RIBOSOME BOUND RNA-DEPENDENT RNA POLYMERASE: CHANGES IN TOTAL AND SPECIFIC ACTIVITY DURING MATURATION OF AVIAN ERYTHROCYTES

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SUMMARY

Ribosome bound RNA-dependent RNA polymerase activity from immature chicken erythrocytes was shown to be predominantly primer-dependent, in contrast to the template-dependent synthesis demonstrated by previous workers (4). During the maturation of the immature avian erythrocyte, the total and specific activities of this ribosome bound enzyme preparation increase 40 and 9.4 times respectively.

1. INTRODUCTION

Cytoplasmic, ribosome bound RNA-dependent RNA polymerases (ribonucleoside triphosphate RNA nucleotidyl transferases) have been known to exist for several years (1,2,3). Recently, Downey et al. (4) provided evidence indicating that one of these enzymes might be involved in the specific amplification of globin mRNA in rabbit reticulocytes. We have isolated a similar fraction from immature chicken erythrocytes which shows no predominant template dependent activity and thus no obvious involvement in the possible cytoplasmic amplification of globin messenger. This fraction shows primer-dependent synthesis of polymers of UMP and CMP.

A correlation was found between changes in total and specific activity of this ribosome bound enzyme preparation and changes in globin and RNA synthesis during the maturation of the immature avian erythrocyte, implicating, possibly, the involvement of this enzyme in translational control of globin synthesis.

2. MATERIALS AND METHODS

All radioactive materials were obtained from the Radiochemical Centre, Amersham Calf thymus DNA was purchased from Sigma Chemical Co.; RNase-free sucrose, yeast RNA, dithiothreitol (DTT), poly (A,G), poly (U,C), poly (A), poly (U) and unlabelled ribonucleoside triphosphates from Miles Laboratories Inc.; DEAE-Sephadex A-25 from Pharmacia Fine Chemicals (Uppsala); Biosolv BBS-3 from Beckman Instruments. Chicken erythrocyte DNA was prepared according to Packman et al. (5), and chicken erythrocyte 9S, 18S and 28S RNA from ribosomes,

essentially as described by Lingrel et al. (6). Each RNA fraction was homogeneous as judged by sucrose gradient centrifugation. All other reagents were Analar grade.

2.1 ANIMALS

8 week old cocks (Cornish Game/Plymouth Rock) weighing 1.5 - 2.0 Kg were injected with neutralised phenylhydrazine hydrochloride (5 mg/Kg body weight) for 4 consecutive days; blood was collected from the jugular vein on the day after the last injection (anaemic blood) in an equal volume of a solution containing 2% (w/v) sodium citrate, 2% (w/v) glucose.

For cellular maturation studies, blood was removed daily from the wing vein using heparin as an anticoagulant.

Normal blood was obtained from uninjected animals.

2.2 ISOLATION OF RIBOSOMES

All procedures were carried out at 0 to 4°C unless otherwise stated. Blood cells were filtered through two layers of cheese cloth and washed 3 times by centrifugation at 4 500 g for 10 min in 10 vol. 0.14 M NaCl, 5 mM KCl, 1.5 mM $MgCl_2$. The packed cells were lyzed in 5 vol. of a solution containing 5 mM $MgCl_2$ and 5 mM 2-mercaptoethanol. After 3 min of vigorous shaking, 1 vol. 1.5 M sucrose, 0.15 M KCl, 5 mM 2-mercaptoethanol was added and the lysate centrifuged at 10 000 g for 20 min. Ribosomes were prepared from the post-mitochondrial supernatant by centrifugation through 36% (w/v) sucrose in 30 mM Tris-HCl pH 7.8, 30 mM KCl, 1.5 mM $MgCl_2$ (360 000 g for 1 h in Beckman 60 Ti rotor). This ribosomal preparation, consisting predominantly of monosomes (normal blood) or polysomes (anaemic blood) has an $E_{260\ nm}/E_{280\ nm}$ ratio of 1.63 ± 0.13 (10 preparations), indicating a pure ribosomal preparation (7).

2.3 PREPARATION OF RNA POLYMERASE

The ribosomal pellets were suspended in 50 mM Tris-HCl, pH 7.8, 1.0 mM DTT, 1.0 mM EDTA, 0.25 M sucrose, 0.5 M KCl. RNA polymerase was extracted with KCl and precipitated with $(NH_4)_2SO_4$ (at 4°C) as described by Downey et al. (4). This precipitate was collected by centrifugation, dissolved in 50 mM Tris-HCl, pH 7.8, 0.1 mM EDTA, 1.0 mM DTT, 30% (v/v) glycerol, 5.0 mM $MgCl_2$, (TM-30), and dialyzed against the same buffer for 3 hours.

The dialyzed extract was applied to a DEAE-Sephadex column and eluted as described in Fig. 1. The fractions containing the eluted RNA polymerase activity were pooled and stored at -150°C.

2.4 ASSAY OF RNA- AND DNA POLYMERASE

RNA synthesis: [^{3}H]-UTP was included in an incubation mixture, as described by Downey et al. (4) except that 30 µg 18S RNA was used as template. After incubation for 30 min at 37°C, the reaction was stopped with 2 ml 10% (w/v) trichloroacetic acid (containing 1 mg/ml carrier UTP); 0.1 ml total yeast RNA (2 mg/ml) was added as a carrier. Acid precipitable radioactivity was collected on Whatman GF/C glass fibre filters, washed (100 ml 5% trichloroacetic acid, followed by 20 ml 96% (w/v) ethanol), hydrolyzed (0.75 ml 0.25 M NaOH for 45 min at 100°C), buffered (0.2 ml 5% (v/v) glacial acetic acid),

Table 1 : Preparation of RNA-dependent RNA polymerase from 385 ml of anaemic blood.

Fraction	Total protein (mg)	Total activity (units)	Specific activity (units/mg protein	Enzyme recovery (%)	Purification	$\frac{E_{280\ nm}}{E_{260\ nm}}$
Postmitochondrial supernatant	10248	53128	5.2	100	1	1.10
Ribosome suspension	77.51	29208	376	55	72	0.62
60% saturated $(NH_4)_2SO_4$ precipitate in TM-30 buffer	36.50	14530	398	27	76	0.78
DEAE-Sephadex chromatography (fractions 38-50)	11.00	9064	824	17	158	1.91

solubilized in scintillator (0.5% PPO, 10% Biosolv BBS-3 toluene) and counted. All incorporation figures are the average of duplicate incubations.

DNA synthesis : The assay was carried out as in (8) except that [^{3}H]-dTTP (8 µM) was used at a specific activity of 250 Ci/mol and incubation time was 30 min. 1 unit of polymerase activity is defined as that amount of enzyme which incorporates 1 pmole of UMP (or dTMP) under the assay conditions mentioned above.

Protein was determined by the method of Lowry et al. (9) and $(NH_4)_2SO_4$ with Nessler's reagent. Ribosome yield was evaluated from the relationship :

$$1.0 \text{ mg ribosomes/ml} \longrightarrow A^{1\ cm}_{260\ nm} = 10.$$

3. RESULTS AND DISCUSSION

An RNA polymerase was isolated from ribosomes of immature chicken erythrocytes and enriched 158-fold (Table 1). The KCl-extraction and subsequent precipitation with $(NH_4)_2SO_4$, although effecting little increase in specific

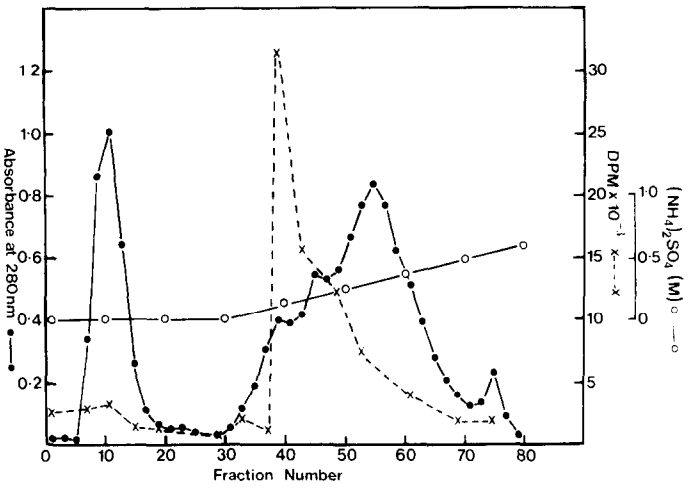

Fig. 1 : DEAE-Sephadex chromatography of RNA-dependent RNA polymerase.

A 60% saturated- $(NH_4)_2SO_4$ precipitate (prepared from ribosomes of an anaemic blood type), dissolved in TM-30 and dialysed against the same buffer (see Materials and Methods), was applied to a 1.3 x 11 cm column of DEAE-Sephadex A-25, previously equilibrated with TM-30 buffer. Protein ($OD_{280\ nm}$ ●——●) was eluted with a 60 ml linear gradient of 0 - 1.0 M $(NH_4)_2SO_4$ in TM-30 at a flow rate of 34 ml/h.

1.0 ml fractions were collected and 100 µl aliquots assayed for enzyme activity in standard incubation mixtures with no added $(NH_4)_2SO_4$ (dpm incorporated x 10^{-2} x----x).

enzyme activity, is nevertheless important as it solubilizes this RNA-dependent RNA polymerase activity (no activity was found in the KCl-extracted ribosome pellet), facilitating further purification on DEAE-Sephadex. Chromatography of this avian RNA polymerase on phosphocellulose, a method used by Downey et al. (4) for the purification of the rabbit enzyme, was unsuccessful in our hands.

RNA-dependent synthesis of RNA by the enriched enzyme preparation (fractions 38-50, Fig. 1) is almost completely dependent on added RNA; 18S RNA, 28S RNA and 9S globin mRNA stimulate the incorporation of [^{3}H]-UTP into RNA (Table 2), whereas DNA, under conditions of RNA synthesis, is inactive. Although DNA-dependent DNA polymerase activity has been isolated from rabbit reticulocyte ribosomes (8), the chicken RNA polymerase in this study effects no incorporation of DNA precursors into acid-insoluble material using etiher added DNA or 18S RNA (Table 2). This enzyme preparation is thus distinguished from DNA-dependen DNA polymerase activity.

Table 2 : Nucleic acid requirements of RNA-dependent RNA polymerase under conditions of RNA and DNA synthesis.

Reaction conditions for RNA and DNA synthesis were as described in Materials and Methods except for addition of nucleic acids as indicated (20 µg/assay) and 25 µg of enzyme preparation (fractions 38-50, Fig. 1) per assay. A background of 950 dpm (endogenous activity) has been subtracted to obtain the above figures.

Nucleic acid	[^{3}H]-precursor	dpm	Relative activity (%)
18S rRNA	UTP	9570	100
9S globin mRNA	UTP	8820	92
28S rRNA	UTP	6130	64
Chicken erythrocyte DNA	UTP	0	0
18S rRNA	TTP	0	0
Chicken erythrocyte DNA	TTP	0	0

It is important, in determining the function of this enzyme, to ascertain whether the stimulation of RNA synthesis by added RNA is a primer or template dependent phenomenon. We tested this by measuring the incorporation into RNA of the individual tritiated triphosphates in the presence of various ribonucleotide polymers (Table 3). It is clear from these data that this enzyme preparation incorporates predominantly [^{3}H]-CTP and [^{3}H]-UTP into TCA-precipitable material regardless of which ribonucleotide polymer is present. (Poly(A) is the most effective polymer in this respect, a finding for which

Table 3 : [^{3}H]-ribonucleoside triphosphate incorporation into RNA using various ribonucleotide polymers.

Reaction conditions as described in Methods and Materials except for addition of individual [^{3}H]-ribonucleoside triphosphates (all at a specific activity of 250 Ci/mol) and ribonucleotide polymers (70 µg/assay) as indicated (amount of enzyme added as in Table 2).

Ribonucleoside triphosphate present	Ribonucleotide polymer			
	Poly(U)	Poly(A)	Poly(A,G)	Poly(U,C)
	Incorporation (dpm)			
[^{3}H]-ATP	224	756	810	751
[^{3}H]-GTP	0	951	2945	911
[^{3}H]-CTP	2779	28253	21684	8951
[^{3}H]-UTP	2795	38377	18803	12406

we have no explanation at present). This suggests a primer-dependent synthesis of a polynucleotide, in contrast to the template dependent synthesis of RNA shown by Downey and co-workers(4).We have not excluded the possibility that our enzyme preparation might be a mixture of template and primer-dependent activities, the primer-dependent RNA polymerase being the dominant one. Further investigation should clarify this point.

The function of a primer-dependent, ribosome bound RNA polymerase is obscure. In this study, this activity is located on ribosomes committed to the predominan synthesis of globin (10); therefore, if this enzyme activity were necessary for translation, one might expect changes in total and specific activity of RNA-dependent RNA polymerase concurrent with changes in globin and RNA synthesis during erythrocyte maturation. The results of such an experiment are shown in Fig. 2. Treatment with phenylhydrazine results in a large increase in the number of circulating, immature erythrocytes (reticulocytes). Concurrently, RNA and protein synthesis increase 4 and 36 times respectively; that most of the protein synthesized is globin, was confirmed by a 90% incorporation of TCA-precipitable radioactivity, both into the two major components of chicken

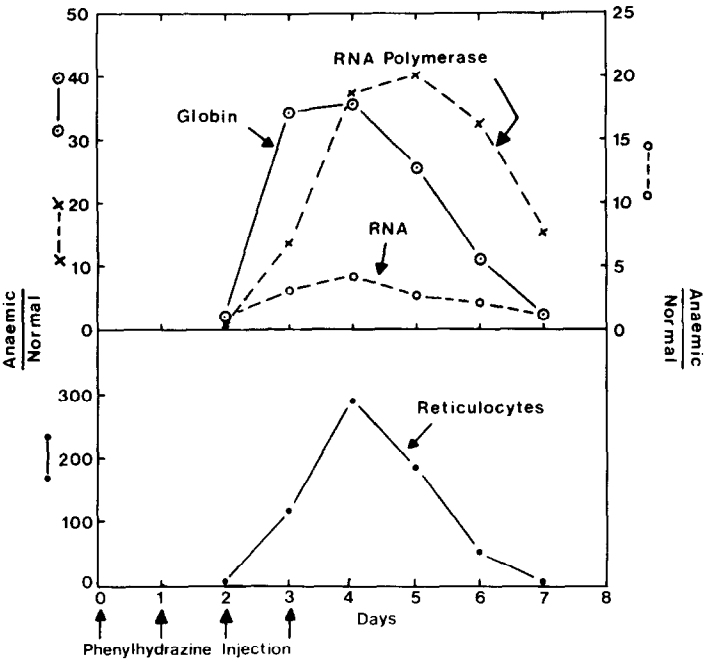

Fig. 2 :

RNA synthesis, globin synthesis and ribosome bound RNA polymerase activity in red blood cells during anaemia. Average values of 3 control and 3 experimental animals. Blood was removed daily and ribosomes isolated as described. The ribosomal pellets were suspended in 50 mM Tris-HCl, pH 7.8, 1.0 mM DTT, 1.0 mM EDTA, 0.25 M sucrose and RNA polymerase activity determined in the standard incubation mixture (no added 18S RNA). Globin and RNA synthesis: 0.1 ml aliquots of blood were incubated with 1 µCi [3H]-histidine (55 $Ci/_{mmol}$) and 1 µCi [3H]-uridine (3.33 $Ci/_{mmol}$) respectively for 1 hour at 37°C. TCA-precipitates were collected, washed and counted as in (19). All results are expressed as a ratio of the parameters found in anaemic and normal animals.

⊙——⊙ globin synthesis (dpm/10^9 cells)

o----o total cellular RNA synthesis (dpm/10^9 cells)

x----x RNA-dependent RNA polymerase activity (units/10^9 cells)

●——● Reticulocyte count (Brilliant cresyl blue). Values from 0.2% (Normal) to 70.0% (anaemic, day 4).

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haemoglobin, as separated on carboxymethyl cellulose (20), and into HCl-acetone precipitated globin, chromatographed as described by Dintzis (11). (Chicken globin elutes as a single peak under these conditions). Total ribosome bound RNA-dependent RNA polymerase activity (units of enzyme activity/10^9 cells) rises significantly during anaemia (40 times on day 5). The specific activity

(units/mg ribosomes) also increases, reaching a maximum on day 7 (anaemic/normal ratio of 9.4) and declining to 3.4 times normal values on day 12.

The increased synthesis of total cellular RNA and globin during phenylhydrazine induced anaemia is well documented (12,13); that the bulk of this activity occurs in the immature erythrocyte is also well known (14). Many constituents closely associated with translation have also been found to undergo a concomitant increase (total ribosome yield increases from 0.0108 mg/10^9 cells (normal) to 0.1079 mg/10^9 cells (anaemic day 5, Fig. 1); polyribosome content (15); 9S RNA content (16)). The change in ribosome bound RNA polymerase activity reported here could therefore reflect the involvement of this enzyme in the translational control of globin synthesis by some mechanism other than messenger amplification, since the enzyme exhibits a predominantly primer-dependent activity. At the present time we can offer no evidence from which to propose a more specific function for this primer-dependent activity. Two speculative possibilities for the function of such an enzyme are in the synthesis of U,C-rich regions in rRNA, necessary for mRNA recognition (17), or in the synthesis and modification of translational control RNA (18).

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