Deoxyribonucleic Acid-Dependent Ribonucleic Acid Polymerases from Murine Spleen Cells

INCREASED AMOUNTS OF THE NUCLEOLAR SPECIES IN LEUKAEMIC TISSUE

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1. In the spleens of infected mice, the Friend leukaemia virus induces a sharp increase in the ability of subsequently isolated nuclei to incorporate exogenous UTP into an acid-insoluble product. Inhibitor studies indicate that the incremental RNA synthesis proceeds from a DNA template and that both nucleolar and nucleoplasmic activities are involved. 2. The partially purified DNA-dependent RNA polymerases from control and virus-infected tissue are indistinguishable with respect to chromatographic mobility, dependence on bivalent cations, ionic strength, pH and their susceptibility to α -amanitin. The RNA polymerases of the murine spleen resemble the enzymes of other mammalian tissue in these properties. 3. A comparison of the amount of polymerase solubilized from normal and infected tissue correlates with the activity observed in assays of the respective nuclei. These experiments indicated that the increase in nucleolar RNA synthesis after infection is mediated by increased extractable polymerase I activity whereas the change in nucleoplasmic RNA synthesis results from an alteration of chromatin or a chromatin-associated factor.

There are several examples of modification of bacterial host-cell RNA polymerase by an infecting bacteriophage, facilitating a controlled expression and replication of the information in the viral genome (Siefert *et al.*, 1969; Travers, 1969, 1970; Naono & Tokuyama, 1970). Although the viruses that infect higher animals perform the essential functions of expressing and replicating their genetic material through an amazing diversity of processes, the vast majority utilize virus-specified polymerases for these purposes (Baltimore, 1971). The RNA tumour viruses stand alone as a class of animal viruses which may use the host cell DNA-dependent RNA polymerase for both the proliferation of viral messenger RNA and progeny RNA (Temin, 1971).

The erythroblastic leukaemia viruses, such as Friend virus, are members of this group whose full expression appears not to occur in homogeneous tissue culture (Boiron *et al.*, 1967). However, in the murine spleen, the pathogenic proliferative changes induced by Friend virus infection and replication are associated with markedly increased rates of RNA synthesis. Such observations have been made with intact spleen cells (Silber *et al.*, 1967), isolated spleen nuclei (Munson *et al.*, 1970) and the aggregate-enzyme preparation from spleen nuclei (Lin & Rich, 1968).

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Recent advances in the solubilization and fractionation of the enzymes responsible for nuclear RNA synthesis in eukaryotic cells (Roeder & Rutter, 1969, 1970*a,b*) have allowed an extension of these studies to the molecular level. In the present report the partially purified DNA-dependent RNA polymerases from normal and Friend virus-infected tissue have been fractionated into subspecies and compared both qualitatively and quantitatively.

Experimental Procedure

Methods

Friend virus infection in mice. Random-bred (21-day-old) ICR/Ha Swiss mice, from the colony maintained in this laboratory, were inoculated intraperitoneally with a leukaemogenic dose of Friend virus by protocols previously established (Rich *et al.*, 1965). At various times after inoculation the mice were killed and the spleens quickly excised. The organs were weighed and placed on ice for not longer than 1 h before use.

Nuclei preparation. All subsequent operations were performed at 0–4°C. The spleens of 3–30 mice (3–10 g of tissue) were minced and homogenized in 3 vol. of 0.32 M-sucrose – 15 mM-MgCl₂ – 1 mM-dithiothreitol – 0.25 mM-spermine. After passage through silk filter cloth, the homogenate was diluted with 2 vol. of 2.3 M-sucrose and subjected to centrifugation at 87000g for 1 h. Nuclei were resuspended and washed

in 0.32M-sucrose–1 mM-MgCl₂–5 mM-dithiothreitol and used immediately. The concentration of nuclei in the final suspension was determined with a Coulter cell counter.

RNA polymerase solubilization and fractionation. The procedure of Roeder & Rutter (1970a) was employed with the following modifications. After sonication and adjustment of the (NH₄)₂SO₄ concentration to 0.1 M, chromatin was removed by centrifugation at 15000g for 20 min; for studies of chromatin the pellet was redissolved in TGMED buffer [0.05 M-Tris-HCl (pH 7.9)–25% (v/v) glycerol–5 mM-MgCl₂–0.1 mM-EDTA–5 mM-dithiothreitol] containing 0.2 M-(NH₄)₂SO₄. The polymerase in the supernatant fraction was precipitated with (NH₄)₂SO₄ and after dialysis against TGMED buffer plus 0.05 M-(NH₄)₂SO₄ was fractionated on DEAE-Sephadex. Enzyme activity in the column fractions was stable for several weeks at –70°C.

DNA, RNA and protein determinations. Protein was measured by the method of Lowry *et al.* (1951) with crystalline bovine serum albumin as the standard. DNA was assayed by the diphenylamine procedure of Burton (1956) with calf thymus DNA as the standard. RNA was measured by the method of Fleck & Begg (1965).

Materials

ATP, GTP, CTP and synthetic polynucleotides were obtained from P-L Biochemicals Inc., Milwaukee, Wis., U.S.A. Calf thymus DNA was purchased from Worthington Biochemical Corp., Freehold, N.J., U.S.A., and was reprecipitated twice before use. Actinomycin D was acquired from Merck, Sharp and Dohme, Rahway, N.J., U.S.A. Henley and Co., New York, N.Y., U.S.A., supplied the α -amanitin used in these studies. Pyruvate kinase and phosphoenolpyruvate were from Sigma Chemical Co., St. Louis, Mo., U.S.A., and [2-¹⁴C]UTP (100 c.p.m./pmol) was a product of New England Nuclear Corp., Boston, Mass., U.S.A.

Results

RNA synthesis by spleen nuclei from normal and Friend virus-infected mice

The nuclei preparations used in these studies exhibited high purity and integrity under electron-microscopic examination (D. F. Babcock & L. Johns, unpublished work). RNA/DNA/protein contents for nuclei derived from normal and infected tissue were 0.1:1.0:3.2 and 0.25:1.1:3.3 mg/10⁸ nuclei respectively. These values indicate freedom from cytoplasmic contamination (Blobel & Potter, 1967), and are consistent with values previously reported for this tissue (Munson *et al.*, 1970).

[2-¹⁴C]UTP incorporation by a suspension of these nuclei proceeds for less than 10 min at 37°C. In assays

at a standard ionic strength [0.1 M-(NH₄)₂SO₄] maximum synthesis was observed at pH 7.9 in the presence of 2 mM-MnCl₂.

The RNA polymerase activity of nuclei prepared from the spleens of mice infected with Friend virus was examined (Fig. 1). Between 3 and 9 days after infection the polymerase activity per nucleus doubles and remains increased until at least 2 weeks after infection. During this period the average spleen weight increases fivefold.

Actinomycin D sensitivity of nuclear RNA synthesis for normal and infected tissue

Evidence that the increased RNA synthesis of nuclei from infected tissue was dependent on a DNA template was obtained from experiments comparing

Fig. 1. Increase in nuclear RNA polymerase as a result of Friend virus infection

Groups of 15 mice received a standard dosage of Friend virus. ●, Average spleen weights; ○, the average of three assays of RNA polymerase activity in the standard reaction, which contained in a volume of 0.2 ml: 10 μ mol of Tris-HCl (pH 7.9), 0.4 μ mol of MnCl₂, 1 μ mol of phosphoenolpyruvate, 1 μ g of pyruvate kinase, 0.1 μ mol each of ATP, CTP and GTP, 0.01 μ mol of [2-¹⁴C]UTP, 20 μ mol of (NH₄)₂SO₄ and 0.2 μ mol of dithiothreitol. Reactions were initiated by addition of 1×10^7 – 5×10^7 nuclei (0.1–0.5 mg of DNA). After 10 min at 37°C the reaction was terminated by the addition of 1 vol. of 10% (w/v) trichloroacetic acid containing 0.04 M-Na₄P₂O₇. The precipitates were collected on a membrane filter, washed, dried and the radioactivity was counted in a toluene-based scintillation fluid (Lin & Rich, 1968). One unit of activity is the incorporation of 1 pmol of [2-¹⁴C]UTP into a 10 min incubation.

the relative actinomycin D sensitivities of nuclei from the two tissues (Fig. 2). Nuclear RNA synthesis at low ionic strength was completely suppressed by the addition of 100 μ g of actinomycin/mg of DNA to the assay mixture. In assays at high ionic strength RNA synthesis of a more resistant nature was observed (Widnell & Tata, 1966); with 0.3M-(NH₄)₂SO₄ and 100 μ g of actinomycin/mg of DNA, [2-¹⁴C]UTP was still incorporated into polymer at 10% of the level observed in its absence (Stern & Friedman, 1971). In assays at either ionic strength, RNA synthesis by nuclei from normal and infected tissue was comparably inhibited by the antibiotic. Thus it appeared unlikely that terminal addition by a hypothetical poly(U)-synthesizing enzyme (Wilkie & Smellie, 1968) or synthesis from an RNA template (Baltimore & Franklin, 1963) could explain the increased activity of infected tissue. These results encouraged us to examine the nuclear DNA-dependent RNA polymerases in more detail.

Fig. 2. Inhibition of nuclear RNA polymerase activity by actinomycin D

Nuclei from infected tissue (●) and normal tissue (▲) were assayed in 5mM-MgCl₂-0.05M-(NH₄)₂SO₄. Nuclei from infected tissue (○) and normal tissue (△) were assayed in 2mM-MnCl₂-0.30M-(NH₄)₂SO₄. Maximum incorporations were respectively: 1044, 447, 2422 and 1657 c.p.m.

(NH₄)₂SO₄ dependence of nuclear RNA polymerase activity

Nuclear DNA-dependent RNA synthesis is activated by a high ionic strength (Widnell & Tata, 1966). Fig. 3 compares the ionic strength dependence of UTP incorporation by nuclei from normal tissue and tissue harvested 10 days after Friend virus infection. Nuclei from both tissues show two maxima, at approximately 0.08M- and 0.30M-(NH₄)₂SO₄. This biphasic response is thought to correspond to activation of nucleolar and nucleoplasmic RNA synthesis (Johnson *et al.*, 1969). The activity observable in assays at 0.1M-(NH₄)₂SO₄ was chosen for subsequent comparison as it represents a point close to the physiological ionic strength and contains both nucleoplasmic and nucleolar components (see below).

α -Amanitin-resistant activity in nuclei from normal and infected tissue

The bicyclic octapeptide, α -amanitin, at a concentration of 10nM gives half-maximum inhibition of RNA synthesis by isolated spleen nuclei and maximum inhibition at concentrations greater than 1 μ M. Nuclei from other mammalian tissues show a similar response (Stirpe & Fiume, 1967; Novello & Stirpe, 1969; Zylber & Penman, 1971).

Fig. 3. Effect of ionic strength and α -amanitin on nuclear RNA synthesis

Nuclei from infected tissue (●) and normal tissue (▲) were assayed in the absence of α -amanitin. Nuclei from infected tissue (○) and normal tissue (△) were assayed in the presence of 1 μ M- α -amanitin.

Fig. 3 also compares the extent of UTP incorporation at various $(\text{NH}_4)_2\text{SO}_4$ concentrations in the presence of $1\text{ }\mu\text{M}$ - α -amanitin. Nuclear RNA synthesis was 50% resistant to α -amanitin at low ionic strength, and a 2.0-fold difference in α -amanitin-resistant RNA synthesis by nuclei from normal and infected tissue was apparent at all salt concentrations.

Since nucleolar RNA synthesis *in vitro* is resistant to both α -amanitin and high salt concentrations (Jacob *et al.*, 1970), total activity minus the α -

amanitin-insensitive activity equals nucleoplasmic RNA synthesis at any ionic strength. Clearly nucleolar RNA synthesis is increased twofold in nuclei from infected tissue. In assays at 0.1 M - $(\text{NH}_4)_2\text{SO}_4$ the amount of α -amanitin-sensitive nucleoplasmic RNA synthesis is also increased twofold whereas at 0.3 M - $(\text{NH}_4)_2\text{SO}_4$ rates of nucleoplasmic RNA synthesis are the same for the two tissues.

The mechanism underlying the $(\text{NH}_4)_2\text{SO}_4$ activation of nuclear RNA synthesis is unclear (Chambon

Fig. 4. Fractionation of RNA polymerase activity on DEAE-Sephadex

The dialysed extract (Table 2) was applied to a column ($1.2\text{ cm} \times 10\text{ cm}$) of DEAE-Sephadex equilibrated against TGMED buffer + 0.05 M - $(\text{NH}_4)_2\text{SO}_4$. The column was washed with 20 ml of this solution and a 100 ml linear 0.05 – 0.80 M - $(\text{NH}_4)_2\text{SO}_4$ gradient was applied. Fractions of 2 ml were collected and portions assayed for polymerase I and II activity at the optimum conditions described. ●, Polymerase activity; ○, E_{280} ; △, $(\text{NH}_4)_2\text{SO}_4$ concentration. (a) Enzymes from infected tissue and (b) enzymes from normal tissue.

et al., 1968), but possibly involves dissociation of chromosomal proteins, allowing unrestricted synthesis from the chromatin template. The similar activity of nuclei from normal and infected tissue in assays at high ionic strength may thus indicate that comparable amounts of the nucleoplasmic RNA polymerase are present in the two preparations.

Solubilization and characterization of nuclear RNA polymerase

To determine if the differences in nuclear RNA synthesis described above correspond to differences in the respective nucleolar and nucleoplasmic enzymes, purification and fractionation of these activities were carried out. Polymerase I (the nucleolar enzyme) and polymerase II (the nucleoplasmic enzyme) were eluted from DEAE-Sephadex at salt concentrations of 0.18M and 0.23M respectively. We found no trace of a polymerase III species (Roeder & Rutter, 1969), even when the salt gradient was extended to 0.7M-(NH₄)₂SO₄. Note that no new, chromatographically distinct RNA polymerase was extracted from nuclei of infected tissue (see Fig. 4).

Table 1 shows the properties of the assay system used for characterization of the partially purified polymerases from spleen nuclei. The reaction was dependent on added nucleoside triphosphate, Mn²⁺ and DNA for full activity. Other synthetic double-stranded ribo- and deoxyribo-nucleotide homopolymers were relatively ineffective templates, as noted by Lentfer & Lezius (1972). Actinomycin D suppressed

incorporation by both enzymes to low values and polymerase II was sensitive to inhibition by α -amanitin (Lindell *et al.*, 1970).

By using modifications of the standard assay, pH and bivalent-cation requirements and the effect of (NH₄)₂SO₄ and calf thymus DNA concentrations were assessed for the partially purified polymerases of the murine spleen. The properties of the enzymes from normal and virus-infected spleen were identical and resembled the enzymes of other mammalian tissue (Roeder & Rutter, 1969). The optimum assay conditions for polymerase I from both sources were: 0.05M-(NH₄)₂SO₄-2mM-MnCl₂ or 4mM-MgCl₂, pH7.9, and 20 μ g of heat-denatured calf thymus DNA. For polymerase II optimum assay conditions were: 0.10M-(NH₄)₂SO₄-2mM-MnCl₂ or 4mM-MgCl₂, pH8.1, and 20 μ g of heat-denatured calf thymus DNA. Thus in chromatographic mobility, template preferences and the above properties the nuclear DNA-dependent RNA polymerases from the two sources are indistinguishable. Final resolution of the question of the identity of the polymerases from normal and infected tissue must await studies of the purified enzymes by using more sophisticated techniques.

Quantitative comparison of solubilized RNA polymerase from normal and infected tissue

Having established that the requirements of the polymerases from normal and infected tissue were the same, it became possible to usefully compare these

Table 1. *Characteristics of the RNA polymerase assay*

Partially purified polymerases in the DEAE-Sephadex column fractions were assayed as described in Fig. 1 (except for the inclusion of 20 μ g of heat-denatured calf thymus DNA and, for polymerase I, adjustment of the (NH₄)₂SO₄ concentration to 0.05M) with the indicated additions or deletions. Abbreviations are as follows: poly(rA), the single-stranded polymer of AMP; poly(rA)·(dT), the polymer of AMP associated with the polymer of dTMP; poly(dA)·(dT), the polymer of dAMP associated with the polymer of dTMP. Results are given as c.p.m. of [2-¹⁴C]UTP incorporated.

Assay	[2- ¹⁴ C]UTP incorporated (c.p.m.)			
	Enzyme from infected tissue		Enzyme from normal tissue	
	Polymerase II	Polymerase I	Polymerase II	Polymerase I
Complete	4119	1278	3307	1053
-Enzyme	83	67	74	69
-GTP	229	149	196	154
-GTP, CTP and ATP	95	93	87	96
-Mn ²⁺	176	224	157	252
-DNA	86	94	96	107
-DNA+poly(rA) (10 μ g)	116	103	100	109
-DNA+poly(rA)·(dT) (2 μ g)	126	92	126	94
-DNA+poly(dA)·(dT) (10 μ g)	152	130	131	127
+Actinomycin D (50 μ g)	335	151	294	148
+ α -Amanitin (1 μ g)	247	1187	211	1023

enzymes in a quantitative manner. Table 2 lists the purification and recovery of polymerase activity during the solubilization and fractionation procedures. Of the RNA polymerase activity observed in assays of nuclei at standard ionic strength, only 25% was found in the sonicated material. This value was increased to 40% by the addition of calf thymus DNA to the assay, but still indicates that sonication results in the loss of structural integrity necessary for maximum synthesis of RNA. Precipitation of chromatin carries with it only negligible amounts of activity (less than 3% of the total). Upon chromatin removal the supernatant becomes dependent on added DNA template. Recovery of activity present in the nuclei of infected tissue was 95% at this stage, but the activity recovered from normal tissue was now 220% of that observed before disruption; after removal of chromatin equivalent amounts of polymerase were found in preparations from normal and infected tissues.

It is noteworthy that the difference in amounts of nucleoplasmic RNA polymerase observed in assays of nuclei at 0.1M-(NH₄)₂SO₄ disappears in assays at salt concentration of 0.3M (Fig. 3) and that the difference in solubilized nucleoplasmic polymerase activity also disappears at the time of chromatin removal. This may mean that the increased nucleoplasmic RNA synthesis of leukaemic cells results from increased template activity of their chromatin

or that the alteration resides in some negative effector of the polymerase of normal tissue (which is removed along with chromatin during polymerase purification).

Comparable recoveries of polymerase activity at each step after chromatin removal indicate that neither inhibitors nor activators were preferentially lost from preparations of normal or infected tissue in subsequent manipulations. Mixing experiments with the DEAE-Sephadex column fractions containing polymerase activity ensured that no effectors of polymerase activity were retained after purification of the enzymes to this extent.

The recovery of each polymerase species applied to DEAE-Sephadex was 120–130% when corrections were made to the polymerase I activity observed in the assay of the applied material at standard ionic strength (65% of maximum activity). The removal of contaminating ribonucleases during DEAE-Sephadex chromatography probably accounts for this stimulation. The apparent overall recovery in the steps after chromatin removal (all assayed on calf thymus DNA) was 80–85%. Purification over the polymerase activity of the sonicated material was approximately 120-fold for polymerase I and 350–700-fold for polymerase II. Further purification on columns of phosphocellulose was achieved only with great loss in stability of the preparations.

A comparison of the DEAE-Sephadex elution

Table 2. *Purification and recovery of RNA polymerase*

Nuclei were prepared from 10g of normal or infected tissue (14 days after inoculation) (8.5×10^8 and 9.8×10^8 nuclei respectively) and the polymerases purified as described. At various stages of purification portions were assayed in the standard reaction mixture.

Assay of:	Infected tissue			Normal tissue		
	Total activity (units)	Protein concentration (mg/ml)	Specific activity (units/mg)	Total activity (units)	Protein concentration (mg/ml)	Specific activity (units/ml)
Nuclei	1180	6.0	34	420	5.3	13.5
Sonicated material	270	3.1	9.7	90	2.3	4.4
Sonicated material + DNA	400	—	—	160	—	—
Chromatin pellet	25	2.7	4.0	20	2.6	3.6
Chromatin supernatant + DNA	1100	0.6	75	955	0.5	75
Chromatin supernatant + DNA + α -amanitin	250	—	—	125	—	—
(NH ₄) ₂ SO ₄ ppt. after dialysis	750	2.0	102	630	2.2	95
(NH ₄) ₂ SO ₄ ppt. after dialysis + α -amanitin	170	—	—	80	—	—
DEAE-Sephadex fraction:						
I	375	0.04	1300	160	0.04	500
II	740	0.04	3500	685	0.03	3700

profiles of polymerases assayed at their respective optima (Fig. 4) reveals that on a per nucleus basis equal quantities of polymerase II were found in normal and infected tissue. The amount of polymerase I was, however, nearly twofold higher in infected tissue. The average relative amounts of polymerase I to total polymerase in the pooled column fractions, determined in two independent experiments, were 19% and 35% for normal and infected tissue, respectively. These values agree well with the ratios of nucleolar to total nuclear RNA synthesis obtained in the assays at 0.3 M-(NH₄)₂SO₄ and are further evidence that the extraction process has not been selective for either polymerase species.

Discussion

RNA/DNA hybridization studies indicate that adenovirus-specific transcription in infected HeLa cells is accomplished by recruiting the existing cellular polymerases (Price & Penman, 1972a). Both α -amanitin-sensitive and -insensitive activities are involved in production of adenovirus mRNA (Price & Penman, 1972b), but experiments with cells transformed by an RNA tumour virus indicate that progeny-virus RNA production resembles mRNA- and differs from rRNA-synthesis in its sensitivity to 5-fluorouridine (Brdar *et al.*, 1972). Thus the increased nucleolar polymerase activity of the Friend virus-infected spleen cells described here is unlikely to be a direct consequence of virus replication in this tissue. Antibiotic studies in our laboratory also support this conclusion (D. F. Babcock & M. A. Rich, unpublished work). We favour instead the idea that the activation of nucleolar polymerase in the leukaemic murine spleen may be of more general significance.

Tumour tissue is often characterized by prominent nucleoli and altered pre-ribosomal RNA synthesis (Busch & Smetana, 1970). In the one case where amounts of the fractionated RNA polymerases were examined, Chesterton *et al.* (1972) reported that the amount of polymerase I in a rat hepatoma cell line was severalfold higher than in preparations from normal rat liver. We propose that the cellular proliferation induced by Friend virus is a further example in which increased nucleolar polymerase is associated with neoplastic growth.

Hersko *et al.* (1971) suggested that in mammalian cells a co-ordinated set of reactions are modulated with respect to the extent that environmental conditions favour cellular growth. One component of this 'pleiotypic response' is pre-ribosomal RNA synthesis, which is mediated by the nucleolar polymerase I. Thus control of pre-ribosomal RNA synthesis in response to the growth state, not only of neoplastic cells but also of normal mammalian cells, may be regulated by the activity of polymerase I.

Evidence is now accumulating that growth-

stimulating hormones do produce increased activity of the nucleolar polymerase I in the responsive tissue (Smuckler & Tata, 1971; Yu & Feigelson, 1971; Sadjel & Jacob, 1971; Mainwaring *et al.*, 1971). These observations are consistent with the demonstration that the nucleolar polymerase is a metabolically labile species that is turned over with considerable rapidity (Yu & Feigelson, 1972; Raynaud-Jammet *et al.*, 1972), making it ideally suited for such a regulatory role. Generalizing from our own findings and those of others (Smuckler & Tata, 1971; Chesterton *et al.*, 1972), the amount of the nucleoplasmic enzyme may be more constant in a given cell with control of nucleoplasmic RNA synthesis being exerted at other levels of organization.

The elevated nucleolar polymerase in the leukaemic spleen is probably associated with adjustment of the metabolism of the Friend virus-transformed target cells to the proliferative changes induced by this agent. Alterations of nucleoplasmic RNA synthesis are probably more complex and may concern virus replication and/or leukaemogenic processes occurring in this tissue.

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