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A Comparison of Methods for Extracting Ribonucleic Acid Polymerases from Rat Liver Nuclei

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Nuclei were prepared from rat liver after homogenization of the tissue in hyperosmotic sucrose and RNA polymerases (EC 2.7.7.6) extracted by two methods applied sequentially. Optimal conditions for washing loosely bound enzymes out of nuclei were determined first, and involved short (10min) incubations at 0°C in the presence of 5mM-Mg^{2+} and 60mM-$(NH_4)_2SO_4$. Subsequent sonication of the residual nuclear pellet after resuspension and lysis at high ionic strength resulted in further release of RNA polymerases. The primary wash yielded about 2×10^4 molecules of RNA polymerases I and III (altogether) and 1×10^4 molecules of form-II enzymes per original nucleus, whereas subsequent sonication released 2×10^4–2.5×10^4 form-I and -III enzyme molecules (altogether) and a further 7×10^3–8×10^3 form-II enzyme molecules, as measured by end-labelling of nascent RNA. RNA polymerase II was partially purified from both types of extracts and shown to initiate very poorly on high-molecular-weight homologous DNA irrespective of the source of the enzyme.

Although there have been innumerable reports of RNA polymerase (EC 2.7.7.6) isolation from eukaryotic cells over the last 15 years, it is possible to categorize the procedures employed into two main types: (1), those relying on simple wash steps, usually with iso-osmotic medium, to release enzymes from isolated nuclei; and (2), more drastic procedures which involve sonication of chromatin at high ionic strength. The former method has the longer history, and though early studies resulted in rather low yields (e.g. Furth & Ho, 1965; Jacob *et al.*, 1968), more recent workers have continued to make enzymes in this way in the belief that they may represent functionally distinct pools (e.g. Yu, 1975*a*). The discovery that sonication of nuclei or chromatin at high ionic strength leads to the release of much more RNA polymerase activity, together with the subsequent resolution of multiple species of enzymes (Roeder & Rutter, 1969, 1970), have resulted in the widespread use of the latter procedure and its application to a huge variety of different cells and tissues.

One interpretation of these observations has been to suggest the existence of different functional states of the RNA polymerases; those molecules readily washed out of nuclei may represent a pool of soluble enzymes not bound to chromatin, whereas those requiring sonication treatment for release may exist as transcription complexes. The concept of free and template-bound pools has received support from the observation that nuclei prepared in viscous hyperosmotic sucrose apparently contain larger pools of free enzymes (Lin *et al.*, 1976) and from experiments using actinomycin D and poly[d(A-T)] to detect such enzymes still within the nuclear preparation (Lampert & Fiegelson, 1974; Yu, 1974). Furthermore there are differences in chromatographic behaviour of the major RNA polymerase species depending on the pool from which they are derived, and at least some of these may be consequent on different subunit structures (e.g. Roeder, 1974; Yu, 1975*b*; Guilfoyle & Key, 1977). It is also of interest that the few studies that have demonstrated some degree of transcriptional fidelity by eukaryotic RNA polymerases (especially forms I and III) *in vitro* have often been made with enzymes derived from soluble pools (e.g. Beebee & Butterworth, 1974; VanKeulen *et al.*, 1975).

In the light of this information it seems important to try to quantify the sizes of the various pools and to establish whether biochemical and functional differences really do exist between them. The experiments described below were carried out in an attempt to gain more information about these issues.

Materials and Methods

Materials

[^{3}H]UTP, [^{3}H]CTP, [^{3}H]GTP and [^{3}H]ATP (all 15–25Ci/mmol) were from The Radiochemical Centre, Amersham, Bucks., U.K. Unlabelled ATP, GTP, UTP and CTP, as well as UMP, uridine, calf thymus DNA, deoxyribonuclease I (electrophoretically pure), ribonuclease A and actinomycin D were from Sigma, Poole, Dorset, U.K. Poly[d(A-T)]

was from International Enzymes, Windsor, Berks., U.K., and polyethyleneimine–cellulose t.l.c. plates (Polygram cell 300) were from Camlab, Cambridge, U.K. α-Amanitin was a kind gift from Professor T. Weiland (Max-Planck Institute, Heidelberg, Germany), and rifamycin AF/0-13 similarly from Dr. R. Cricchio (Gruppo-Lepetit, Milan, Italy).

Preparation of nuclei and isolation of enzymes

Livers were homogenized in 10vol. of hyperosmotic sucrose and nuclei obtained by filtration and centrifugation as described elsewhere (Kellas *et al.*, 1977). Tubes were wiped carefully to remove excess sucrose and nuclear pellets were resuspended in 20mM-Tris/HCl (pH8.0)/1mM-dithiothreitol/5mM-magnesium acetate/60mM-$(NH_4)_2SO_4$ (0.2ml/g of original liver). After standing for 10–15min at 0°C, nuclei were re-pelleted by centrifugation at 500*g* for 10min and the supernatant, after a further centrifugation at 30000*g* for 30min, was used as a source of free enzymes. The nuclear pellets were resuspended in 40mM-Tris/HCl (pH8.0)/1mM-dithiothreitol/5mM-magnesium acetate (0.1ml/g of liver), $(NH_4)_2SO_4$ was added to a final concentration of 0.25M (from a 3M stock), and the viscous material sonicated until droplets formed readily at the tip of a Pasteur pipette (Roeder & Rutter, 1970); this usually involved three or four 3s bursts, all at 0°C, by using an exponential probe of a MSE ultrasonicator at 20μM peak-to-peak amplitude and with intermittent 30s cooling periods. Volumes used were normally 3–4ml. The sonicated preparation was then diluted with 2vol. of 20mM-Tris/HCl (pH8.0)/1mM-dithiothreitol/25% (v/v) glycerol and centrifuged at 30000*g* for 30min. The supernatant was used as a source of chromatin-derived enzymes.

For partial purification of form-II RNA polymerases the free or chromatin-derived fractions were adjusted to 25% (v/v) glycerol, 15mM-2-mercaptoethanol and 0.13M-$(NH_4)_2SO_4$ and passed through a column (2cm×10cm) of DEAE-Sephadex A-25 previously equilibrated with 20mM-Tris/HCl (pH8.0)/25% (v/v) glycerol/0.1mM-EDTA/15mM-2-mercaptoethanol/0.13M-$(NH_4)_2SO_4$. After extensive washing with this buffer, which completely removed RNA polymerase I, RNA polymerases II and III were eluted together with 0.4M-$(NH_4)_2SO_4$ in an otherwise identical buffer. Fractions containing RNA polymerase activity were pooled and $(NH_4)_2SO_4$ was added (from a stock solution) to 60% saturation. After standing for 15min at 0°C, precipitated protein was collected by centrifugation at 43000*g* for 30min. The pellets were each redissolved in 0.3ml of 20mM-Tris/HCl (pH8.0)/1mM-dithiothreitol and carefully layered over 5ml linear gradients of 15–30% (v/v) glycerol in 20mM-Tris/HCl (pH8.0)/1mM-dithiothreitol/0.1mM-EDTA/60mM-$(NH_4)_2SO_4$. Centrifugation for 4.5h at 200000*g* and 0°C in a Beckman SW50.1 rotor was followed by fractionation of the gradients into 0.2ml portions. Those with RNA polymerase activity were pooled and stored at −70°C.

Preparation of high-molecular-weight rat liver DNA

DNA was extracted from nuclei and purified as described by Gross-Bellard *et al.* (1973). Analysis on sucrose gradients and by electron microscopy indicated a size range of 20×10^6–100×10^6 daltons for the double-stranded material, which was stored at −20°C as stock solutions each of which was thawed only once before use.

RNA polymerase assays

These were essentially as described by Chesterton & Butterworth (1971), in duplicates and in final volumes of 100–140μl. Standard assays for soluble enzymes contained: 20mM-Tris/HCl, pH8.0, 2mM-$MnSO_4$, 150mM-KCl, 10mM-2-mercaptoethanol, 10μg of calf thymus DNA, 0.25mM each of the three unlabelled nucleoside triphosphates and 0.05mM-[^{3}H]nucleoside triphosphate (usually at 165c.p.m./pmol). In experiments with high-molecular-weight rat liver DNA, assays contained 4μg of template (a saturating amount) and [^{3}H]UTP at 0.1mM (200c.p.m./pmol). Incubations were for 15min at 37°C. Precipitation with trichloroacetic acid and filtration on GF/C discs were as described by Chesterton & Butterworth (1971).

Estimations of numbers of RNA polymerase molecules and polyribonucleotide elongation rates

These measurements were carried out as described by Coupar & Chesterton (1977) and involved hydrolysis of transcripts labelled *in vitro* by overnight incubation in 0.3M-KOH followed by separation of internally labelled (UMP) and terminally labelled (uridine) residues by t.l.c. on polyethyleneimine–cellulose. Further details are given in the appropriate Table legend.

Results

Release of RNA polymerases by washing nuclei

As shown in Fig. 1, it proved possible to wash out large amounts of RNA polymerase simply by resuspending nuclei in buffer and re-pelleting them. This approach has been described elsewhere (e.g. Yu, 1975*a*), but little effort has previously been reported to investigate the conditions and monitor the effects on release of total protein and DNA. The latter should give some indication of how well chromosomal integrity is maintained during the wash procedures. It was apparent that increasing ionic strength promoted the release of protein (up to about 17% of the total), but not of DNA (less than 2% of the total), from the rat liver nucleus. RNA polymerases were excluded into the wash medium in a biphasic response

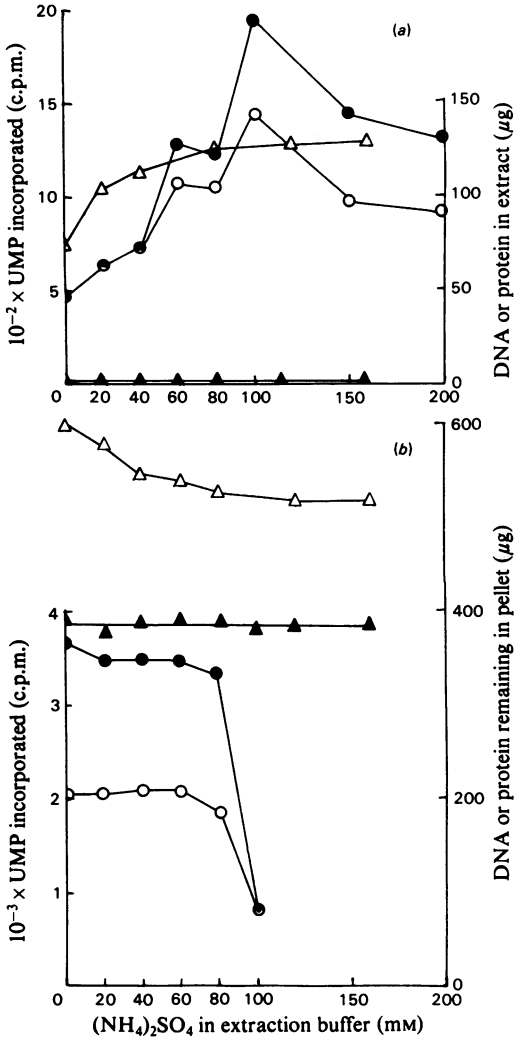

Fig. 1. *Salt-dependence of RNA polymerase extraction*
Nuclei were prepared from 10g of liver and extracted at various salt concentrations but otherwise as outlined in the Materials and Methods section. Samples (20μl) of the supernatant (*a*) or samples (50μl) of the nuclei resuspended after extraction (*b*) were assayed for RNA polymerase activity in the absence (●) or presence (○) of α-amanitin (1μg/ml). Further samples were precipitated with 10% (w/v) trichloroacetic acid, collected by centrifugation and protein (△) and DNA (▲) contents assayed by standard methods (Lowry *et al.*, 1951; Burton, 1956).

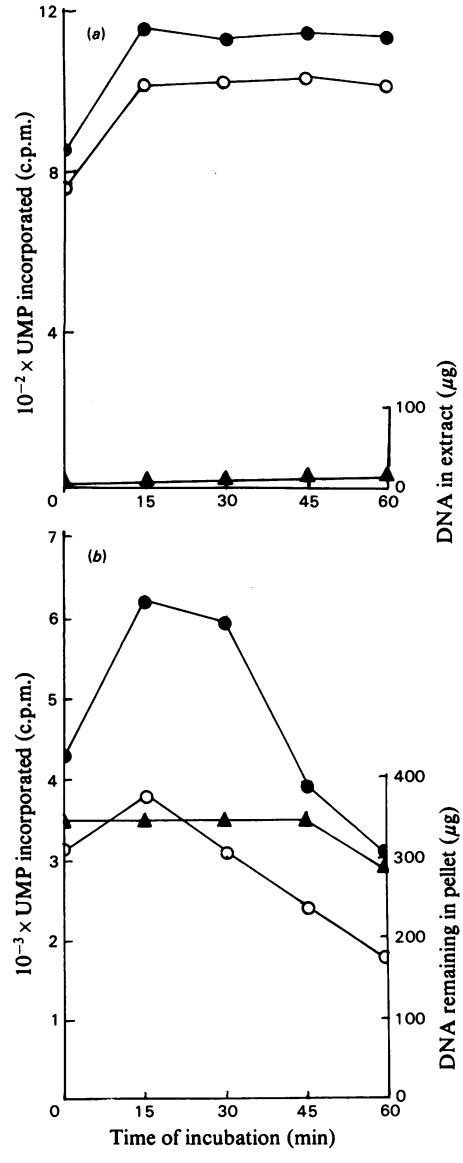

Fig. 2. *Time-dependence of RNA polymerase release*
Nuclei were prepared from 10g of liver and extracted in standard buffer for various times at 0°C. Samples (20μl) of supernatant (*a*) or samples (50μl) of resuspended pellets (*b*) were then assayed for RNA polymerase activities in the absence (●) or presence (○) of α-amanitin (1μg/ml). DNA content was also measured (▲).

to the salt, with an initial peak at around 60mM-$(NH_4)_2SO_4$ and another larger one around 100mM-$(NH_4)_2SO_4$. This proved to be a highly reproducible pattern, with between 15 and 25% of the total activity sensitive to low concentrations of α-amanitin.

However, the second and higher peak of release (at 100mM-salt), unlike the first at 60mM-salt, was associated with visible breakdown of nuclear morphology and decreases in the amounts of residual

(template-bound) RNA polymerase activities in the pellets. The lower salt concentrations therefore seemed more likely to be effecting simple leaching of the organelles and were used in subsequent studies to obtain putative free enzymes.

The effects of Mg^{2+} concentration and pH in the extraction buffer were also investigated (results not shown). Over the ranges 0–20mM-Mg^{2+} and pH 7.0–8.5 there were no significant effects on RNA polymerase release, though less than 3mM-Mg^{2+} resulted in lysis of nuclear structure.

The time course of the extraction is shown in Fig. 2. Once again the release of DNA into the wash was minimal and in no way correlated with the rapid appearance of polymerases within the first few minutes. However, it was notable that endogenous (template-bound) polymerase activities in the nuclear pellets also rose significantly over the same period. Thus, although these observations do not imply that the soluble pool was derived from the template-bound activity, the rise in the latter precludes simple quantitative comparisons. This could have resulted from a coincident removal of nucleases, but equally might have been due to some alterations of chromatin structure. It was therefore decided to examine the two enzyme fractions more closely. It should also be noted that subsequent washes of the nuclear pellet in the extraction buffer failed to release detectable extra amounts of RNA polymerases (results not shown).

Characteristics of the RNA polymerases

Enzymes derived from the nuclear wash and the chromatin pellet (by subsequent sonication at high ionic strength; see the Materials and Methods section) were more than 90% dependent on exogenous DNA and were inhibited by more than 75% by actinomycin D, confirming that true DNA-dependent RNA polymerases were responsible for most of the observed incorporation (Table 1). The lack of involvement of transcription-complex fragments in the activity of the solubilized polymerase fractions was confirmed by using poly[d(A-T)] and actinomycin D in excess. Transcription of this template was in all instances essentially insensitive to actinomycin, inferring that enzymes were not 'reading off' short stretches of DNA before initiation on poly[d(A-T)].

Table 1. *Template-dependence of RNA polymerase activities*

Assays were as described in the Materials and Methods section. Where present, actinomycin D was added to 50μg/ml and poly[d(A-T)] to 100μg/ml. Results are averages of duplicates (variation ±10%).

Assay conditions	[^{3}H]UMP incorporated (c.p.m.)	
	Wash enzyme	Chromatin-derived enzyme
Standard	1132	3571
−DNA	127	265
With actinomycin D	279	378
−DNA, with poly[d(A-T)]	1096	1350
−DNA, with poly[d(A-T)] and actinomycin D	1161	1220
−DNA, with poly[d(A-T)] and α-amanitin (1μg/ml)	602	550
−DNA, with poly[d(A-T)], actinomycin D and α-amanitin (1μg/ml)	575	612

The use of terminal-labelling techniques to ascertain the numbers of endogenous RNA polymerase molecules elongating RNA chains within nuclei has become increasingly popular (e.g. Coupar & Chesterton, 1977; Coupar *et al.*, 1978; Beebee, 1978) and with suitable controls the method should also allow quantification of free enzymes. Table 2 provides data on the first control, an assessment of the base compositions of template-bound nuclear, iso-osmotic-wash and solubilized-chromatin-derived RNA polymerase transcripts (the latter two by using calf thymus DNA as a template). There are some notable features: firstly, nuclei incubated at high ionic strength in the presence of α-amanitin and Mn^{2+} did not exhibit the G+C-rich transcription characteristic of pre-ribosomal RNA that is apparent at low ionic strength with Mg^{2+} *in vitro* (Widnell & Tata, 1966). This may have been due to the existence of significant amounts of RNA polymerase III in these hyperosmotic nuclei, or to the transcription of different sequences by polymerase I under these assay conditions. Secondly, the RNA synthesized by template-bound RNA polymerase II was extremely pyrimidine-rich (72%). Both of these results militate against the simple use of previous results from different assay conditions *in vitro* in subsequent calculations of numbers of enzyme molecules. Thirdly, it was interesting to see that the base compositions of transcripts of calf thymus DNA by free and chromatin-derived RNA polymerases were somewhat different, and these data were taken into account in the calculations of Table 3 (see below).

Another potential problem in applying measurements of terminal nucleosides to soluble enzymes is the time required for initiation *in vitro*. The experiment depicted in Fig. 3 shows that incubation at 37°C for 2min with template, RNA polymerase and all four unlabelled nucleoside triphosphates was sufficient to achieve constant subsequent extents of rifamycin AF/0-13 resistance when the initiation inhibitor and [^{3}H]UTP were added simultaneously. This suggested the attainment of a steady-state situation, and it was notable that the final degree of rifamycin AF/0-13 resistance was more than twice as

Table 2. *Base compositions of transcripts*

Assays for soluble enzymes were carried out under the conditions described in the Materials and Methods section. Nuclei were incubated in the presence of 2mM-Mn^{2+} and 0.2M-$(NH_4)_2SO_4$ in order that only elongating (rather than initiating) polymerases would be active. α-Amanitin was used at 1μg/ml to distinguish polymerase-II contributions from those of enzymes I and III together.

Labelled nucleotide added	Incorporation (mol %)					
	Nuclei		Free enzyme		Chromatin-derived enzyme	
Polymerase ...	I+III	II	I+III	II	I+III	II
ATP	18.6	11.0	18.7	13.6	17.6	28.2
UTP	27.3	34.2	25.9	34.7	20.3	51.5
GTP	27.0	17.1	17.1	40.4	28.0	7.1
CTP	27.1	37.7	38.3	11.3	34.1	13.2

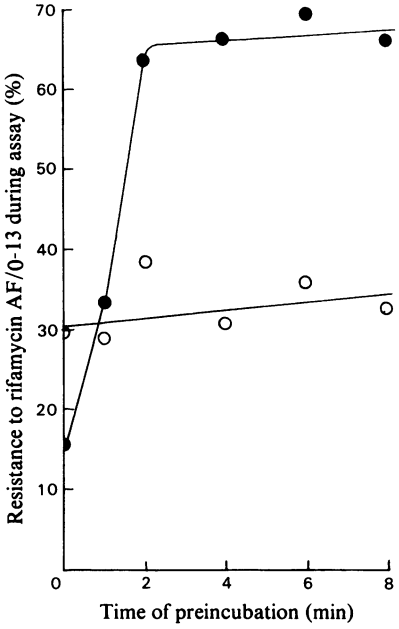

Fig. 3. *Development of rifamycin AF/0-13 resistance*

Assays were as usual with portions of either free or chromatin-derived enzymes but only unlabelled nucleoside triphosphates, ATP, GTP and CTP at 0.25mM and UTP at 0.05mM for various preincubation periods at 37°C as shown. [^{3}H]UTP, either alone or with rifamycin AF/0-13 (to 100μg/ml), was then added and incubations were continued a further 5min before termination. ●, Wash (free) enzyme, 100% activity ranging between 550 and 630c.p.m.; ○, chromatin-derived enzyme, 100% activity ranging between 590 and 760c.p.m.

high for the wash ('free') enzymes (65–68%) as for the chromatin-derived preparation (30–35%). Rifamycin AF/0-13 blocks both DNA binding and initiation, but not elongation (Meilhac *et al.*, 1972). However, no attempts were made to distinguish between the responses of the different RNA polymerase forms in these crude preparations.

Other types of controls essential to this kind of study have been discussed at length (Coupar & Chesterton, 1977; Coupar *et al.*, 1978). Isotope-dilution studies indicated that nuclei prepared in hyperosmotic sucrose contained significant pools of nucleoside triphosphates, necessitating a brief wash in iso-osmotic buffer which decreased endogenous concentrations to less than 1μM in assays. Ionic conditions were those determined experimentally to support the highest rates of RNA synthesis *in vitro*.

The results of attempts to quantify numbers of enzyme molecules are shown in Table 3. Incubations for either 1 or 2min in the presence of [^{3}H]UTP led to essentially similar amounts of labelled uridine, suggesting that the method was sufficiently consistent for estimates of enzyme numbers to be made. Another series of control experiments involved adding excess actinomycin D and α-amanitin for the second minute of a 2-min incubation to determine whether there were any transcription-independent contributions to the labelled-uridine measurements, and the experiments were also performed with twice as much DNA as that used for obtaining the data of Table 3. Essentially similar results were obtained in all these controls (results not shown).

Nuclei isolated by the hyperosmotic-sucrose procedure and washed once in iso-osmotic buffer apparently contained about 3.5×10^4 RNA polymerase II molecules engaged in transcription. More than 10^4 polymerase II molecules were found in the wash, and a further 8×10^3 were released after sonication of the nuclei in high-ionic-strength buffer. A total of around 4.5×10^4 form-II enzymes were computed to exist on a per-cell basis by Cochet-Meilhac *et al.* (1974), using titration with labelled α-amanitin, though this value should probably be

Table 3. *Numbers of polymerase molecules and elongation rates*

Nuclei from 8 g of liver were used as a source of free and chromatin-bound enzymes, and 10% (free) or 5% (chromatin-derived) portions of the extracts were used in duplicate standard assays scaled up to 0.25 ml and each with 50 μg of calf thymus DNA. Incubations were carried out at 37°C, with only unlabelled nucleoside triphosphates present for the first 2 min, after which [^{3}H]UTP was added to a final specific radioactivity of 1.67 Ci/mmol. Assays were terminated after a further 1 or 2 min, and the samples treated exactly as described elsewhere (Coupar & Chesterton, 1977; Coupar *et al.*, 1978). α-Amanitin was used at 1 μg/ml to distinguish between the polymerase activities. In a concurrent experiment nuclei were given a single wash in iso-osmotic buffer to decrease endogenous nucleotide pools, and material from 0.8 g of liver was used in assays with 2 mM-Mn^{2+} and 0.2 M-$(NH_4)_2SO_4$ for 1 or 2 min at 37°C. Allowance was made for 0.4% conversion of UMP into uridine during KOH hydrolysis (Coupar & Chesterton, 1977, and also measured directly) and for the fact that RNA recoveries were around 90% from assays of nuclear and free enzyme, but only 50% from assays of chromatin-derived enzyme. Data are given as the mean values of duplicates after these corrections, and variations were never greater than 10% of the averages. Counting efficiency was 25%, and numbers of enzyme molecules were calculated as described by Coupar *et al.* (1978) by using DNA content as a measure of nuclear numbers. Note that the values for chromatin-derived enzymes are doubled at this final stage because only half of one equivalent of nuclei was used in the initial assays.

	RNA polymerases I+III					RNA polymerase II				
Assay	Uridine (pmol)		Total molecules of terminal nucleoside	No. of enzyme molecules per nucleus	UMP (pmol)	Elongation rate (nucleotide molecule/s)	Uridine (pmol)		Total molecules of terminal nucleoside	No. of enzyme molecules per nucleus	UMP (pmol)	Elongation rate (nucleotide molecule/s)
Nuclei, 1 min	1.89	av. →	6.87	2.7×10^4	46.7	0.41	2.99	av. →	9.03	3.5×10^4	95.7	0.53
Nuclei, 2 min	1.86				98.5	0.44	3.09				200.1	0.54
Free enzyme, 1 min	1.35	av. →	5.14	2.0×10^4	19.4	0.24	1.24	av. →	2.94	1.2×10^4	11.6	0.15
Free enzyme, 2 min	1.31				32.1	0.21	1.02				25.2	0.21
Chromatin enzyme, 1 min	0.59	av. →	3.05	2.4×10^4	2.7	0.08	0.51	av. →	0.99	7.8×10^3	8.4	0.28
Chromatin enzyme, 2 min	0.63				8.7	0.11	0.51				30.9	0.51

revised downwards to about 3.5×10^4 to compensate for more recent estimates of the DNA content of rat liver nuclei (Coupar *et al.*, 1978). It is these latter values (5 pg/nucleus) on which the estimates of the present paper were made. The numbers of engaged form-II enzymes reported here are considerably higher than those observed by Coupar & Chesterton (1977), who calculated that only around 0.5×10^4–1.5×10^4 such molecules were present in the rat liver nucleus. These differences may have arisen simply because their more prolonged isolation procedures for nuclei, involving homogenization in iso-osmotic sucrose, lead to permanent inactivation of either a proportion of the nuclei or of the transcription complexes within them.

No attempt has been made to differentiate between RNA polymerases I and III, but the data suggest that there must have been a total of at least 4.7×10^4 molecules of the two altogether in the rat nucleus. As many as 2.0×10^4 (43%) were easily washed out of the nucleus, and the recovery of the remaining enzymes from transcription complexes by sonication in high-salt medium resulted in much larger recoveries than for form II (around 90% rather than 20%).

Measurements of elongation rates in these experiments were of limited interest because they were probably severely restricted by substrate concentration, especially that of [^{3}H]UTP, which was present at only 20 μM. Internal comparisons do, however, infer that elongation rates of solubilized enzymes on calf thymus DNA were lower than those engaged on chromatin, though the former values could of course be affected by time taken up by initiation *in vitro*; there is some suggestion of acceleration on these added templates, particularly by chromatin-derived RNA polymerase II.

Partial purification of RNA polymerase II

The RNA polymerase fractions were chromatographed on DEAE-Sephadex in order to ascertain the enzyme species present in each (Fig. 4). The sonicated chromatin preparation contained forms Ia, Ib, IIb and IIIa in significant amounts, whereas the first wash contained large amounts of form Ib together with forms IIa, IIb, IIIa and IIIb. The occurrence of form Ia primarily as a template-bound form and forms IIa and IIIb in soluble pools is in accord with several previous studies (Roeder, 1974; Yu, 1975*b*; Guilfoyle & Key, 1977; Kellas *et al.*, 1977). Chromatography of the two fractions recombined together suggested that the individual patterns were not grossly distorted by differential distributions of contaminants such as nucleases. The sonication procedure affects the pattern of microheterogeneity in RNA polymerase I (Kellas *et al.*, 1977), but sonication of the wash fraction caused no change in the profile shown in Fig. 4 (results not shown), though overall activities were decreased.

It will be of interest to determine whether significant differences exist between the catalytic properties of RNA polymerases derived in the two different ways. However, to do so requires at least partial purification of the enzymes such that contaminating nucleases (especially deoxyribonucleases, which can cause haplotomic breaks in DNA and create artificial promoters) are essentially removed. Another important consideration in the purification strategy adopted below was the avoidance of anionic-exchange resins, which might simulate DNA and cause the release of important subunits, such as occurs when prokaryotic RNA polymerase is chromatographed on phosphocellulose (Burgess *et al.*, 1969).

The results of the procedure used for partial purification of RNA polymerase II from the two types of extract are shown in Table 4. Yields were similar from both extracts, though the final specific activity of the chromatin-derived enzyme was higher owing to the greater purification achieved at the DEAE-Sephadex step. In both cases the final preparations contained no detectable ribonuclease or deoxyribonuclease contamination, the latter being judged by its ability to stimulate transcription by *Escherichia coli* RNA polymerase on double-stranded DNA.

As expected from the results of Fig. 4, the final fractions also contained significant amounts of RNA polymerase III activity. Despite the observation that RNA polymerase III from some cell types is eluted at lower salt concentrations from DEAE-cellulose than from DEAE-Sephadex (e.g. Austoker *et al.*, 1974), use of the former failed to improve the situation and led to lower specific activities in the final fractions. Chromatography on anionic resins has been used to separate RNA polymerases II and III, but as mentioned above it was deemed more important to avoid such procedures than to eliminate RNA polymerase III. The main purposes of the purification were to remove contaminants that might interfere with RNA polymerase assays and to allow recovery of a significant proportion of the enzyme activity, and in both of these respects the procedure was satisfactory.

Glycerol-gradient centrifugation during the final purification step revealed that enzyme activity sedimented as single discrete peaks and at similar rates irrespective of whether the enzyme was isolated by gentle washing or by high-salt sonication. Table 5 shows that after this step about 50% of the polymerase activity from nuclear washes and 70% of that from nuclear sonicated preparations were sensitive to low concentrations of α-amanitin when calf thymus DNA was used as a template. However, it was clear that the apparent proportion of RNA polymerase II depended on the template provided, and that irrespective of source the form-II enzyme was virtually incapable of transcribing high-molecular-weight rat liver DNA and demonstrated an absolute

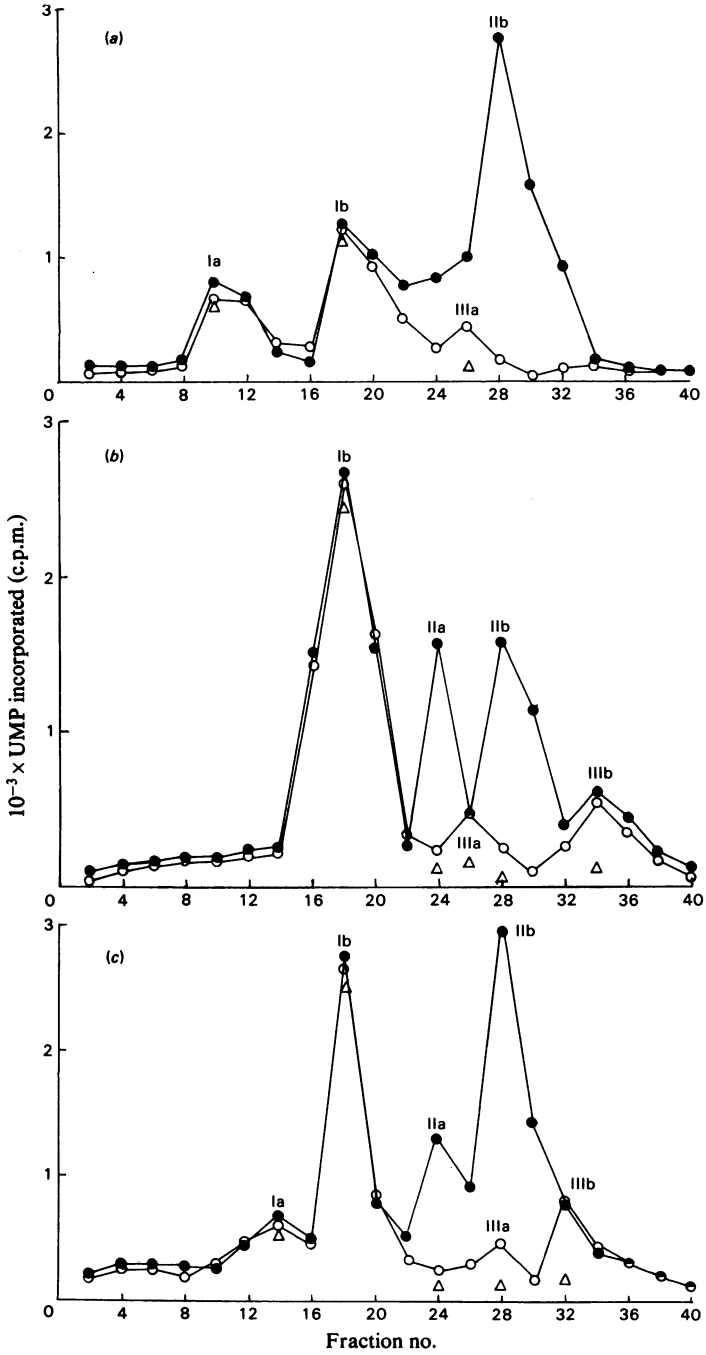

Fig. 4. *DEAE-Sephadex chromatography of RNA polymerases*

Free and chromatin-derived enzymes from 18g of liver were chromatographed on identical columns (1.6cm×7.5cm) of DEAE-Sephadex A-25 previously equilibrated as described in the Materials and Methods section, but with only 0.05M-$(NH_4)_2SO_4$. Enzymes were eluted with a linear gradient of 0.05–0.5M-$(NH_4)_2SO_4$; 1.5ml fractions were collected and 100μl portions assayed for RNA polymerase activity. ●, Total activity; ○, with 1μg of α-amanitin/ml; △, with 150μg of α-amanitin/ml. (*a*) Chromatin-derived enzymes; (*b*) wash enzymes; (*c*) wash and chromatin-derived enzymes derived from 10g of liver in the usual way and pooled before chromatography.

Table 4. *Partial purification of RNA polymerase II from wash and chromatin-derived enzyme fractions*

RNA polymerases were isolated from nuclei derived from 30g of liver and partially purified as described in the Materials and Methods section. Activity was measured as c.p.m. of [^{3}H]UMP incorporated by samples from each step and corrected for total volumes before presentation. Protein was determined on the same basis as described in Fig. 1. Exoribonuclease activity was measured by assays under standard conditions except that [^{3}H]UTP was replaced by 10ng of ^{3}H-labelled 28S RNA (10^5c.p.m./μg). Deoxyribonuclease contamination was assayed by incubation under standard conditions but without [^{3}H]UTP, followed by heating at 65°C for 10min. After cooling on ice, 10μl of *E. coli* RNA polymerase together with the usual amount of [^{3}H]UTP were added and RNA synthesis was measured in the usual way after a subsequent 15-min incubation.

(*a*)

	Wash enzyme					Chromatin-derived enzyme				
	Activity (c.p.m.)					Activity (c.p.m.)				
Fraction	Total	+α-amanitin (1μg/ml)	(mg of protein)	(c.p.m./μg of protein) (form II)	Yield of form II (%)	Total	+α-amanitin (1μg/ml)	(mg of protein)	(c.p.m./μg of protein) (form II)	Yield of form II (%)
Homogenate	—	—	723	—	—	—	—	723	—	—
Nuclear extract	1551500	1241200	6.40	48.5	100	615700	184700	12.7	34.0	100
DEAE-Sephadex A-25 fraction	492300	243600	1.25	199	78	565700	169700	0.85	466	92
$(NH_4)_2SO_4$ precipitate	147900	73600	0.74	101	23	142800	42800	0.36	278	23
Glycerol-gradient fraction	125500	62800	0.16	385	20	121900	36600	0.06	1422	20

(*b*)

	Wash enzyme		Chromatin-derived enzyme	
Fraction	^{3}H-labelled 28S RNA at end of incubation (c.p.m.)	[^{3}H]UMP incorporated by *E. coli* RNA polymerase (c.p.m.)	^{3}H-labelled 28S RNA at end of incubation (c.p.m.)	[^{3}H]UMP incorporated by *E. coli* RNA polymerase (c.p.m.)
Blank control	1077	890	1111	986
Ribonuclease A (5pg)	502	—	600	—
Deoxyribonuclease I (10pg)	—	3204	—	3076
Deoxyribonuclease I (100pg)	—	534	—	601
Nuclear extract	625	899	878	1972
DEAE-Sephadex A-25 fraction	905	877	1055	1232
$(NH_4)_2SO_4$ precipitate	1060	880	1123	900
Glycerol-gradient fraction	1099	876	1099	999

Table 5. *Template requirements of partially purified RNA polymerases*
Samples of RNA polymerases purified through glycerol gradients were tested for their abilities to synthesize RNA under various conditions.

	Activity (c.p.m. of [^{3}H]UMP incorporated)			
	Wash enzymes		Chromatin-derived enzymes	
Conditions	Total	+α-amanitin (1 μg/ml)	Total	+α-amanitin (1 μg/ml)
Without DNA	25	0	40	0
With calf thymus DNA (standard assay)	2767	1495	4266	1681
With rat liver DNA and $MnSO_4$ (2mM)	1567	1256	2715	2242
With rat liver DNA and $MgCl_2$ (10mM)	783	769	905	903
With denatured rat liver DNA and $MnSO_4$ (2mM)	4796	1550	5759	1584

requirement for Mn^{2+} rather than Mg^{2+} in the assays. Both preparations were highly active on denatured rat DNA.

Discussion

Since effective methods for solubilizing eukaryotic DNA-dependent RNA polymerases have become available, many workers have reported changes in amounts of extractable enzymes under conditions where RNA synthesis appears to be regulated *in vivo* (e.g. Roeder, 1974; Yu, 1975*b*; Fuhrman & Gill, 1976; and very many others). However, little attempt has been made to estimate recoveries of the enzymes in most of these studies, which rely on the assumption that relative recoveries should be similar in the various different situations. There appear to be no specific grounds for this premise.

There are a number of possible ways of measuring RNA polymerase activity, all of which have their difficulties. Direct measurement of RNA synthesis *in vitro* is unsatisfactory owing to the incomparability of the native chromatin template and exogenously added DNA and because of the unknown effects of nucleases and other contaminants. Attempts to remove the latter by purification of the polymerases usually leads to losses of activity that are larger than the apparent physiological changes in enzyme activity. More sophisticated methodology has involved immunoprecipitation with antisera to the various classes of enzymes (e.g. Buhler *et al.*, 1976; Guialis *et al.*, 1977); this is limited by a certain degree of cross-reactivity between the polymerase types, at least in some tissues, and more importantly because it will be difficult to use the technique to measure template-bound enzymes in nuclei or chromatin. Thus it is useful only after the crucial first solubilization step. Titration with labelled α-amanitin is a very sensitive method (Cochet-Meilhac *et al.*, 1974), but obviously can only be used with RNA polymerase II; the effects of RNA polymerase III on α-amanitin-binding studies have not been reported.

The strategy employed in this paper involved an attempt to quantify numbers of RNA polymerase molecules by end-labelling of nascent RNA chains. Nuclei were isolated in hyperosmotic sucrose because this procedure has been shown to maximize enzyme recoveries, presumably by limiting diffusion into the homogenization medium. In this study even higher concentrations of sucrose than those used by Lin *et al.* (1976) were used (2.4M rather than 2.2–2.3M) as well as higher ratios of buffer/liver (10:1 rather than 7:1). No polymerase activity was detectable in the homogenate after nuclear removal by centrifugation, though there are considerable practical difficulties in dealing with large volumes of viscous sucrose with high protein concentrations, and it is possible that some enzymes were lost at this step.

Washing nuclei in iso-osmotic buffer caused the release of considerable amounts of RNA polymerase activity without obvious morphological changes in nuclear structure. Whatever the functional significance of this enzyme fraction it is clearly advisable to remove it in this way during routine preparations before the use of sonication, since this has been shown to denature soluble RNA polymerases (Yu, 1975*a*; Kellas *et al.*, 1977). Optimal conditions for such washing were determined. Sonication at high ionic strength released a further batch of enzymes, and it should be noted that the combination of circum-

stances used by most previous workers involving isolation of nuclei in iso-osmotic media followed immediately by high-salt sonication must have selected strongly for this second RNA polymerase fraction.

The use of end-labelling allowed estimates to be made of the numbers and proportions of enzymes released and subsequently active on exogenously added DNA. The data obtained are in line with previous estimates of numbers of RNA polymerases per cell based on α-amanitin titration (Cochet-Meilhac *et al.*, 1974) and on recovery of enzyme protein (Schwartz & Roeder, 1974, 1975). This suggests that isolation of nuclei in hyperosmotic sucrose led to the retention of the vast majority of the RNA polymerases present in the liver cells. The data also imply that whereas recovery of template-bound RNA polymerases I and III was high (over 90%), that of form II was much lower (around 20%). It may be that this part of the procedure can be improved, either by more limited sonication or by abandoning it altogether and simply allowing high ionic strength to disrupt transcription complexes.

The results suggest, but do not prove, the existence of pools of RNA polymerases within rat liver nuclei which differ in their degree of association with the chromatin template. The ease with which some activity is washed out without decreasing the endogenous (template-bound) transcription supports this view, as do the different rifamycin AF/0-13 resistances and the different patterns of enzyme sub-species observed after chromatography of low- and high-salt extracts on DEAE-Sephadex. However, although salt had a biphasic effect on RNA polymerase release, other interpretations rather than free and chromatin-bound pools are possible. It is now well established that transcriptionally active genes are more vulnerable to nuclease and direct physical attack than is bulk chromatin DNA (e.g. Gavel & Axel, 1977; Beebee & Butterworth, 1977), and it is possible that the apparently free pools were at least partly derived by such degradation during nuclear isolation. It might be expected, however, that any nuclease attack would continue during later incubation of the nuclei at 0°C, yet all the polymerase available for release could be washed out after only a few minutes without more enzyme appearing over 1 h subsequently. This situation is quite unlike earlier studies with nuclei obtained by iso-osmotic homogenization and incubated at 37°C, where polymerases were released over long time periods presumably coincident with template degradation (e.g. Jacob *et al.*, 1968; Chesterton & Butterworth, 1971).

Since RNA polymerases presumably have to recycle between termination and initiation sites, the existence of a proportion of the enzymes unattached to the chromatin seems virtually inevitable. The situation will become of major interest only if the soluble pool is shown to have properties different from those of the engaged enzymes, a condition not necessitated by a recycling model but for which a clear precedent exists with prokaryotic RNA polymerase and σ protein. Investigation of such possibilities requires at least partial purification of the enzymes, and this has been attempted for RNA polymerase II. Preparations from both extraction procedures were alike in their almost complete inability to transcribe high-molecular-weight homologous DNA. The present study therefore suggests that if there are differences between RNA polymerase II enzymes isolated by the different procedures they are either of too subtle a type to show up with the template used or reside in factors so weakly bound that they were removed by the minimal purification procedures used. Both of these problems may exist, since RNA polymerase II has been shown to prefer supercoiled SV-40 DNA (Chambon, 1975), and S protein, a factor stimulating elongation by RNA polymerase II *in vitro*, has been implicated by studies with antisera in transcriptional events in isolated nuclei (Ueno *et al.*, 1979), but is not co-purified with RNA polymerase II. The major objectives of the work described in the present paper were to make some quantification of methods for releasing RNA polymerases from nuclei, and purify RNA polymerase II to a state where differences dependent on source might be investigated. It remains to be seen as to whether such differences exist.

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