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RNA synthesis in maturing avian erythrocyte nuclei

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Abstract. The rate of RNA synthesis and its inhibition by α-amanitin in the nuclei of mature and immature avian erythrocytes are increased with the increase in ionic strength of incubation medium. Polyacrylamide gel electrophoresis indicates that heterogeneous species of RNAs are synthesised in the mature and immature erythrocyte nuclei. However, a large number of high molecular weight RNAs are synthesised in the nuclei of immature erythrocytes. Elution profiles on poly(U)-sepharose chromatography indicate that the RNAs synthesised in the nuclei of two types of cell contain poly(A) segments. Sixteen per cent of mature erythrocyte nuclear RNA syntbesised are polyadenylated, while it is 13% in immature erythrocyte nuclei. However, the total RNA synthesised is 2–3 fold higher in immature erythrocyte nuclei than that in mature erythrocyte nuclei.

Keywords. RNA synthesis; polyadenylated RNA; avian erythrocyte.

Introduction

Isolated nuclei from a variety of sources offer a good system to study the synthesis of RNA as well as the processing and transport of the synthesised RNAs (Widnell and Tata, 1964; Zylber and Penman, 1971; Smith and Arnstein, 1972; Murzluff *et al*., 1974; Jacobsen *et al*., 1974). Transcription of rRNA by RNA-polymerase in isolated nuclei of *Xenopus laevis* (Reeder and Roeder, 1972) and HeLa Cell (Zylber and Penman, 1971) has been described. Recently Ernest *et al*. (1976) have suggested that bulk of the RNA synthesised in the isolated nuclei of hen oviduct may be the ovalbumin mRNA. Viral specific RNAs (Price and Penman, 1972; Harris and Roeder, 1978) are synthesised in the nuclei isolated from virus infected cells. Synthesis of poly(A) sequences found in heterogeneous nuclear RNA has also been demonstrated (Jelinek, 1974; Kieras *et al*., 1978) using the isolated nuclei system.

Unlike mammalian erythrocytes, the avian erythrocytes are nucleated. The precursor erythroid cells are highly active in RNA synthesis and as these cells mature there is concomitant decrease in RNA synthesis. Fully mature avian erythrocytes, although nucleated, are able to synthesise only small amounts of RNA (Scherrer *et al*., 1966; Attardi *et al*., 1970; Madgwick *et al*., 1972; Mandal *et al*.,

1974). Therefore, maturing avian erythrocytes provide a model system to study the control of transcription. In this paper, we have attempted to compare the RNA synthesis in the nuclei isolated from mature and immature erythrocytes of white leghorn cock.

Materials and methods

Materials

White leghorn cocks were purchased from local poultry. ATP, GTP, CTP, UTP, α-amanitin and actinomycin D were obtained from Sigma Chemicals, St. Louis, MO, USA. Heparin I.P. was purchased from Biological Evans, Hyderabad. DNase (RNase-free) was a product of Worthington Biochemical Corporation, Freehold, NJ, USA. Poly(U)-sepharose was the product of PL, Biochemicals, Milwaukee, WI, USA. [^{3}H]-Uridine was purchased from the Isotope Division Bhabha Atomic Research Centre, Trombay, Bombay. [^{3}H]-UTP and [^{3}H]-GTP were obtained from the Radiochemical Centre, Amersham, England. All other reagents were of commercially available analytical reagent grade.

Methods

Collection of blood : To obtain blood rich in immature erythrocytes, anemia was induced in the cocks by subcutaneous injection of neutralised Phenylhydrazine (10 mg/kg body wt) for three consecutive days. Blood was collected 24 h after the third hydrazine injection from the wing veins using heparin (10–20 units/ml) as the anticoagulant.

Isolation of nuclei : The blood was centrifuged at 1100g for 10 min in MSE refrigerated centrifuge. The plasma and the buffy coat containing leucocytes were removed and the erythrocytes were washed with 0·14 M NaCl. The washed cells were suspended in 10 vol of 0·14 M NaCl containing 0·2% Triton X–100 at 4°C for 30 min (van der Westhuyzen *et al*., 1973). The nuclei were collected by centrifugation at 10,000 g for 5 min and washed twice with 10 mM Tris-HCl buffer pH 8·0, containing 0·14 M NaCl, 5mM $MgCl_2$, 0·1 mM EDTA and 5mM 2-mercaptoethanol.

Incorporation of [^{3}*H*]*-UTP into isolated nuclei* : RNA synthesis in isolated nuclei was measured using the reaction condition described by Reeder and Roeder (1972). The reaction mixture contained in a total volume of 50 μl : 50 mM Tris-HCl buffer pH 8·0, 5mM $MgCl_2$, 1 mM $MnCl_2$, 50 mM $(NH_4)_2SO_4$, 0·6 mM each of ATP, GTP, CTP and 0·06 mM [^{3}H]-UTP (sp. act. 45 cpm/pmol) and indicated amounts of nuclei. The samples were incubated at 30° C for specified period of time with shaking. The reaction was stopped by soaking a 40 μl aliquot fraction on Whatman 3 MM filter paper discs. The discs were then processed to measure the trichloroacetic acid precipitable radioactivity as described by Ernest *et al*. (1976).

Isolation of [^{3}*H*]*-RNA synthesised by the isolated nuclei* : The reaction mixture described above was scaled upto 1 ml and incubated at 30° C for 60 min. These

nuclei were further incubated at 30° C for 10 min after adding 20 μg DNase (RNase-free). The reaction was stopped with sodium dodecyl sulphate (Sod. dod. SO_4) (final concentration 0·5%). RNA was extracted with equal vol of water saturated phenol at 60° C for 5 min with vigorous shaking (Sehener and Darnell, 1962), chilled quickly in an ice bath and re-extracted at room temperature for 5 min. The emulsion was centrifuged at 10,000 g for 5 min. RNA was precipitated from the aqueous phase after adding 2 vol of ethanol and sodium acetate (final concentration 2%). The precipitate was dissolved in 1 ml of 10 mM Tris-HCl buffer pH 7·4 containing 5 mM $MgCl_2$, incubated with 20 μg DNase (RNase-free) at 4° C for 60 min. and RNA was extracted with equal vol of water-saturated phenol at room temperature. The aqueous phase was dialysed against 10 mM Tris-HCl pH 7·4 for 2 h at 4°C with 2 changes and precipitated by adding ethanol-sodium acetate as described above.

Polyacrylamide gel electrophoresis of RNA : Electrophoresis of RNA was carried out in 3% polyacrylamide gel according to Bishop *et al*. (1967). After electrophoresis, the gels were stained for 1 h with 0·1 % toluidine blue in 1% acetic acid and destained by repeated washing with 1% acetic acid until the background was clear. For radioactivity measurement, the gels were cut into 2 mm slices and the slices were individually digested with 0·025 ml of 20% sod. dod. SO_4 and 0·3 ml of ammonia solution at 60° C for 3 h in the counting vials. Radioactivity was measured by adding 5 ml of dioxan-based liquifiuor and counted in a Beckman LS-100 spectrometer.

Analysis of poly(A) containing RNA : The amount of poly(A) containing RNA was determined by poly(U)-sepharose chromatography of the labelled RNA following the method of Lindberg and Persson (1972). The RNA sample was diluted to 0·3 ml by the addition of high salt buffer (0·5 M NaCl, 10 mM Tris-HCl pH 7·4, 0·2% sod. dod. SO_4, 2mM EDTA) and=applied to a poly(U)-sepharose column (0·5 × 4 cm) equilibrated with the same buffer. The column was washed with three column vol of the high salt buffer to remove the non-poly(A) containing RNA. The poly(A) containing RNA was eluted with three column vol of the elution buffer (10 mM Tris-HCl pH 7·4, 90% formamide, 2 mM EDTA). Fractions (0·5 ml) were collected and the trichloracetic acid-precipitable radioactivity in each fraction was determined.

Results and discussion

Incorporation of [^{3}H]-UTP into RNA.

The time course of [^{3}H]-UTP incorporation into RNA by the isolated nuclei of mature and immature erythrocytes is shown in figure 1 and this is a measure of RNA synthesised by ribosomal and non-ribosomal RNA polymerases. The incorporation of [^{3}H]-UTP was higher in the immature erythrocytes than in mature erythrocytes. RNA synthesis in the isolated nuclei reached a plateau after 30–40 min of incubation. In the presence of α-amanitin, while the RNA synthesis in the mature erythrocyte nuclei was reduced by 50–60% throughout the incubation period, significantly larger inhibition was observed in the nuclei of immature erythrocytes. However, the total α-amanitin resistant RNA synthesis in the immature erythrocyte nuclei was also higher than that in

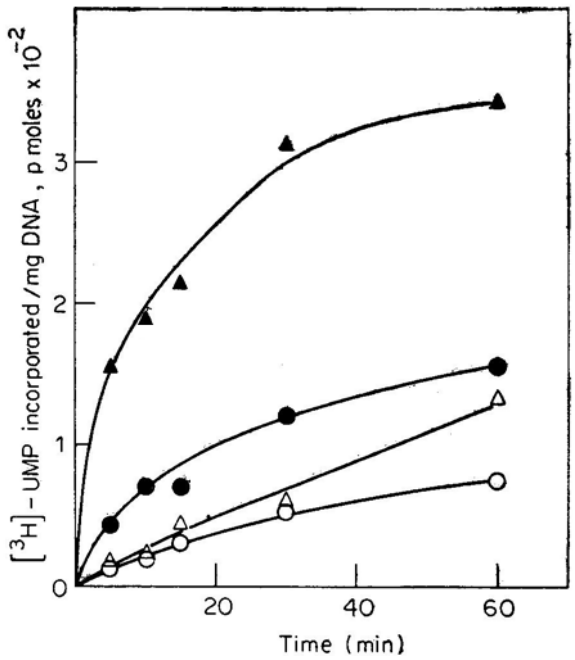

Figure 1. Time-course RNA synthesis in the nuclei of mature and immature erythrocytes at low salt concentration.

Nuclei isolated from mature and immature erythrocytes were incubated using the reaction condition described in Methods. The amount of nuclei used were equivalent to 1·2–1·6 mg DNA/ml. Ammonium sulphate and α-amanitin were used at 50 mM and 2·5mg/ml respectively. The incubation was carried out at 30° C and at time intervals indicated in the figure and 40 μl aliquot was soaked onto Whatman 3 MM circles and the trichloroacetic acid-precipitable radioactivity was measured. ● Mature erythrocyte nuclei; ○ Mature erythrocyte nuclei + α-amanitin; ▲ Immature erythrocyte nuclei; Δ Immature erythrocyte nuclei + α-amanitin.

mature erythrocyte nuclei. It is premature to speculate at the present time on the nature of the α-amanitin resistant-RNA synthesised by these nuclei. Since α-amanitin (2·5 μg/ml) inhibits selectively purified RNA polymerase II from different eukaryotic sources (Chambon, 1974), the α-amanitin-sensitive activity in these nuclei may reflect the activity of the RNA polymerase II.

An increase in [^{3}H]-UTP incorporation by the nuclei of both types of cell was observed (figure 2) when the ionic strength of the incubation system was increased to that of 100 mM $(NH_4)_2SO_4$ in agreement with observations in other systems also (Jacob *et al*., 1970). In the presence of α-amanitin, RNA synthesis was inhibited by 80% in mature erythrocyte nuclei and over 90% in immature erythrocyte nuclei at this ionic strength. This finding corroborates with the results of Jacob *et al*. (1970). However, the ionic strength used in the experiment shown in figure 2 was higher than the physiological ionic strength in the nuclei (~ 0·18). It has been observed that the extent of RNA synthesis in the nuclei isolated from the two types of cell and the extent of anemia developed in the birds vary from batch to batch. The inhibition by actinomycin D (table 1) and α-amanitin (figures 1 and 2) suggests that the RNA synthesis takes place in the isolated nuclei of maturing avian erythrocytes.

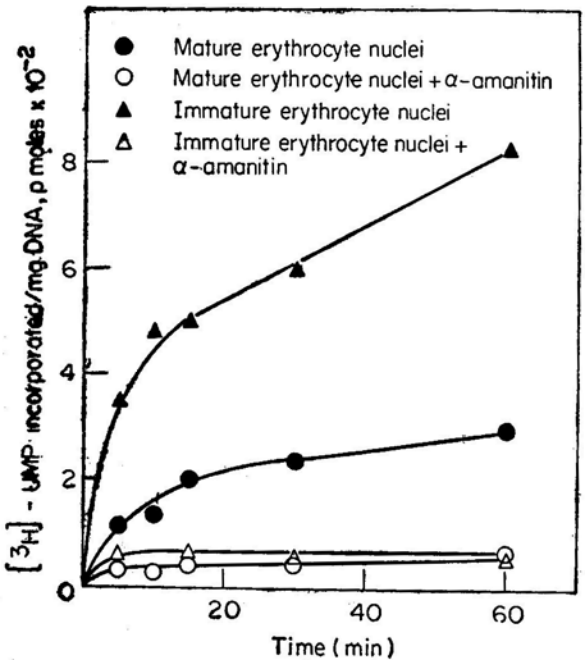

Figure 2. Time-course of RNA synthesis in the presence of high sali by nuclei of mature and immature erythrocytes. Ammonium sulphate (100 mM) was used.

Table 1. Efect of actinomycin D on nuclear RNA synthesis.

Experiment	[³H]-UMP residues incorporated (p mol/mg DNA)		Inhibition (%)	
	30 min	60 min	30 min	60 min
Mature erythrocyte nuclei : Complete	240	313	0	0
Complete + actinomycin D	49	90	80	71
Immature erythrocyte nuclei : Complete	603	828	0	0
Complete + actinomycin D	159	185	74	78

The complete assay system is same as described in Materials and Methods except that $(NH_4)_2SO_4$ was used at 100 mM, actinomycin D was used at 15 μg/ml and the amounts of nuclei used were equivalent to 1·2–1·6 mg DNA/ml. Trichloroacetic acid precipitable radioactivity was measured.

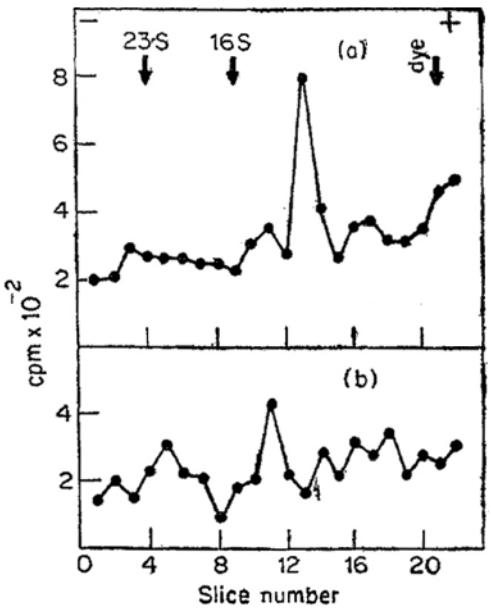

Figure 3. Polyacrylamide gel electrophoretic pattern of labelled RNA synthesised in isolated mature erythrocyte nuclei.

[^{3}H]-RNA was synthesised in mature erythrocyte nuclei in absence (a) and presence (b) of α-amanitin (2·5 μg/ml) as described in Methods, except that [^{3}H]-UTP was used at a sp. act. of 100 cpm/pmol. The RNAs equivalent to 8250 cpm (figure 3a) and 5880 cpm (figure 3b) were electrophoresed on 3% Polyacrylamide gel (Bishop *et al*., 1967). After the electrophoresis the gels were sliced and radioactivity in each slice was measured.

The nature of RNA synthesised in the nuclei of mature and immature erythrocytes examined by polyacrylamide gel electrophoresis showed heterogeneity in size classes (figures 3 and 4). Both mature and immature erythrocyte nuclei synthesise high molecular weight RNA, though the immature erythrocyte nuclei synthesise higher molecular weight RNA than the mature erythrocyte nuclei. The high molecular weight RNA (> 16S) synthesised by immature erythrocyte nuclei may be either ribosomal or precursor of the cytoplasmic globin mRNA type. Using cDNA as a probe, recently Strair *et al*. (1971) have demonstrated that 70% of nuclear globin mRNA (which are polyadenylated) sedimented at 16·5S and the rest of the nuclear globin mRNA sedimented at 10S region. The decrease in the radioactivity peak of RNA (4S–16S) synthesised was comparatively less when the mature nuclei were incubated in the presence of α-amanitin than the marked reduction observed in low molecular weight (4S–16S) RNA synthesised by immature erythrocyte nuclei. Since polyacrylamide gel electrophoresis did not yield any quantitative indication as to the nature of RNA synthesised by the erythrocyte nuclei, an alternative method was used to isolate poly(A)-containing RNA.

The. amount of poly(A)-containing RNA synthesised in the nuclei of mature and immature erythrocytes in the presence and absence of α-amanitin were deter-

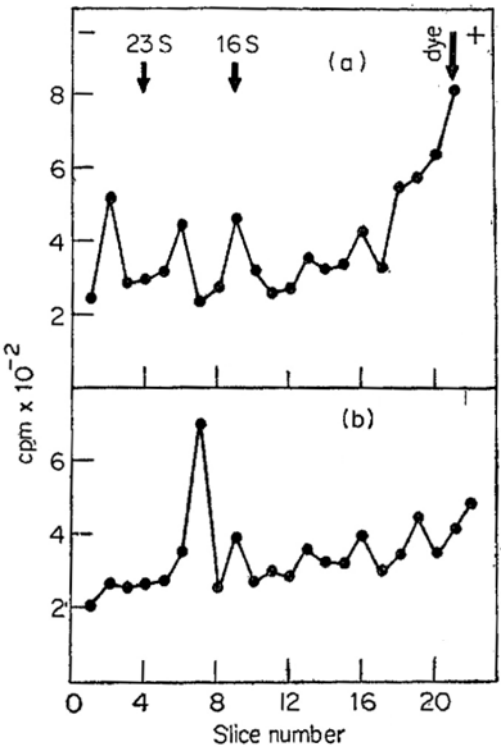

Figure 4. Polyacrylamide gel electrophoretic pattern of labelled RNA synthesised in isolated immature erythrocyte nuclei.

[³H]-RNA was synthesised in immature eryt hrocyte nuclei in absence (a) and presence (b) of α-amanitin (2·5 μg/ml). The RNAs equivalent to 10,000 cpm (figure 4a) and 8,500 cpm (figure 4b) were elactrophoresed on 3% polyacrylamide gel following the method of Bishop *et al.* (1967). All other conditions were same as described in the legend to figure 3.

mined by poly(U)-sepharose column chromatography of the labelled RNAs. The result of poly(U)-sepharose chromatography is summarised in table 2. The amount of poly(A)-containing RNA synthesised in the nuclei of mature and immature erythrocytes are 16% and 13% respectively of the total [^{3}H]-RNA synthesised. α-Amanitin inhibited (40%) the synthesis of poly(A)-containing RNA m the mature erythrocyte nuclei and by about 60% in the immature erythrocyte nuclei. A 1·5-fold increase in poly(A)-containing RNA synthesis by immature erythrocyte nuclei has been noted.

If 8–12 S RNA synthesised constitutes mainly messenger RNA, then in the mature erythrocytes the synthesis of mRNA is larger than in immature cells. This is apparently contradictory as the immature cells are expected to synthesise more messenger RNA as well as other types of RNA. But the main assumption in this type of calculation is that in polyacrylamide gel electrophoresis and m poly(U)-sepharose chromatography there is a clear separation of mRNA from non-messenger as well as all the mRNAs including the pre-mRNAs are polyadenylated. It is possible to calculate from the electrophoretic separation of RNAs synthesised in

Table 2. Poly(U)-sepharose chromatography of [^{3}H]-RNA synthesised in the nuclei of mature and immature erythrocytes.

RNA synthesised in	Total unbound RNA (cpm)	Total bound RNA (cpm)	% poly(A) containing RNA
Mature erythrocyte nuclei	3317	630	16
Mature erythrocyte nuclei + α-amanitin (2·5 μg/ml)	1616	187	10
Immature erythrocyte nuclei	6219	879	13
Immature erythrocyte nuclei + α-amanitin (2·5 μg/ml)	3615	193	5

[^{3}H]-RNAs were synthesised in the nuclei of mature and immature erythrocytes using the reaction condition described in Methods. The RNAs were isolated by hot phenol-sod. dod. SO_4 method and subjected to poly(U)-sepharose column chromatography as described.

the presence and absence of α-amanitin by mature and immature erythrocyte nuclei, that 26% of RNA in the region beyond 16S appears to be mRNA type in the case of immature erythrocytes (figure 3) whereas this is only 16·5% in the case of mature erythrocytes (figure 4). In the presence of α-amanitin, however, there is also an inhibition in the synthesis of RNAs greater than 16S species as separated on polyacrylamide gel (figure 4). This is also observed in the case of unbound species of RNA chromatographed on poly(U)-sepharose column suggesting that higher molecular weight heterogeneous RNA might contain messenger type of RNA devoid of long poly(A) stretches along with the rRNAs or pre-rRNAs.

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