DNA-DEPENDENT RNA POLYMERASE IN MATURING AVIAN ERYTHROCYTES

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Received 2 January 1973

1. Introduction

Multiple forms of RNA polymerase generally exist in eukaryotic organisms (for references see [1]). These forms presumably play an important role in the selective transcription of the genome. The α-amanitin insensitive RNA polymerases AI and AII [2] occur in the nucleolus and appear to function in the transcription of nucleolar RNA [3], whereas the α-amanitin sensitive RNA polymerases BI and BII [4, 5] are nucleoplasmic enzymes and probably function in the synthesis of the many classes of messenger and heterogeneous nuclear RNA. We have investigated whether the extent of RNA synthesis in the nucleated chicken erythrocyte during its differentiation into an almost completely repressed cell is directly correlated to the activity of RNA polymerases. Though the RNA polymerase activity was found to be significantly greater in the immature erythrocyte and to decline during maturation, cellular RNA synthesis had returned to normal at a time when the RNA polymerase level was still 4 times above controls. The soluble RNA polymerase was found to be almost exclusively RNA polymerase B form.

2. Materials and methods

2.1. Animals

White leghorn cocks were injected with phenylhydrazine (8 mg/Kg body weight) for 3 consecutive days and killed 2 days after the last injection (anaemic blood type). Blood was collected in 2% (w/v) sodium citrate, 2% (w/v) glucose.

2.2. Isolation and incubation of nuclei

All procedures were carried out at 0 to 4° unless otherwise stated. Blood cells were washed 3 times by centrifugation at 10,000 *g* for 2 min in 10 vol 0.15 M NaCl and nuclei were isolated by the following methods:

Method (a): Cells were lysed in 10 vol 0.2% (v/v) Triton X-100, 0.15 M NaCl for 30 min and the suspension was spun at 10,000 *g* for 5 min. The nuclear pellet was washed 3 times by centrifugation in 10 vol 0.15 M NaCl.

Method (b): Cells were lysed in 20 vol 0.05% (w/v) digitonin (BDH), 0.15 M NaCl for 5 min, and the nuclear pellet was washed once in 20 vol 0.25 M sucrose, 0.05 M Tris-HCl, pH 7.9, 5 mM $MgCl_2$, 25 mM KCl.

Chromatin was prepared by lysing the nuclei in 10 vol of 0.01 M Tris-HCl, pH 7.9 and washing the insoluble residue 3 times in the same buffer.

DNA was determined according to Dische [6], protein by the method of Lowry [7] and $(NH_4)_2SO_4$ with Nessler's reagent.

RNA polymerase activity in intact nuclei and chromatin was determined in 0.5 ml 0.05 M Tris-HCl, pH 7.9, 1 mM dithiothreitol (DTT), 2 mM $MnCl_2$, 0.2 M sucrose, 1 mM ATP, GTP and CTP, 0.16 mM [^{3}H]UTP (25 mCi/mmole), nuclear suspension (500–1000 μg DNA), and 0.05 M or 0.40 M $(NH_4)_2SO_4$. The reaction was terminated after 30 min incubation at 37° by the addition of 3.0 ml 5% (w/v) trichloroacetic acid and acid precipitable radioactive material was determined as described in fig. 1.

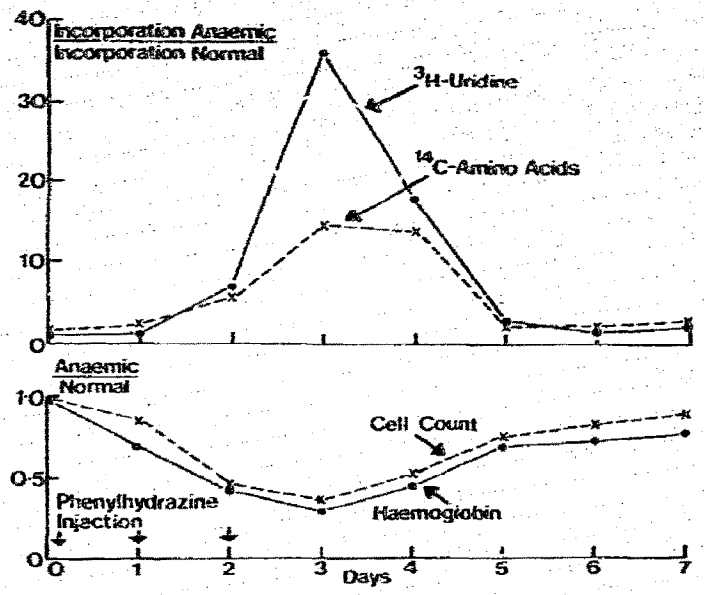

Fig. 1. RNA and protein synthesis in red blood cells during anaemia. Average values of 3 controls and 3 experimental animals. Blood was removed daily from the wing vein using heparin as anticoagulant and incubated *in vitro* for 20 min at 37° as described by Schall and Turba [8] with 2 μCi [^{3}H]uridine (400 mCi/mmole) and 0.14 μCi/mAtom carbon). Trichloroacetic acid precipitates were collected and washed by centrifugation with 5% trichloroacetic acid, solubilized in 0.5 ml 0.25 N NaOH for 45 min at 100°, decolourized with H_2O_2, and counted in a toluene-based scintillator (0.8% TLA, 15% Bio Solv BBS-3, Beckman Instruments). Results are expressed as a ratio of the incorporation per 10^6 cells from anaemic to the incorporation per 10^6 cells from normal chickens.

2.3. Extraction and assay of RNA polymerase

RNA polymerase was solubilized similarly to the methods of Roeder and Rutter [9]. Nuclear pellets were suspended in 3 vol 0.05 M Tris-HCl, pH 7.9, 30% (v/v) glycerol, 5 mM $MgCl_2$, 0.1 mM EDTA, 5 mM DTT. 25 ml aliquots were made 0.30 M with respect to $(NH_4)_2SO_4$ and sonicated with a Bronwell, Biosonik 111 (intermediate tip, intensity 60) for three 30 sec periods. The suspension was immediately mixed with 2 vol of 0.05 M Tris-HCl, pH 7.9, 30% glycerol (v/v), 5 mM $MgCl_2$, 0.1 mM EDTA, 1 mM DTT (medium A) and spun at 45,000 *g* for 45 min. The clear supernatant was taken as the solubilized enzyme (fraction 1). Calf thymus RNA polymerase was extracted similarly from whole thymus tissue, and the enzyme precipitated at 58% $(NH_4)_2SO_4$ saturation from the supernatant (fraction 2).

Soluble RNA polymerase was determined in 0.5 ml 0.05 M Tris-HCl, pH 7.9, 1 mM DTT, 2 mM $MnCl_2$, 0.32 mM ATP, GTP and CTP, 0.08 mM [^{3}H]UTP (25 mCi/mmole), 0.10 M $(NH_4)_2SO_4$, enzyme sample and 100 μg calf thymus DNA (Sigma). The reaction was terminated after 20 min incubation at 37° by the addition of 3.0 ml 5% (w/v) trichloroacetic acid. Acid precipitable radioactivity was collected and washed on a Whatman GF/C glass fibre filter with 5% trichloroacetic acid, the precipitate was hydrolyzed and counted as above. 1 unit soluble RNA polymerase corresponds to 1 pmole [^{3}H]UMP incorporated into RNA.

All radioactive materials were obtained from the Radiochemical Centre, Amersham. The following were also used where indicated: α-amanitin (Boehringer), 1 μg per assay; calf thymus DNA denatured by heating at 100° for 5 min followed by rapid cooling; *Micrococcus lysodeikticus* DNA (Miles-Seravac).

3. Results and discussion

The levels of RNA and protein synthesis in blood cells during the development of anaemia and the subsequent recuperation phase were determined (fig. 1) in order to relate them to the levels of RNA polymerase. As reported previously [10, 11] the incorporation of [^{3}H]uridine and [^{14}C]amino acids is significantly higher in cells from maximally anaemic animals than in cells from normal animals, 15 and 36 times, respectively. Although cell populations contained the usual small percentage of leucocytes (~1%), the total level and proportions of the different types do not change appreciably during phenylhydrazine treatment and the bulk of RNA synthesis in the total population occurs in the erythrocytes [11].

The RNA polymerase activity in nuclei isolated from anaemic blood (day 4) is 3.6 times greater than in nuclei from normal blood when assayed at 0.05 M $(NH_4)_2SO_4$ and 3.5 times greater when assayed at 0.40 M $(NH_4)_2SO_4$ (table 1). At 0.05 M $(NH_4)_2SO_4$ histones do not dissociate from chromatin [12, 13] and no derepression occurs [13], while the molarity allows activity measurements of both class A and B soluble animal RNA polymerases [5]. At 0.40 M $(NH_4)_2SO_4$ there is a substantial dissociation of chromosomal proteins from chromatin [12, 13] and the activity is most likely largely a reflection of the amount of RNA polymerase present.

Table 1
RNA polymerase activity in blood cells.

Fraction	Blood type	$(NH_4)_2SO_4$ (M) 0.05	0.10	0.40	No. of experiments
		(pmoles [^{3}H]UMP/mg DNA)			
Nuclei	anaemic	36 ± 11		380 ± 90	8
	normal	10 ± 4		110 ± 31	8
Chromatin	anaemic	–	12	–	2
	normal	–	3	–	2
		(units/mg nuclear DNA)			
Soluble RNA polymerase	anaemic	–	82 ± 5	–	5
	normal	–	26 ± 5	–	5

RNA polymerase activity with standard deviations in fractions from nuclei isolated by method (a) from blood cells of normal and anaemic chickens.

The endogenous RNA polymerase activity of the chromatin of erythrocytes from anaemic chickens similarly has a greater activity than that from chromatin of normal animals (4 times), the activities per mg DNA being lower than those in intact nuclei (table 1).

The RNA polymerase activity (fraction 1) extracted from nuclei of anaemic blood was 3.2 times greater than that from normal blood (table 1). This soluble activity was almost completely dependent (90%) on added template DNA and yielded on DEAE–Sephadex chromatography one peak of activity corresponding to calf thymus RNA polymerase B (fig. 2a). Nuclei isolated by a very rapid procedure (method b) yielded larger amounts of total RNA polymerase activity (table 2). The activity from anaemic animals on days 4,6,8 and 12 were 6.3, 3.6, 3.1 and 1.1 times greater than normal activity. As in the previous series this activity consisted almost exclusively of RNA polymerase B (table 2, fig. 2). This activity was found to correspond to animal RNA polymerase B by the following criteria: inhibited more than 95% by α-amanitin [14]; eluted at approx. 0.24 M $(NH_4)_2SO_4$ from DEAE-Sephadex [9, 15], typical ionic concentration dependence with an optimum at 0.10 M $(NH_4)_2SO_4$ [15, 16]; typical dependence on Mn^{2+} and Mg^{2+} with optima at 2 mM and at over 6 mM, respectively [15]; ratio of activity at optimal Mn^{2+} and Mg^{2+} concentration equal to 4 [15, 16]. No significant differences between the properties of the normal and anaemic RNA polymerase were

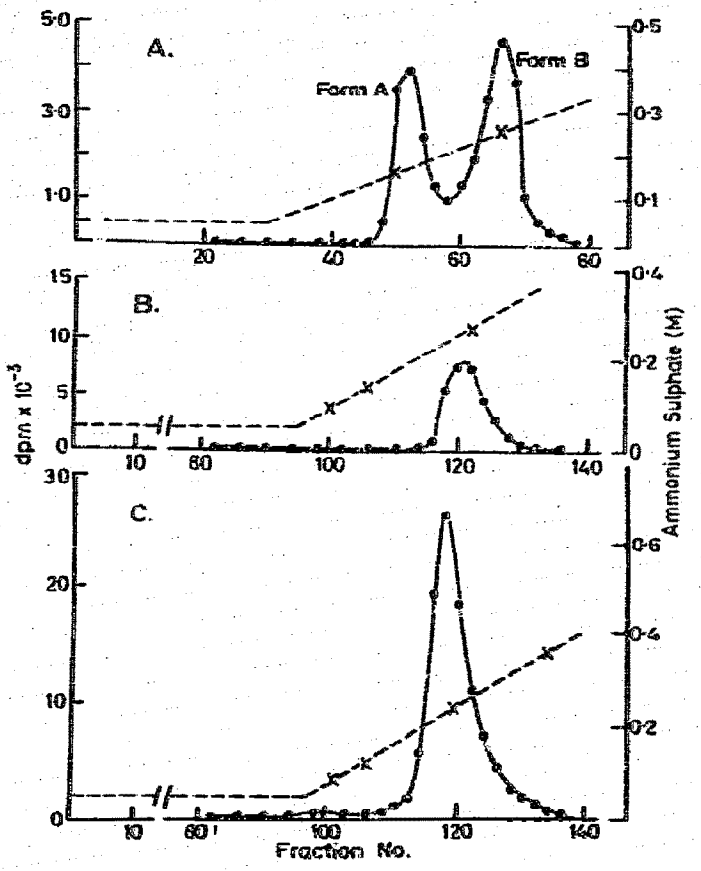

Fig. 2. DEAE-Sephadex chromatography of RNA polymerase. Fraction 1 from avian erythrocytes or fraction 2 from calf thymus (15–30 mg protein) diluted with medium A to a final $(NH_4)_2SO_4$ concentration of 0.05 M, was loaded onto a 1.3 × 12 cm column of DEAE-Sephadex (A-25) equilibrated with 0.05 M $(NH_4)_2SO_4$ in medium A, and eluted with a 60 ml linear gradient of 0.05 M to 0.50 M $(NH_4)_2SO_4$ in medium A. Flow rate: 15 ml/hr. Fraction volume: 1 ml. 200 μl aliquots assayed in standard incubation mixtures containing to additional $(NH_4)_2SO_4$. A) calf thymus; B) normal blood cells (60 mg tissue DNA); C) Anaemic blood cells (60 mg tissue DNA).

detected. RNA polymerase B from both blood types was completely dependent on added template DNA and displayed the same relative activities with the different DNA templates tested (table 3). RNA polymerase B transcribed heat-denatured DNA more efficiently than native DNA.

Erythrocytes of anaemic chickens provide a convenient source for RNA polymerase B, 373 units per mg tissue DNA (table 2). DEAE-Sephadex chromatography resulted in a 20 times purification of RNA polymerase B from anaemic blood yielding a fraction having a spe-

Table 2
Levels of soluble RNA polymerase in erythrocytes.

Day of anaemia	α-Amanitin (1 μg)	Units per mg nuclear DNA
0	–	59
	+	0
4	–	373
	+	30
6	–	210
8	–	181
12	–	66

RNA polymerase activity in pooled extracts from erythrocyte nuclei (isolated by method (b)) from 3 normal and 3 anaemic chickens each.

Table 3
RNA polymerase B activity with different DNA templates.

DNA and enzyme source	Relative activity
Calf thymus DNA, native	
Form B, anaemic	1.0
Form B, normal	1.0
Calf thymus DNA, denatured	
Form B, anaemic	2.1
Form B, normal	2.1
M. lysodeikticus DNA, native	
Form B, anaemic	1.5
Form B, normal	1.4
No DNA	
Form B, anaemic	0
Form B, normal	0

RNA polymerase B (after DEAE-Sephadex chromatography of fraction 1) from normal and anaemic chicken blood cells, assayed with 100 μg DNA as template.

cific activity of approx. 11 units per mg protein.

The blood cells of anaemic chickens were predominantly (> 90%) late polychromatic and mature erythrocytes, neither of which possess nucleoli [17]. The absence of an RNA polymerase A in mature and immature erythrocytes correlates well with this absence of nucleoli, and is also in agreement with the conclusion [11] that rRNA synthesis terminates before DNA-like RNA synthesis during erythrocyte maturation.

If the decrease in the level of soluble RNA polymerase activity during maturation (table 2) is a result of RNA polymerase B catabolism in the erythrocyte following complete shutdown of protein synthesis, the half-life of the enzyme is 77 hr. Though the enzyme activity on the 6th day of anaemia is still 3.6 times greater than normal activity, the RNA synthesis in blood cells at the corresponding time had returned to the normal level. This indicates that the termination of RNA synthesis in the mature erythrocyte is not closely correlated to the level of RNA polymerase B. Investigations into the template properties of erythrocyte chromatin with RNA polymerase B are in progress.

Acknowledgements

We thank the University of Cape Town Research Committee and the Council for Scientific and Industrial Research (South Africa) for their support. D.R. v.d. W. holds the Taeuber and Corssen Postgraduate Scholarship, M.C.D.B. thanks the C.S.I.R. for a postgraduate bursary.

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