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The Use of Rat Liver Nucleoplasm for the Characterization of Heterogeneous Nuclear Ribonucleic Acid Synthesis *in vitro*

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1. A nucleoplasmic fraction rich in endogenous RNA polymerase II activity was isolated from rat liver nuclei and conditions were determined under which elongation of RNA molecules initiated *in vivo* continued at maximal rates *in vitro*. 2. Elongation rates *in vitro* were calculated to be about 0.25 nucleotide/s and there were about 7×10^3 RNA molecules in the process of being elongated by form-II RNA polymerase per original nucleus. 3. Evidence was obtained suggesting that transcription-dependent release of RNA polymerase II molecules from the template occurred during the incubations *in vitro*. 4. The nascent RNA was tightly associated with protein and banded as ribonucleoprotein in caesium salt gradients. 5. RNA molecules labelled *in vitro* were up to 13000 nucleotides in length, but consisted of long unlabelled chains transcribed *in vivo* with only short labelled sequences added *in vitro*, and without significant polyadenylation. 6. Hybridization of transcripts in the presence of a vast excess of DNA demonstrated that both form-II RNA polymerase and another enzyme, resistant to low α-amanitin concentrations, were synthesizing RNA molecules complementary to both reiterated and unique DNA sequences in the genome.

Despite the multitude of studies on chromatin transcription *in vitro* that have appeared over the last 20 years, many fundamental aspects of the process have yet to be investigated. Thus, for example, controversy remains as to whether prokaryotic RNA polymerases are useful tools for such work or whether the homologous eukaryotic enzymes need always be used (see, e.g., Chan *et al.*, 1976; Biessman *et al.*, 1978). Most studies have involved either gross measurements of RNA synthesis or, more recently, probes for the transcription of specific genes. Little has yet been done to characterize the populations of RNA molecules synthesized *in vitro* with respect to numbers, elongation rates or sequence complexity, although suitable techniques are now available. Such studies as have been carried out on these aspects of chromatin transcription have yielded valuable insights into the fidelity of the process *in vitro* (e.g. Bacheler & Smith, 1976; Coupar & Chesterton, 1977).

One approach to the isolation of a system for chromatin transcription *in vitro* might be based on an initial characterization of the populations of RNA molecules being synthesized at any particular point in time *in vivo* and the use of this information as a reference for the fidelity of transcription initiated *in vitro*. Comparisons with nuclear RNA directly are of limited value, owing to the presence of several distinct RNA classes synthesized by different polymerases and to the probable existence even within heterogeneous nuclear RNA of sub-populations with different turnover rates (Levis & Penman, 1977). Thus sequence analysis of nuclear RNA may not necessarily reflect the sequences under transcription at any particular point in time.

The system chosen for the following study was a nucleoplasmic fraction from rat liver. The choice was based on the wealth of comparative information already available for this tissue, the ease of obtaining large amounts of material and the enrichment of RNA polymerase II activity consequent on fractionation of nucleoplasm from whole nuclei (Roeder & Rutter, 1970). Assays were carried out at high ionic strength to prevent initiation and to maximize the rates of RNA synthesis (Johnson *et al.*, 1971). Ribonuclease activity is also minimized under these conditions (Beebee & Butterworth, 1975). Thus, although quite unsuitable for study of the initiation of transcription owing to the fragmented state of the DNA template after nucleoplasm preparation and the high ionic strength used *in vitro*, it was expected that information would be obtained on the RNA molecules initiated *in vivo* and elongated *in vitro* mainly by RNA polymerase II for later comparisons with systems involving initiation of transcription *in vitro*.

Experimental

Materials

[^{3}H]UTP (10–20Ci/mmol) and [^{14}C]orotic acid (57mCi/mmol) were from The Radiochemical Centre (Amersham, Bucks., U.K.). Poly(A) was from Miles Laboratories (Elkhart, IN, U.S.A.). UTP, ATP, CTP, GTP, ribonuclease A, deoxyribonuclease I, protease VI, uridine, UMP and actinomycin D were from Sigma (Poole, Dorset, U.K.). Polyethyleneimine-cellulose (Polygram Cel 300 PEI) t.l.c. plates were from Camlab (Cambridge, U.K.). Poly(dA-dT) was from International Enzymes (Windsor, Bucks., U.K.). Sephadex G-75, SP (sulphopropyl)-Sephadex C50 and poly(U)–Sepharose were from Pharmacia (Uppsala, Sweden). α-Amanitin was a kind gift from Professor T. Weiland (Max-Plank Institute, Heidelberg, Germany), and rifamycin AF/0-13 similarly from Dr. R. Cricchio (Gruppo Lepetit, Milan, Italy).

All glassware used in hybridization reactions was silicone-treated by using dimethyldichlorosilane (BDH Chemicals, Poole, Dorset, U.K.) before use.

Preparation of nucleoplasm

Livers from male rats (200–250g wt.) were minced and homogenized in 5vol. of 2.4M-sucrose/3mM-magnesium acetate/25mM-KCl, filtered through two layers of muslin and diluted to 10 liver volumes with the above buffer. Nuclei were pelleted by centrifugation at 100000*g* for 1h, resuspended in 0.5% (v/v) Triton X-100/5mM-magnesium acetate/0.25M-sucrose (0.5ml/g of liver) and centrifuged at 1000*g* for 10min. The pellets from this centrifugation were suspended in 0.25M-sucrose (0.4ml/g of liver) and sonicated for 4×10s periods (interspersed with 30s cooling periods) at a peak-to-peak amplitude of 6μm by using a MSE ultrasonicator with 9.5mm-diameter probe. The sonicated preparation was underlaid with an equal volume of 0.88M-sucrose, centrifuged for 20min at 1500*g* to pellet nucleoli and heterochromatic particles, and the translucent top layer used as the nucleoplasmic fraction. All operations were at 0–4°C.

Assay of RNA synthesis

Assays were carried out in final volumes of 0.25ml, in duplicate, for 15min at 37°C unless otherwise stated. Each contained nucleoplasm from 250mg of liver, 2mM-$MnSO_4$, 0.2M-$(NH_4)_2SO_4$, 20mM-Tris/HCl, pH8.0, 15mM-2-mercaptoethanol, 0.3mM-ATP, -GTP and -CTP and 0.05mM-[^{3}H]UTP (78.4mCi/mmol). Incubations were terminated by addition of 1ml of 10% (w/v) trichloroacetic acid/5% (w/v) tetrasodium pyrophosphate, the precipitates filtered on Whatman GF/C discs, dried, and their radioactivity was measured as described elsewhere (Chesterton & Butterworth, 1971). Counting efficiencies were approx. 25% for ^{3}H and 80% for ^{14}C.

Release of RNA polymerase

Amounts of free (template-unbound) RNA polymerase were measured as described elsewhere (Kellas *et al.*, 1977). Elongation by template-bound enzymes was inhibited by the inclusion of high concentrations (150μg/ml) of actinomycin D, and unbound enzymes were assayed by their ability to initiate on and transcribe added poly(dA-dT) after dilution to 80mM-$(NH_4)_2SO_4$ (to permit initiation).

Size analysis of transcripts

Labelled RNA was isolated from nucleoplasm after incubation for 3min at 37°C with 10mM-magnesium acetate and 10μg of deoxyribonuclease I/ml (still at 0.2M-$(NH_4)_2SO_4$) and then a further 30min in the presence of 0.5% (w/v) sodium dodecyl sulphate and 400μg of protease/ml. The mixture was extracted with an equal volume of water-saturated phenol at room temperature, and after centrifugation at 500*g* for 10min, the aqueous layer was precipitated at −20°C for 18h with 2vol. of ethanol. RNA was pelleted by centrifugation (15000*g* for 30min) and resuspended in small volumes (0.1–0.2ml) of either buffer A [20mM-sodium acetate (pH5.0)/0.1mM-EDTA/0.1M-NaCl] or buffer B [10mM-Tris/HCl (pH8.0)/1mM-EDTA/70% (v/v) deionized formamide]. In the former case (non-denaturing conditions) the RNA was layered over 13ml linear gradients of 5–40% (w/v) sucrose in buffer A and centrifuged for 16h at 74000*g* in a Beckman SW40 rotor at 10°C. In the second instance, RNA was denatured by heating at 70°C for 5min and then overlaid on 13ml gradients containing 2–10% (w/v) sucrose in buffer B with 85% (v/v) deionized formamide. Centrifugation in a SW40 rotor was for 17h at 225000*g* and at 25°C. Gradients were fractionated, RNA was precipitated with 10% (w/v) trichloroacetic acid and filtered on GF/C discs before radioactivity measurement. ^{3}H-labelled rRNA molecules labelled *in vivo* were used as molecular-weight markers and as hybridization controls; they were isolated from peripheral lymphocytes incubated overnight in the presence of [^{3}H]uridine and separated on sucrose gradients.

Elongation rates and numbers of active RNA polymerases

The numbers of RNA polymerases engaged on the endogenous template and the rate at which RNA synthesis was occurring *in vitro* were determined essentially as described by Coupar & Chesterton (1977) and Coupar *et al.* (1978). Brief 2min incubations *in vitro* were terminated by addition of 50μl of $HClO_4$; pelleted material was washed thoroughly by

four successive resuspensions in 300μl of 0.3M-$HClO_4$ and one in 300μl of 10% (w/v) trichloroacetic acid, and hydrolysed overnight with 70μl of 0.3M-KOH at 37°C in the presence of excess of unlabelled uridine and UMP. After neutralization and removal of $KClO_4$ precipitates (1000g for 10min), the hydrolysates were carefully streaked on plates (20cm×20cm) of polyethyleneimine-cellulose and these were developed successively with methanol, water and 2M-sodium formate, pH3.45. Standards (uridine, UMP and UTP) were chromatographed on the same plate. After drying, lines were drawn on the plate at 5mm intervals at right-angles to the direction of development and 25mm-wide sections of cellulose between lines were scraped away and prepared for radioactivity determinations. Numbers of elongating RNA polymerase II molecules were calculated by subtracting the number of pmol of uridine obtained in the presence of α-amanitin from those in its absence, and by using published values of the base compositions of preribosomal and heterogeneous nuclear RNA (Widnell & Tata, 1966). It was assumed that a RNA polymerase molecule would terminate at a particular nucleotide with the frequency expected from the relative abundance of that nucleotide, a premise supported by control experiments using labelled GTP as well as UTP (Coupar *et al.*, 1978). Elongation rates were calculated from [^{3}H]uridine/[^{3}H]UMP ratios.

Caesium salt-gradient centrifugation

Incubations *in vitro* were scaled up to 0.5ml, and terminated by direct addition to ice-cold mixtures of saturated CsCl (2.0ml), saturated Cs_2SO_4 (2.0ml) and 0.1M-Tris/HCl, pH8.0 (0.5ml). Final density was adjusted with water to 1.735g/ml and the mixtures were centrifuged at 146000g for 16h at 4°C in a 60Ti Beckman fixed-angle rotor. Fractions (0.25ml) were collected, precipitated with 10% (w/v) trichloroacetic acid and filtered on GF/C discs before radioactivity determination.

Purification of transcripts synthesized in vitro

Labelled RNA was isolated from nucleoplasmic incubations as described above as far as the phenol extraction, but for some experiments a greater degree of purity was required. In these instances, the aqueous layer was first chromatographed on a column (1.6cm×30cm) of Sephadex G-75 equilibrated in 50mM-NaCl to remove low-molecular-weight material. RNA eluted in the void volume was then bound to DEAE-cellulose in a 1cm×5cm column, washed with 0.3M-NaCl and eluted with 0.75M-NaCl. The eluate was applied to a column (2cm×20cm) of SP-Sephadex C-50 in 0.3M-NaCl/20mM-sodium acetate, pH5.0, and washed through with the same buffer. The RNA was finally precipitated with 2vol. of ethanol and collected as described above, dried *in vacuo* and redissolved in small volumes of buffer or water before storage at −20°C.

Isolation of unlabelled nucleic acids

DNA for hybridization studies was isolated from rat liver nuclei essentially as described by Paul & Gilmour (1968) and sheared by sonication (20×10s bursts at peak-to-peak amplitude of 20μm) down to about 500 base-pairs in size (as determined by agarose-gel electrophoresis). In other instances, DNA was prepared by techniques that minimize shear damage (Gross-Bellard *et al.*, 1973).

Unlabelled nuclear RNA was purified essentially as described for transcripts synthesized *in vitro*.

Poly(U)–Sepharose chromatography

RNA synthesized *in vitro* and purified as described above was passed through a column (1.6cm×5cm) of poly(U)–Sepharose equilibrated in 50mM-Tris/HCl (pH7.5)/0.7M-NaCl/10mM-EDTA/25% (v/v) deionized formamide at room temperature. The column was subsequently eluted with 10mM-Tris/HCl (pH7.5)/10mM-EDTA/0.2% (w/v) lauroylsarcosine/90% (v/v) deionized formamide and fractions were precipitated with 10% (w/v) trichloroacetic acid, filtered on GF/C discs and radioactivity was measured in the usual way (Chesterton & Butterworth, 1971).

Nucleic acid hybridization

Assay vials contained, normally in 75μl: sheared DNA (3.3–15.0mg/ml), 1% (w/v) sodium dodecyl sulphate, 1mM-EDTA, various amounts of [^{3}H]RNA (specific radioactivity $>10^6$c.p.m./μg) and 0.12M-NaH_2PO_4/Na_2HPO_4 (equimolar mix), pH6.8. All were overlaid with 0.1ml of paraffin oil, heated at 100°C for 7min and transferred to 65°C for incubation. Hybrid formation was determined after diluting each sample into 8.0ml of 40mM-Tris/HCl (pH8.0)/0.3M-NaCl and dividing into four equal portions. All were incubated at 37°C for 30min, two batches alone and two in the presence of 25μg of ribonuclease A/ml. Each was then precipitated with 10% (w/v) trichloroacetic acid, filtered, and ribonuclease-resistance assessed from radioactivity remaining on the GF/C discs.

Results

Preliminary assessment of nucleoplasmic RNA synthesis

Initial studies investigated the extent to which endogenous nuclear form-II RNA polymerase was retained in the nucleoplasmic fraction and the degree to which the properties of the enzyme remained similar to those seen in nuclei. Table 1 shows that high yields of nuclei (above 80%) were obtained as a routine after the initial centrifugation through hyper-

Table 1. *Characterization of the fractionation procedure*

Nuclei were prepared and washed and nucleoplasm was isolated from the pooled livers of two rats. Yields of nuclei were determined by haemocytometer counting, RNA polymerase activities by incorporation of [^{3}H]UMP in the absence or presence of 1 μg of α-amanitin/ml, and DNA was measured by the method of Burton (1956) after washes in trichloroacetic acid to remove sucrose. Values are means ± S.D. for duplicate assays.

	10^{-8} × No. of nuclei/g of liver	DNA (mg/g of liver)	RNA polymerase activity (pmol of [^{3}H]UMP incorporated/min per g of liver)	Proportion of form-II enzyme (%)
Whole liver	2	1.10 ± 0.21	—	—
Nuclei after sucrose step	1.64 ± 0.04	0.96 ± 0.11	10.9 ± 0.31	55
Nuclei after Triton wash	1.18 ± 0.11	0.95 ± 0.12	10.9 ± 0.03	39
Nucleoplasm	—	0.55 ± 0.07	7.6 ± 0.25	75

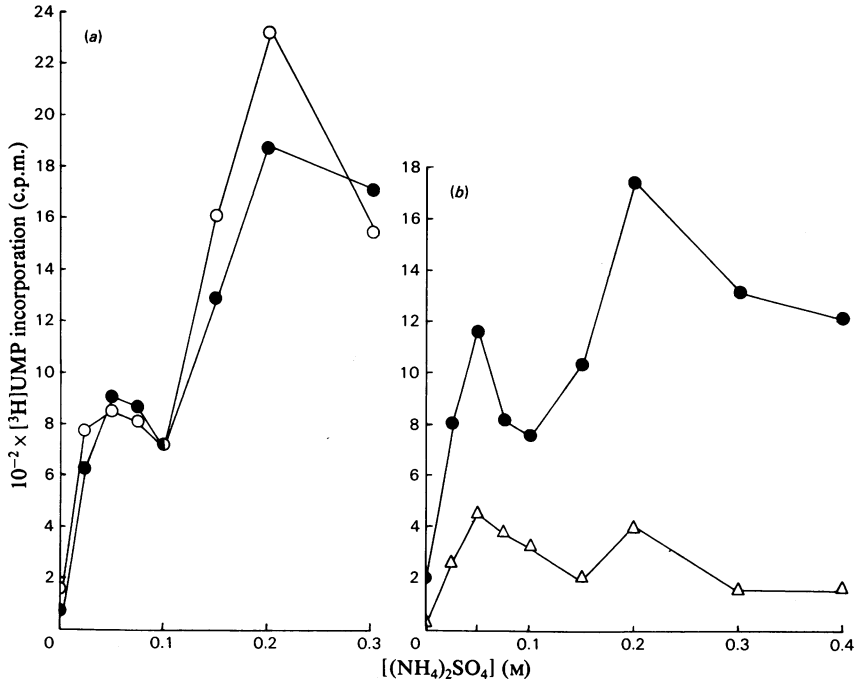

Fig. 1. *Effects of salt and rifamycin AF/0-13 on nucleoplasmic RNA synthesis*

Assays were as described in the Experimental section. Rifamycin AF/0-13, when present, was at 100 μg/ml and α-amanitin at 1 μg/ml. ●, Total RNA synthesis; ○, RNA synthesis with rifamycin AF/0-13; △, RNA synthesis with α-amanitin.

osmotic sucrose. The apparent loss of nuclei, but not of net RNA polymerase II activity, after the wash in Triton X-100 was largely explained by a changed morphology of many of the nuclei and consequent difficulties in counting them efficiently. Less than 2% of the DNA and 1% of the RNA were solubilized by this step. The final recovery of form-II RNA polymerase activity, as measured by incorporation studies with nucleoplasm, varied somewhat between experiments, but was usually about 95% of that seen in nuclei from an equivalent amount of liver. The major losses of RNA polymerase at this time involved form I, which pellets in the nucleolar fraction (Grummt & Lindigkeit, 1973; Beebee & Butterworth, 1975), resulting in the relative enrichment of form II exemplified in Table 1. The loss of DNA observed after sonication was not reflected by the presence of large amounts in the nucleolar pellets (which norm-

ally contained less than 10% of the total), but may have arisen from the drastic shearing process rendering much of the DNA unprecipitable by trichloroacetic acid. This precipitation was necessary to avoid sucrose contamination, which interferes with the diphenylamine assay procedure.

The data of Fig. 1 confirm that high ionic strength stimulated RNA polymerase II activity in nucleoplasm as it does in intact nuclei (Johnson *et al.*, 1971). A biphasic response to increasing salt concentration was always seen. Studies with rifamycin AF/0-13, an inhibitor of initiation by eukaryotic RNA polymerases (Meilhac *et al.*, 1972), confirmed that no detectable initiation of RNA synthesis was occurring *in vitro* under any of the ionic conditions investigated and that all the observed incorporation must be due to the elongation of RNA molecules initiated *in vivo*.

The RNA polymerase activities of nucleoplasm at 0.2M-$(NH_4)_2SO_4$ were further characterized as shown in Fig. 2. About 75% of the incorporation was inhibited by 1 μg of α-amanitin/ml and was therefore due to RNA polymerase II (Lindell *et al.*, 1970). A further 13% was inhibited over the concentration range 5–150 μg/ml, diagnostic of RNA polymerase III, and a residual amount was probably catalysed by RNA polymerase I. Form III should be inhibited by about 70–75% at 150 μg of α-amanitin/ml (Seifart *et al.*, 1972). Synthesis was linear for about 30 min in either the presence or absence of α-amanitin.

Characteristics of the system

The results of Table 2 indicate that there were about 2.8×10^{12} form-II RNA polymerase molecules engaged in elongation per mg of precipitable DNA in the nucleoplasmic fraction. Taking account of losses of measurable DNA during the nucleoplasm preparation, this value is the equivalent of about 7×10^3 enzyme molecules per original nucleus. Elongation rates by the form-II polymerase were apparently around 0.25 nucleotide/s. Because the α-amanitin-resistant activity was probably constituted by more than one type of enzyme transcribing different types of genes, no attempt was made to carry out similar calculations on these data.

A number of controls is necessary to establish the validity of this kind of experiment. Endogenous UTP pools, by diluting the concentration of added label, could seriously affect the calculations. Estimation by isotope dilution suggested that after the wash in Triton X-100 less than 1 μM-UTP was present in the preparation. Nucleases and phosphatases, acting together, could generate spurious 3′-termini and lead to the numbers of polymerase molecules being overestimated. Premature termination, occurring on a large scale, could also cause the elongation rate to be underestimated. Control experiments were carried out in which either actinomycin D and α-amanitin were present during the second minute of incubation (at 100 and 1 μg/ml respectively), or the reaction was terminated after only 1 min (see Table 2). The former conditions should indicate the significance of nuclease and phosphatase activity, the latter the degree of time-dependent chain termination. In neither instance were the results apparently different from those recorded in the normal assays.

Actinomycin D and poly(dA-dT) were used in experiments to determine whether the amounts of RNA polymerase II not associated with the endo-

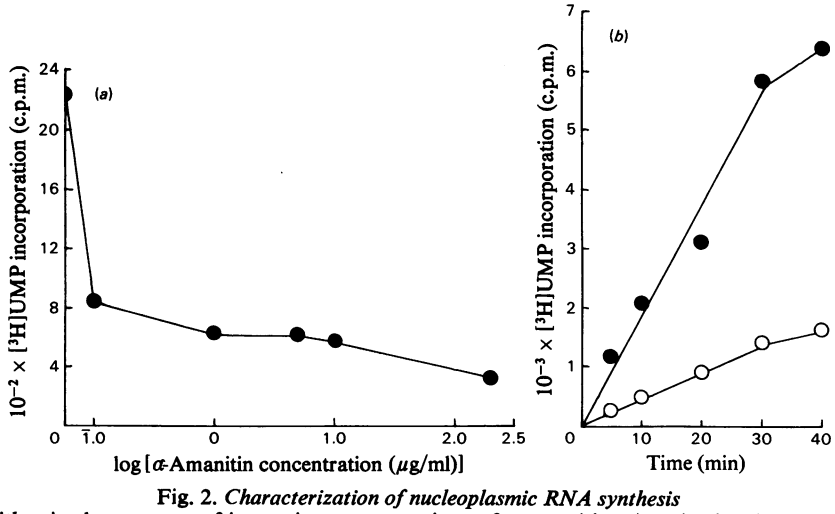

Fig. 2. *Characterization of nucleoplasmic RNA synthesis*

Assays were either in the presence of increasing concentrations of α-amanitin (*a*) or in the absence (●) or presence (○) of α-amanitin (1 μg/ml; *b*).

Table 2. *Estimations of uridine and UMP incorporation by nucleoplasm*

Assays contained nucleoplasm from 2.5g of liver in final volumes of 1.25ml, with components as described in the Experimental section, except that [^{3}H]UTP was present at a specific radioactivity of 10Ci/mmol. Incubations were for 1 or 2min at 37°C after prewarming the nucleoplasm to this temperature before starting the reaction (owing to the large volumes involved). Duplicate assays were carried out either in the presence or absence of α-amanitin, or with α-amanitin and actinomycin D present only during the second minute. Recovery of labelled RNA was greater than 95% during the initial washes, and counting efficiency of ^{3}H extracted from polyethyleneimine-cellulose was 30%. Sections of cellulose between the uridine and UMP spots produced backgrounds of less than 200c.p.m., whereas there were typically up to 40000c.p.m. in a UMP spot and up to 1000c.p.m. in a uridine spot. Numbers of terminal uridine residues were calculated from these data by allowing for 0.4% conversion of UMP into uridine (Coupar *et al.*, 1978). DNA in the $HClO_4$ precipitate was determined by the method of Burton (1956). Values are means ± S.D. for two experiments.

	[^{3}H]Uridine (pmol/mg of nucleoplasmic DNA)	[^{3}H]UMP (pmol/min per mg of nucleoplasmic DNA)
Normal assay (2min)	1.80 ± 0.09	24.82 ± 2.33
With α-amanitin	0.65 ± 0.08	7.35 ± 1.09
Normal assay (1min)	1.83 ± 0.07	25.55 ± 2.57
With α-amanitin	0.66 ± 0.07	8.00 ± 1.11
Assay (2min) with α-amanitin and actinomycin D present during second minute only	1.79 ± 0.09	23.86 ± 2.53

Table 3. *Activities of free enzymes during incubation*

Duplicate assays contained initially nucleoplasm from 200mg of liver in final volumes of 0.125ml. Addition of poly(dA-dT) to 100μg/ml, actinomycin D to 150μg/ml and [^{3}H]UTP at the start or after 20min increased the volumes to 0.375ml and lowered the ionic strength to 80mM-$(NH_4)_2SO_4$. Incubations were then continued for a further 15min, all at 37°C. Values are means ± S.D. for duplicate assays.

	Free enzyme activity (c.p.m. of [^{3}H]UMP incorporated)	
	0–15min	20–35min
Standard assay	495 ± 40	389 ± 36
With α-amanitin (1μg/ml)	218 ± 27	151 ± 21
ATP, GTP, CTP and unlabelled UTP added with [^{3}H]UTP instead of at start	425 ± 37	144 ± 22
As above with α-amanitin	210 ± 26	101 ± 20

genous template (free enzyme) varied during the course of the incubations. Preliminary experiments were carried out to establish that the poly(dA-dT) concentration used was sufficient to be unlimiting of free enzyme activity, and that the actinomycin D concentration was high enough to prevent concurrent enzyme release from the endogenous templates (Kellas *et al.*, 1977) (results not shown). The results of Table 3 imply that little change occurred in the total amount of poly(dA-dT)-dependent polymerase activity through the course of the incubation. However, under conditions where transcription was prevented (by lack of substrates) very much less enzyme was apparent at later times, suggesting that a lability of free enzyme may have been masking the effect of polymerase release. The data demonstrate only changes in the free enzyme pool and can give no information on the size of this pool relative to the numbers of template-bound RNA polymerases, since the specific activities of the enzymes on the two templates may be very different.

Further characterization of the transcripts was carried out by centrifugation of the material in caesium salt gradients. After synthesis in either the absence or the presence of α-amanitin, the RNA demonstrated buoyant densities significantly lower than that of purified 28S RNA (Fig. 3). The transcripts exhibited heterodisperse profiles with peaks of radioactivity around 1.756g/ml (synthesis without α-amanitin) and 1.735g/ml (with α-amanitin): 28S rRNA banded at 1.765g/ml. These characteristic profiles were reproducible and indicated that the nascent RNA molecules might be attached in some way to other materials forming complexes of lower buoyant density which were stable at high ionic strength.

Treatment of the nucleoplasmic transcripts with either deoxyribonuclease I or protease revealed that

the lower buoyant densities were due to the association of the nascent RNA with proteins; deoxyribonuclease did not affect the banding profiles, whereas protease decreased or abolished the differences between 28S RNA and the transcripts. The RNA synthesized in the absence of α-amanitin banded around 1.762g/ml, and that synthesized in the presence of α-amanitin at around 1.767g/ml (Fig. 3*b*).

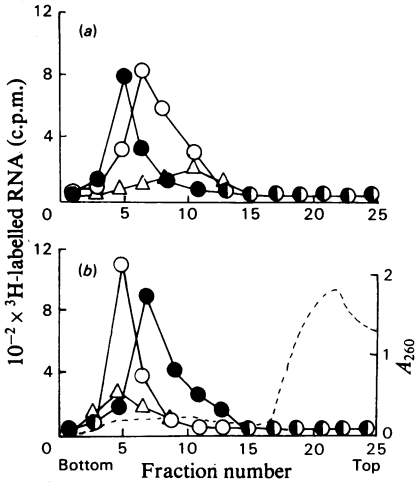

Fig. 3. *Isopycnic centrifugation of nucleoplasmic RNA* Gradients were prepared, centrifuged and analysed as described in the Experimental section. (*a*) ●, ^{3}H-labelled 28S rRNA; ○, nucleoplasmic RNA labelled *in vitro*; △, nucleoplasmic RNA labelled in the presence of α-amanitin (1μg/ml). (*b*) ●, Transcripts treated with deoxyribonuclease I (10μg/ml for 2min at 37°C in 10mM-magnesium acetate); ○, transcripts treated with protease [400μg/ml in 0.5% (w/v) sodium dodecyl sulphate for 5min at 37°C] and extracted with phenol; △, transcripts synthesized in the presence of α-amanitin and treated with protease. ----, A_{260}.

Nature of the transcripts

The sizes of RNA molecules isolated from nucleoplasm after incubation *in vitro* are shown in Fig. 4. Similar profiles were obtained whether aqueous or denaturing (formamide) conditions were used, and the size ranges were also similar for RNA molecules labelled *in vivo* and *in vitro*. A small peak of material around 45S was apparently synthesized by a form-I RNA polymerase component; heterodisperse RNA in the range 18–40S was primarily transcribed by RNA polymerase II, and a large peak of material sedimenting between 12S and 18S seems to have been produced by all types of polymerases and may include breakdown products arising during manipulation. Low-molecular-weight RNA synthesized by RNA polymerase III was also apparent. Since the elongation rates determined in Table 2 dictate that during the time period of the incubations an average of only about 150 nucleotides could have been added to each RNA molecule being elongated by RNA polymerase II, it is clear that the size profiles were dictated essentially by the lengths of unlabelled RNA molecules previously synthesized *in vivo*.

Passage of nucleoplasmic RNA labelled *in vitro* through a column of poly(U)–Sepharose resulted in very little retention under conditions whereby pure poly(A) was quantitatively adsorbed (Fig. 5). This observation suggests that at the high ionic strength of the incubation there was little or no addition of poly(A) sequences to the newly synthesized RNA. Less than 3.5% of the RNA was retained on the column despite the fact that 75% of the labelled material was synthesized by RNA polymerase II and some 20% of heterogeneous nuclear RNA is polyadenylated *in vivo* (Levis & Penman, 1977). This result confirms that poly(A) addition in this system is unlikely to have interfered with estimates of numbers of elongating RNA molecules based on quantification of terminal uridine residues as outlined in Table 2.

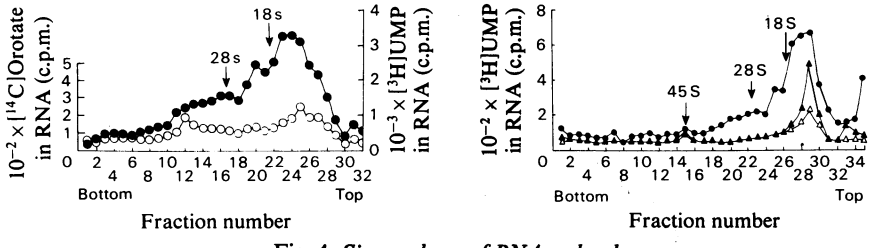

Fig. 4. *Size analyses of RNA molecules*

RNA was isolated from nucleoplasm either immediately (material labelled *in vivo*) or after incubation *in vitro* for 10min at 37°C and centrifuged through either 5–40% (w/v) sucrose gradients (*a*) or 2–10% (w/v) sucrose gradients in 85% (v/v) formamide (*b*). RNA was labelled *in vivo* by intraperitoneal injection of 20μCi of [^{14}C]orotic acid 30min before the rat was killed. ○, RNA labelled *in vivo*. ●, RNA labelled *in vitro*. ▲, RNA labelled *in vitro* in the presence of α-amanitin (1μg/ml). △, RNA labelled *in vitro* in the presence of α-amanitin (150μg/ml).

The labelled RNA population was also characterized by hybridization in a vast excess of DNA to determine the nature of the template sequences from which it had been transcribed (Melli *et al.*, 1971). Fig. 6 shows the rates with which the RNA molecules hybridized to DNA and the overall extent of hybrid formation. It did not prove possible to drive more than 40% of the RNA into duplex even in the presence of 8-fold greater excess of DNA than was used in these experiments, although altering the DNA/RNA ratio did markedly affect the hybridization of the rRNA control. These data are shown in Table 4, which also provides evidence that hybrid formation was not due to the labelled RNA annealing with the unlabelled nuclear RNA which co-purifies with it.

The data clearly show a biphasic hybridization reaction for transcripts of form-II RNA polymerase and for those of an α-amanitin-resistant enzyme also. Interpretation is complicated by the presence of endogenous (unlabelled) RNA sequences, possibly acting as competitors, and the low extents of hybridization with form-I polymerase products. However, both repeated and unique DNA sequences were clearly under transcription by more than one RNA polymerase species in the nucleoplasm preparation.

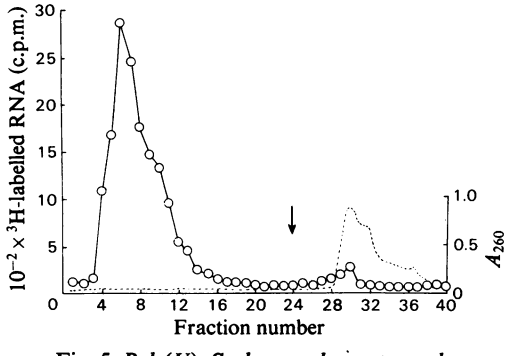

Fig. 5. *Poly(U)–Sepharose chromatography*
Transcripts were synthesized *in vitro* and chromatographed on poly(U)–Sepharose as described in the Experimental section. Nucleoplasm from 2.5g of liver was used. Alternatively the column was loaded with 100μg of poly(A) and binding monitored by measurements of A_{260}. ○, Transcripts; ----, poly(A). Elution buffer was added at the arrow.

Discussion

The main objectives of this study were to isolate a nuclear fraction enriched in endogenous RNA polymerase II enzymes capable of elongating RNA chains *in vitro* that were initiated *in vivo*, and to make a preliminary characterization of the system including the nascent RNA molecules present in the chromatin. RNA polymerase II is thought to be responsible for pre-mRNA synthesis *in vivo* (for a review see Chambon, 1975) and it is therefore of interest to understand the nature of the transcription process mediated by this enzyme and to discover the types of sequences synthesized by it. Whereas considerable effort has been expended in obtaining chromatin fractions with high template activities, e.g. by nuclease treatment and subsequent fractionation (Marushige & Bonner, 1971; Matsui & Busch, 1977), and also in isolating nucleoli for investigation of

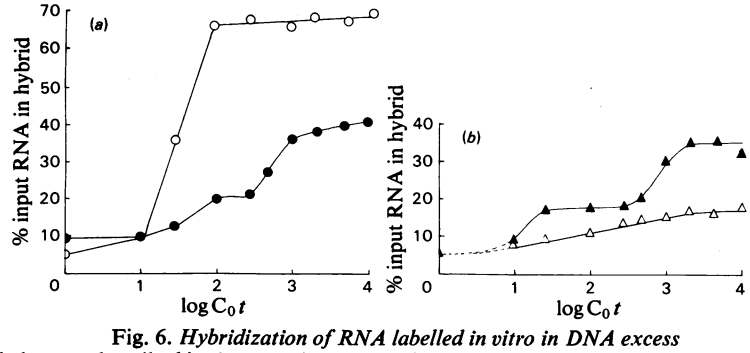

Fig. 6. *Hybridization of RNA labelled in vitro in DNA excess*
Assays were carried out as described in the Experimental section, each containing 1μg of total nuclear RNA (including that labelled *in vitro* for 15min) or 20ng of 28S rRNA (>10^5 c.p.m./μg) as a control, and extent of hybridization was ascertained at various C_0t (DNA concentration × time) values. DNA/RNA mass ratios were: DNA/total RNA = 165:1 for nuclear RNA or 6900:1 for 28S RNA; DNA/[^{3}H]UMP-labelled component of nuclear RNA = 150000:1. (*a*) ●, Total RNA synthesized *in vitro*; ○, 28S rRNA. (*b*) ▲, RNA synthesized in the presence of 1μg of α-amanitin/ml; △, RNA synthesized in the presence of 150μg of amanitin/ml. Degradative loss of RNA was negligible over the time periods of incubation. Maximum hybridization values were 350–400c.p.m. per duplicate assay. At $C_0t = 10^4$, about 80% of the DNA had reannealed as judged by absorption measurements.

Table 4. *Characterization of hybridization reaction*

Hybridization assays were as outlined in the Experimental section and were incubated for the appropriate times. Excess of DNA is given as the factor compared with that used in normal incubations and ranged between 165:1 and 1320:1 (see the legend to Fig. 6), varied by increasing the amount of DNA. Data are radioactivity (c.p.m.) in hybridized RNA, with percentages of totals in parentheses. Lower total numbers of c.p.m. at high excess of DNA resulted from increased quenching effects of the large amounts of material on the filters. Experiments with an excess of RNA were similar, except that unlabelled nuclear RNA replaced DNA in the hybridization assays (mass ratio unlabelled RNA/DNA = 125000:1). C_0t, DNA concentration (μg/ml)×time of incubation (h); r_0t = as for C_0t but with RNA concentration.

	^{3}H-labelled nucleoplasmic RNA in hybrid (c.p.m.)			^{3}H-labelled 28 S rRNA in hybrid
Conditions	$C_0t = 100$	$C_0t = 1000$	RNA excess	($C_0t = 100$)
DNA excess				
×1	130 (12.9)	252 (30.1)	—	299 (70.1)
×2	139 (12.8)	262 (30.0)	—	325 (74.4)
×4	121 (13.7)	205 (29.7)	—	316 (77.2)
×8	108 (13.3)	200 (29.6)	—	300 (80.6)
RNA excess				
0 time	—	—	163 (9.7)	
r_0t 100	—	—	112 (6.4)	
r_0t 1000	—	—	88 (4.9)	

endogenous form-I RNA polymerase activity (Grummt & Lindigkeit, 1973; Beebee & Butterworth, 1975, 1977) there have as yet been few attempts to examine specifically and in detail the endogenous form-II activity. Tata & Baker (1978) have succeeded in fractionating nuclei by using micrococcal nuclease and obtained fragments rich in endogenous RNA polymerase II associated with nucleosomes, though as yet there is no information on the nature of the sequences under transcription. Neither method has been able to eliminate completely the presence of other polymerase activities, but the complications caused by these components have been minimized and the contribution of the form-II enzyme is markedly enriched.

A number of features of the nucleoplasmic preparation are notable. There was a high recovery of template-bound RNA polymerase II molecules, at least as far as can be judged by comparisons with nuclei. This was confirmed by incorporation studies and by analysis of numbers of elongating RNA chains; the yields in nucleoplasm, equivalent to about 7×10^3 polymerase II molecules per original nucleus, were very close to those observed by Coupar *et al.* (1978) in intact nuclei. Conversely, the procedure caused extensive damage to the DNA template and the nucleoplasmic preparation is unlikely to be of value in studies on the initiation of RNA synthesis, owing to artefacts that arise when RNA polymerases react with small or damaged DNA molecules (Flint *et al.*, 1974). Sucrose-gradient analysis of DNA isolated from nucleoplasm by the method of Gross-Bellard *et al.* (1973) demonstrated that most of the material had a double-stranded molecular weight of only about 3×10^6, with single strands of around 1.6×10^6 mol.wt., indicating a preponderance of small fragments but with few internal single-strand breaks (results not shown). However, the near-quantitative recoveries of form-II polymerase and the concurrent enrichment do make the system potentially valuable for elucidating the nature of the sequences under transcription *in vivo* and merely elongated a short distance *in vitro*, since this process seems to have been little affected by the decrease in template size.

The investigations also confirm two rather puzzling features of transcription in isolated nuclei (Coupar & Chesterton, 1977; Coupar *et al.*, 1978), namely the small number of RNA polymerase II molecules engaged in elongating RNA chains, and the very slow elongation rates. Since of the order of 1.4×10^4–2.4×10^4 genes are probably expressed in rat liver (Coupar *et al.*, 1978; Savage *et al.*, 1978), the implications are that either only a small fraction of the transcription complexes active *in vivo* is re-activated *in vitro*, or that many genes for which mRNA molecules exist in the cytoplasm are not continuously transcribed. This observation is emphasized by electron-microscope studies, which have confirmed that, although not packed as closely as the enzymes transcribing ribosomal genes, the polymerases tentatively identified as being associated with heterogeneous nuclear RNA synthesis are also present in numbers on individual active genes (Foe *et al.*, 1976). A packing ratio of only 10 enzymes per cistron would permit the simultaneous expression of only 700 genes, 3–5% of the apparent total reflected in the mRNA population, if the end-group analyses are correct. Independent estimates of the total RNA polymerase II content of liver cells made by titration with labelled α-amanitin and adjusted to recent measure-

ments of nuclear DNA content (Cochet-Meilhac *et al.*, 1974; Coupar *et al.*, 1978) and by direct protein purification methods (Schwartz & Roeder, 1975) suggest a total complement of no more than 2.5×10^4–3.5×10^4 molecules per diploid genome. Even if all of these enzymes were template-bound, an average packing ratio of no more than two per cistron could result if all genes reflected in the mRNA population are permanently transcribed (assuming that all pre-mRNA is synthesized by RNA polymerase II). An alternative explanation, that much of the form-II enzyme is free and not bound to DNA, has been indicated by the work of several laboratories (e.g. Schwartz *et al.*, 1974; Tata & Baker, 1978). Further work on these issues is clearly required.

Elongation rates for the form-II RNA polymerase were rather slower in this study than those observed in whole nuclei under different assay conditions (Coupar *et al.*, 1978). The reasons for these small differences, and the much larger ones of about 100-fold between rates observed generally *in vivo* and *in vitro*, remain as major problems awaiting resolution. Whether the isolation of subcellular fractions such as nuclei inevitably results in the loss of elongation factors awaits further investigation, though it is of interest that, at least for pre-rRNA transcription *in vivo*, there is some evidence of regulation at the level of elongation (Franze-Fernandez & Fontanive-Sanguesa, 1975).

Centrifugation of nucleoplasmic RNA in caesium salt gradients demonstrated that the nascent molecules, though not necessarily the sequences actually transcribed *in vitro*, were tightly associated with proteins. The strong nature of the interaction, reminiscent of messenger ribonucleoprotein particles, which are also stable in caesium gradients (Greenberg, 1976), and the different buoyant densities observed with RNA synthesized in the absence and presence of α-amanitin, suggest that some specificity may attach to these interactions, though the nature of the proteins involved has not yet been investigated further. The observations do, however, demonstrate another characteristic property of transcription in eukaryotic cells which may be sought in systems in which initiation occurs *in vitro* and used as a measure of the integrity of such a system. It will also be of interest to identify the particular proteins involved and their role, if any, in the transcription reaction.

Analysis of transcripts of endogenous RNA polymerase II enzymes by hybridization in DNA excess has not previously been reported, and the results indicate that, unlike the situation with endogenous form-I enzyme in nucleoli, which transcribes almost entirely reiterated DNA sequences (Beebee & Butterworth, 1976), nucleoplasmic form II synthesized RNA from both reiterated and unique regions of the genome. This result provides more evidence that differences must exist in the specificity of template–RNA polymerase interaction between the various eukaryotic enzymes. Although no more than 40% of the transcripts entered into duplex formation, this proportion is nevertheless surprisingly high in view of the large amounts of unlabelled nuclear RNA present (the mass ratios of unlabelled/labelled RNA molecules were calculated to be about 900:1 in these experiments). These observations tend to confirm the implications mentioned above that the sequence complexity of nuclear RNA may not reflect that of the genes under transcription at any particular point in time. Recent hybridization studies using labelled DNA derived from the non-repetitive portion of the rat genome have indicated that liver nuclei contain about 9×10^4 different species of RNA molecules, but that there are on average only 0.1–0.3 copy of each per cell and a total of only 2.2×10^4 RNA molecules in any particular nucleus (Chikaraishi *et al.*, 1978). Together with the results of Table 2 these data suggest that only about 33% of the heterogeneous nuclear RNA in a particular nucleus may be under transcription at any time.

Low overall extents of hybridization seem to be a feature of heterogeneous nuclear RNA in DNA excess (Lewin, 1975), though the reasons remain unclear. In part it may be caused by the presence of different abundance classes of RNA rendering achievement of an excess of DNA very difficult; certainly this kind of situation can lead to the kinds of results shown in Fig. 6 (Muto, 1977), but attempts to improve the DNA/RNA ratio failed to alleviate the situation. The RNA molecules remained greater than 120 nucleotides in average length at the end of the hybridization reaction (results not shown).

The rather high (10%) intrinsic ribonuclease resistance of the transcripts is also notable and could be related to extensive intramolecular base-pairing of the sort demonstrated for heterogeneous nuclear RNA (Fedoroff *et al.*, 1977). No evidence of intermolecular interactions between the RNA molecules was observed.

It was apparent that another RNA polymerase apart from form II was transcribing unique sequences in the genome, though owing to the low degree of hybridization of transcripts synthesized in the presence of high α-amanitin concentrations it remains unclear as to whether polymerase I or III was responsible. RNA polymerase I has been implicated in the synthesis of low-molecular-weight RNA in the nucleoplasm of HeLa cells (Benecke & Penman, 1977), whereas form III has been shown to transcribe unique sequences in calf thymus chromatin (Atikkan & Furth, 1977). This problem also warrants further investigation in cell-free systems.

The experiments described in the present paper therefore represent an initial confirmation that it is possible to obtain a nucleoplasmic fraction from rat liver nuclei enriched in RNA polymerase II, and

have also highlighted specific features and questions about the transcription events mediated by that enzyme.

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