyme activity is present in intact cells, its product should be detectable amongst those RNA species synthesized in the presence of actinomycin D.

Actinomycin D was found to inhibit the incorporation of [^{3}H]-uridine into RNA isolated from ribosomes in intact cells by 94.5%, confirming the degree of inhibition of cellular RNA synthesis previously reported (11,12). Analysis of ribosomal RNA extracted from immature erythrocytes incubated with [^{3}H]-uridine (Fig. 1) revealed a characteristic, radioactively labelled, 9S RNA, corresponding to globin mRNA (13,14). Little or no labelled 18S and 28S rRNA was detected. The cells of the anaemic blood used in these investigations were found to be predominantly (79%) mid- and late polychromatic erythrocytes (classification according to Lucas and Jamroz (15)).

* It should be emphasized that the ribosomal RNA fraction as isolated contains total ribosomal and polysomal RNA plus newly synthesized radioactively labelled RNA product ([^{3}H]-pRNA, see ref. (7)).

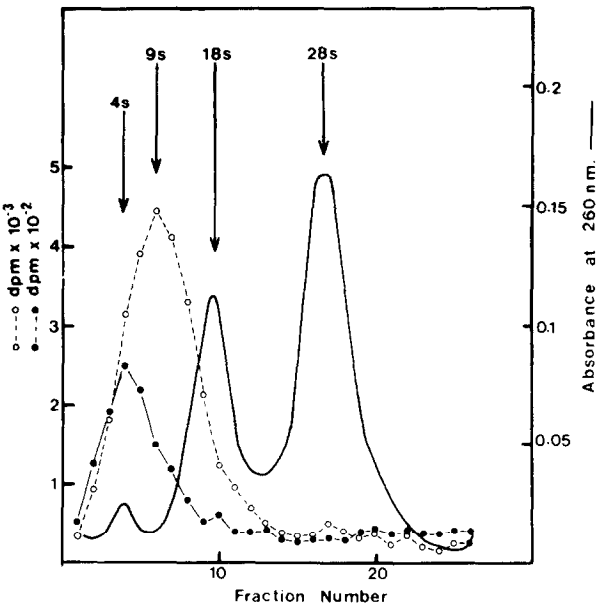

Fig. 1 : Analysis by sucrose density gradient centrifugation of ribosomal RNA prepared from immature erythrocytes incubated with [^{3}H]-uridine in the presence and absence of actinomycin D.

0.50 $OD_{260\ nm}$ units of ribosomal RNA was applied to 15-30% sucrose gradients and centrifuged at 42 000 g for 3 h at 20°C in a Beckman SW 65LTi rotor. The gradient was analyzed at 260 nm, 0.2 ml fractions were collected and TCA-precipitable radioactivity was determined. (●——●) actinomycin D present, (o----o) actinomycin D absent.

Both these cell types lack nucleoli and are not able to synthesize rRNA (16). More immature erythrocytes (basophilic erythroblasts) in the blood preparations used by Evans and Lingrel (17) and Spohr et al. (14) might account for the synthesis of rRNA observed by these workers.

Analysis of ribosomal RNA extracted from immature erythrocytes incubated with [^{3}H]-uridine in the presence of actinomycin D (Fig. 1) revealed, in contrast, a single low molecular weight peak of radioactive RNA sedimenting in the 4-5S region of the sucrose gradient. Eason and co-workers (11) have shown, using Krebs II ascites tumour cells incubated with [^{3}H]-uridine, the appearance of radioactively labelled 4S material in the presence of actinomycin D. These authors demonstrated that the major portion of this radioactivity, after hydrolysis of 4S RNA (obtained by extraction of whole cells with phenol) with alkali and separation of 2'(3')-monophosphates, was found as [^{3}H]-CMP. They attribute most of

Table 1 : Distribution of radioactivity in ribonucleosides and ribonucleotides obtained after alkaline hydrolysis of ribosomal RNA a) from immature erythrocytes incubated in the presence of actinomycin D and [^{3}H]-uridine and b) from ribosomes incubated with [^{3}H]-UTP *in vitro*.

Alkaline hydrolysis of ribosomal RNA ([^{3}H]-pRNA) and high voltage electrophoresis of the products was carried out as described in Materials and methods

		Hydrolysis products	% of total radioactivity	nucleotide / nucleoside
a)	Intact cells incubated	UMP	85.08	5.50
		Uridine	15.47	
		GMP,AMP,CMP	0	
b)	Ribosomes incubated	UMP	85.90	6.09
		Uridine	14.11	
		GMP,AMP,CMP	0	

this actinomycin D-insensitive RNA synthesis to addition of [^{3}H]-CMP to the 3'-OH terminus of tRNA. This type of oligonucleotide synthesis has also been reported or presumed to occur in reticulocytes by a number of workers (13,18).

In order to investigate the nature of the radioactively labelled 4S RNA isolated from ribosomes of immature erythrocytes incubated with [^{3}H]-uridine, total ribosomal RNA was digested with alkali and the products of digestion separated by thin layer electrophoresis. In three such experiments all the radioactivity was found as UMP and uridine (Table 1a). The ratio of UMP/uridine was 5.50. As this ribosome associated terminal ribonucleotidyl transferase is capable of hetero-oligoribonucleotide synthesis (4), the chain length of the oligonucleotide product cannot be determined from these data. However, as no conversion to [^{3}H]-CMP was detectable it is clear that this ribosome-associated incorporation of UTP into 4-5S RNA, in intact cells, is a different and distinct activity from the pCpCpAOH addition to tRNA. Atypical incorporation of UTP by tRNA nucleotidyl transferase is not known to occur *in vivo* (19).

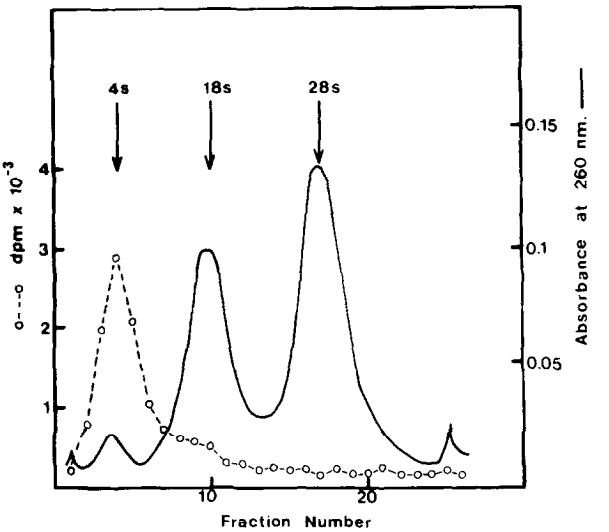

Fig. 2 : Sucrose density gradient centrifugation analysis of ribosomal RNA from ribosomes incubated with [^{3}H]-UTP *in vitro*.

Centrifugation and subsequent analysis as described in legend to Fig. 1.

Ribosomal RNA isolated from immature erythrocyte ribosomes incubated with [^{3}H]-UTP was analyzed by density gradient centrifugation. All of the incorporated TCA-precipitable radioactive material was found in the 4-5S region of the sucrose gradient (Fig. 2). This is in agreement with the labelling pattern shown by previous workers (1). A comparison of Figs. 1 and 2 reveals that the position of the single peak of radioactivity from the profile of ribosomal RNA isolated from intact cells (Fig. 1), and ribosomes incubated *in vitro* (Fig. 2) is exactly the same. In addition, analysis of the alkaline digestion products of ribosomal RNA from ribosomes incubated with [^{3}H]-UTP *in vitro* revealed a UMP/uridine ratio of 6.09 (Table 1b) (radioactivity was found associated with UMP and uridine only), in close agreement with the nucleotide/nucleoside ratio obtained from the hydrolysis of ribosomal RNA isolated from ribosomes of immature erythrocytes incubated with [^{3}H]-uridine in the presence of actinomycin D.

This close similarity of sedimentation properties and UMP/uridine ratios between ribosomal RNA from intact cells and ribosomes incubated *in vitro* strongly suggests that this radioactively labelled RNA, present in ribosomal RNA isolated from immature erythrocytes incubated in the presence of actinomycin D, might be the product of the ribosome associated terminal ribonucleotidyl transferase activity observed *in vitro*.

McReynolds and Penman (20) have demonstrated the presence of radioactively labelled low molecular weight RNA in HeLa cell nuclei incubated in the presence of actinomycin D. These authors, in addition, have found this labelled RNA product to have a UMP/uridine ratio of less than ten. They attribute the presence of this RNA to the low levels of synthesis of polynucleotide by a nuclear DNA-dependent RNA polymerase between blocked guanylic acid residues (the site of inhibition by actinomycin D (21)). In order to exclude the possibility that nuclear leakage of such an 'abortive' RNA species, during cell lysis, could account for the presence of low molecular weight, radioactively-labelled RNA in the ribosome fraction of immature erythrocytes incubated in the presence of actinomycin D, we extracted total RNA from a nuclear fraction of immature erythrocytes incubated with [^{3}H]-uridine and actinomycin D (see Materials and methods) and analyzed the distribution of radioactivity in nucleotides and nucleosides following alkaline digestion. The UMP/uridine ratio of this RNA preparation was found to be 11.4. The nucleotide/nucleoside ratio therefore indicates that there was no nonspecific adsorption of this nuclear RNA to ribosomes during ribosomal preparation. This adds further support to our conclusion that the appearance of radioactively-labelled RNA in the ribosome fraction of immature erythrocytes is due to a ribosome-associated actinomycin D-insensitive terminal ribonucleotidyl transferase activity.

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