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Ribonucleic Acid Synthesis in Isolated Rat Liver Nuclei under Conditions of Ribonucleic Acid Processing and Transport

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A cell-free system is described which permits a significant and prolonged synthesis of RNA in isolated rat liver nuclei, under conditions previously demonstrated to support normal nuclear processing and transport of both rRNA and mRNA. The system contains cytosol but not $(NH_4)_2SO_4$ or other non-physiological components. Evidence is presented for cytosol factors which stimulate ribosomal, and to a lesser degree, non-ribosomal RNA synthesis.

Although there are several cell-free systems derived from eukaryotic cells which support RNA synthesis, it is customary to exclude cytosol proteins and to include in the assay relatively high concentrations of $(NH_4)_2SO_4$ to activate RNA polymerase II and hence mRNA synthesis (Widnell & Tata, 1964; Novello & Stirpe, 1970; Soll & Sussman, 1973). In the absence of $(NH_4)_2SO_4$, these systems synthesize primarily ribosomal-like RNA, the RNA polymerase I in the nucleolus apparently maintaining significant activity under more physiological conditions. However, several studies (Seifart *et al.*, 1973; Higashinakagawa *et al.*, 1972; Froehner & Bonner, 1973; Thompson & McCarthy, 1968; Banks *et al.*, 1974; Chang & Goldwasser, 1973; Stein & Hausen, 1970) suggest that the cytoplasm contains a factor or factors, which stimulate RNA synthesis; one of these factors was found to lower the ionic requirements of the medium in order to achieve maximal RNA polymerase II activity (Stein & Hausen, 1970). Unfortunately, there does not appear to be any general consensus to date as to the nature or function of the components isolated in different laboratories. However, it is important to note the desirability (Younger & Gelboin, 1970) of including in the assay at least that fraction of the cytosol which contains the natural ribonuclease inhibitor, thus protecting the newly synthesized RNA from rapid breakdown.

Although some of the more recent studies do recognize that RNA synthesis may be subject to cytoplasmic modulation, no attempt was made to demonstrate that post-transcriptional nuclear events proceeded normally. This question is of interest, since nuclear post-transcriptional and transcriptional processes may be coupled. Previous papers from this laboratory (Yu *et al.*, 1972; Schumm & Webb, 1973, 1974*a*,*b*; Schumm *et al.*, 1973*a*,*b*) described a cell-free system, subject to modulation by cytoplasmic proteins, which supports nuclear processing and transport of pre-formed mRNA and rRNA and which consequently exhibits normal nuclear restriction (Schumm & Webb, 1974*b*). The present paper describes the adaptation of this system to the study of RNA transcription in isolated nuclei and initial studies on cytoplasmic factors which effect transcription.

Materials and Methods

Nuclei and dialysed cytosol were prepared from the liver of Sprague–Dawley rats (250g) as previously described (Yu *et al.*, 1972; Schumm & Webb, 1973).

The standard medium which supports RNA processing and transport in isolated nuclei contains 50mM-Tris–HCl buffer, pH7.5, 2.5mM-$MgCl_2$, 2.0mM-dithiothreitol, 0.5mM-$CaCl_2$, 0.3mM-$MnCl_2$, 5.0mM-NaCl, 2.5mM-Na_2HPO_4, 5.0mM-spermidine, 2.5mM-ATP, 2.5mM-phosphoenolpyruvate, 0.5mg of yeast RNA/ml, 35 units (μmol/min) of pyruvate kinase, dialysed cytosol protein (usually 10mg/ml) and nuclei in a final volume of 1.0ml. For measurement of RNA-synthetic activity, this medium was further supplemented with 0.8mM-GTP, 0.8mM-CTP and 0.2mM-[^{14}C]UTP (4Ci/ml); 0.5mM-L-cysteine was also included in the assay to inhibit partially non-specific phosphatase, which degraded the nucleoside triphosphate. After the addition of freshly prepared nuclei, the incubation was performed on a shaking water bath at the designated temperature.

The amount of RNA synthesized during the period of incubation was measured as the total trichloroacetic acid-precipitable radioactivity (Schumm & Webb, 1973). The protein concentration of the cytosol was measured with the biuret reagent (Gornall *et al.*, 1949), with bovine serum albumin as standard. The size distribution of the RNA synthesized *in vitro* was estimated by first purifying the RNA from the assay with phenol, then separating the species according to size on 28ml linear (10–30%, w/v) sucrose gradients in 50mM-Tris, pH7.5; the latter

were centrifuged in an SW 25.1 rotor of a Beckman ultracentrifuge at 51000$g_{av.}$ for 16h as previously described (Yu *et al.*, 1972). The size distributions were also confirmed by separation of the purified RNA in 2.5% (w/v) polyacrylamide–agarose gels (Summeis, 1970), followed by radioassay of the fractions (Yu *et al.*, 1972). The gradients and gels were standardized with nuclear and cytoplasmic RNA species of known size. RNA containing poly(A) was isolated by chromatography of the phenol-extracted RNA on cellulose columns as described by De Larco & Guroff (1973).

The experiments on competitive DNA–RNA hybridization were carried out by the procedure of Gillespie & Spiegelman (1965) except that the incubations between RNA and DNA were carried out for 18h in 4×SSC (SSC = 0.15M-NaCl–0.015M-sodium citrate, pH7.0) at 67°C. Competitor unlabelled RNA was prepared by hot phenol extraction of purified ribosomes. The RNA was treated with deoxyribonuclease, re-extracted with phenol and precipitated three times with 95% ethanol (Schumm & Webb, 1974*a*). The DNA was purified from rat liver by the method of Ono *et al.* (1971). This procedure has been described previously (Schumm & Webb, 1974*b*). Hybridization mixtures contained 40μg of rat liver DNA, labelled RNA extracted from 1×10^7 nuclei and, where indicated, 5mg of purified ribosomal RNA/ml. Saturation curves (c.p.m. in hybrid versus mg of added competitor RNA) showed these to be of sufficiently high concentration for complete competition with this amount of labelled RNA.

Among the techniques used to fractionate and purify the transport factor(s) from the cytosol, DEAE-cellulose column chromatography was most successful. Undialysed freshly prepared regenerating liver cytosol was passed through a DEAE-cellulose column, pre-equilibrated to pH8.5 at 4°C. At this pH almost half of the proteins in the cytosol fraction bind to the column, but the more basic ones pass through. The absorbed proteins were eluted in a stepwise fashion with 0.6M-KCl, pH7.5. Both fractions were extensively dialysed against TKM buffer [50mM-Tris (pH7.8) – 25mM-KCl – 2.5mM-$MgCl_2$] to remove salts, before assay.

Results

The concentration-dependence curve for nuclei and the time-course of the incorporation of label from [^{14}C]UTP into RNA is shown in Fig. 1. With an incubation period of 10min at 30°C, the incorporation of label into acid-precipitable RNA was linear over a wide range of concentration of nuclei (Fig. 1*a*). Similarly, except for an initial rapid incorporation

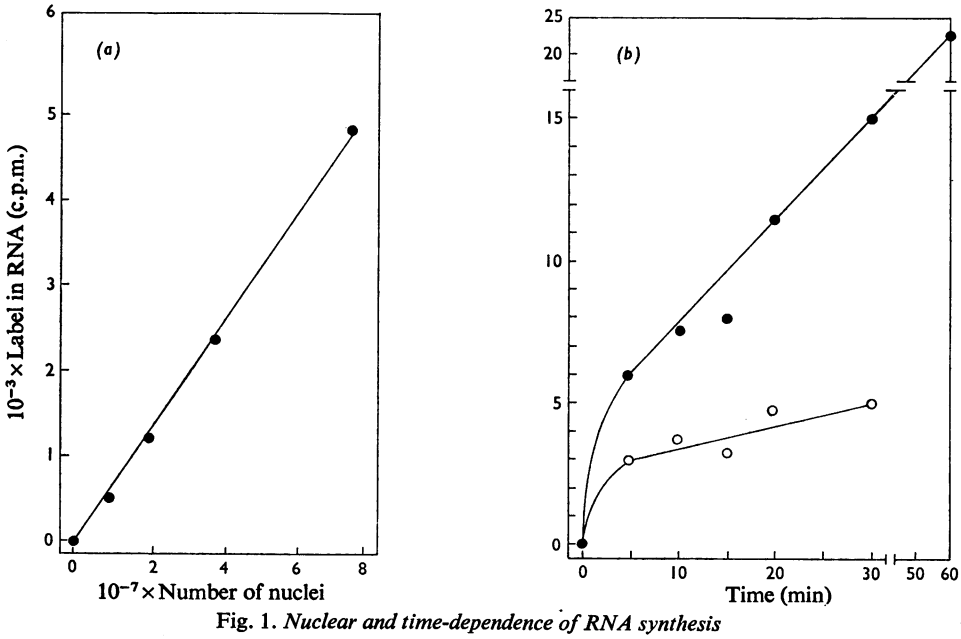

Fig. 1. *Nuclear and time-dependence of RNA synthesis*

(*a*) Dependence of RNA synthesis in normal rat liver nuclei on the concentration of nuclei. The incubation medium contained 10mg of normal liver cytosol/ml. After incubation of the assays at 30°C for 10min the RNA was precipitated with trichloroacetic acid, then washed and counted for radioactivity in liquid scintillant (Schumm & Webb, 1973). (*b*) Time-course of incorporation of label from [^{14}C]UTP into RNA in the absence (●) and presence (○) of 5.0μg of α-amanitin/ml. Each assay contained 0.37×10^8 nuclei or 0.25×10^{-3}mg of DNA/ml. Other conditions were as for (*a*).

during the first 10min of incubation, the incorporation of label into RNA is linear with time up to at least 60min incubation at 30°C (Fig. 1*b*). In contrast, at 25°C the rate of incorporation was linear with time only for 10min and reached a plateau by 15min, and at 37°C the rate of incorporation, although linear beyond 5min, was much lower (results not shown). The higher initial rate of incorporation may be due to completion of partially synthesized RNA chains during the first 5min of incorporation.

Also shown in Fig. 1(*b*) is the effect of adding α-amanitin (5.0μg/ml) to the assay. At this concentration the fungal toxin specifically inhibits RNA polymerase II (Weinmann & Roeder, 1974), and thereby inhibits mRNA synthesis. The residual RNA synthesis, which represents approx. 50% of the control value, represents primarily rRNA synthesis.

The size distribution of the nRNA labelled in the presence and absence of α-amanitin is shown in Fig. 2. The prominent peak at approx. 45S in both cases probably represents primarily ribosomal precursor RNA. α-Amanitin appears to inhibit preferentially the synthesis of the lighter RNA species; however, there also appear to be some species with sedimentation coefficients equal to and greater than 45S whose synthesis is sensitive to the inhibitor. In the absence of cytosol virtually no label is incorporated into species with sedimentation coefficients greater than 18S. This marked dependence of size of RNA on the presence of cytosol in the incubation mixture was confirmed by polyacrylamide-gel electrophoresis (results not shown).

Analysis of the synthesized RNA to determine the presence of RNA containing poly(A) demonstrated that 14.7±1.0% (three separate assays) of the labelled RNA contained poly(A).

In the above experiments the concentration of dialysed cytosol protein in the assay mixture was 10mg/ml. The concentration-dependence curve shown in Fig. 3 indicates that in either the absence or the presence of α-amanitin there is a measurable but not absolute dependence of RNA synthesis on the concentration of cytosol protein. Between a cytosol concentration of approx. 2.5 and 10.0mg/ml the relationship is linear, the rate in the presence of α-amanitin being approximately two-thirds of that in its absence. This indicates that the cytosol-mediated enhancement of rRNA synthesis is twice that of non-ribosomal RNA synthesis.

The greater response of rRNA synthesis to non-dialysable factors in the cytosol is reflected in the data from competitive DNA–RNA hybridization experiments, the results of which are summarized in Table 1. Thus the proportion of rRNA synthesized *in vitro*

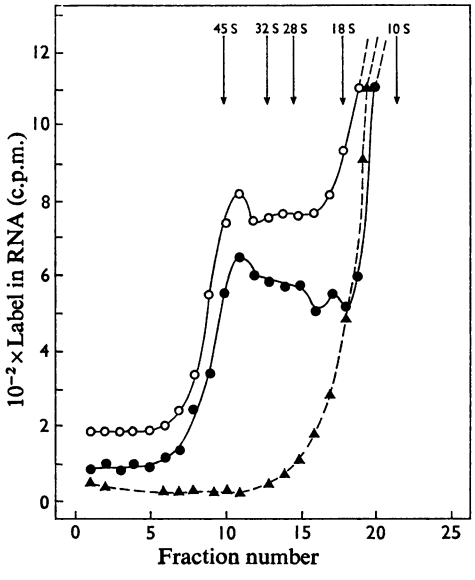

Fig. 2. *Size-distribution analysis of labelled RNA synthesized by isolated nuclei in the absence* (○) *and presence* (●) *of α-amanitin*

The phenol-purified RNA was centrifuged through 28ml linear (10—30%) sucrose gradients and 1.0ml fractions were collected. Other conditions were as for Fig. 1(*b*). The size distribution of RNA synthesized in the absence of cytosol is also shown (▲). The top of the gradient is to the right.

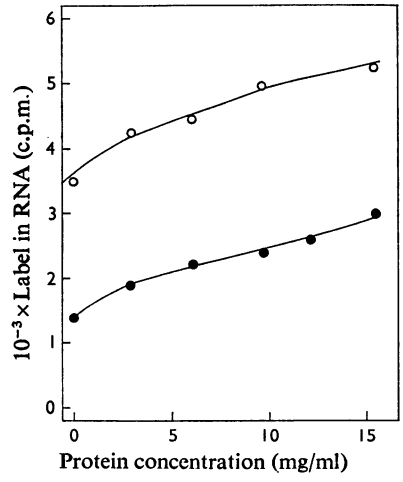

Fig. 3. *Concentration-dependence of RNA synthesis in isolated nuclei on cytosol protein*

The nuclei were incubated in media containing the indicated amounts of proteins for 20min at 30°C in either the absence (○) or the presence (●) of 5.0μg of α-amanitin/ml. Other conditions were as for Fig. 1(*b*).

Table 1. *Estimation by DNA–RNA competitive hybridization of the proportion of rRNA synthesized in the absence and presence of α-amanitin*

%rRNA represents the percentage inhibition of hybridization of newly synthesized labelled RNA to DNA in the absence and presence of excess of unlabelled rRNA.

Additions to the assay		
Cytosol protein (mg/ml)	α-Amanitin (μg/ml)	% rRNA
2.0	—	27
2.0	5.0	48
10.0	—	67
10.0	5.0	70

Table 2. *Some properties of the factor(s) enhancing RNA synthesis in isolated rat liver nuclei*

The conditions of fractionation on DEAE-cellulose are given in the text.

Fraction or treatment	RNA synthesized (% of control)
Control cytosol	100
Non-adsorbing DEAE-cellulose fraction	159
Adsorbing DEAE-cellulose fraction	64
Cytosol heated for 10min at 50°C*	136
Diaflo fraction <50000 mol.wt.*	139
Diaflo fraction >50000 mol.wt.*	72

* Activity based on protein concentration before heating and before Diaflo ultrafiltration.

rose from 48 to 70% in the presence of α-amanitin on increasing the cytosol protein concentration from 2.0 to 10.0mg/ml, and in the absence of the RNA polymerase II inhibitor, the increase due to cytosol was from 27 to 67%, an increase of approx. 250%.

The nature of the non-dialysable cytosol factor(s) which stimulates RNA synthesis was studied further. As indicated by the data in Table 2 the factor(s) is positively charged at pH7.5, since it appears in the flow-through fraction of the DEAE-cellulose column. It is also stable to heating at 50°C for 10min, and has an apparent molecular weight greater than 50000 as determined by Diaflo ultrafiltration.

Discussion

The RNA-synthetic system described in this paper presents certain unique features which distinguish it from other cell-free systems developed for the same purpose. Most significantly, it is a simple modification of a characterized system which supports the normal nuclear processing and transport of pre-formed rRNA and mRNA (Yu *et al.*, 1972; Schumm & Webb, 1973, 1974*a*,*b*; Schumm *et al.*, 1973*a*,*b*). The processing and release of these RNA species in this system has an obligatory requirement for both energy and cytosol. In contrast with other systems, it does not utilize $(NH_4)_2SO_4$ or high concentrations of other salts. Further, the integrity and functional stability of the isolated nuclei are maintained over a prolonged period of time at 30°C. Since this system has greatly facilitated the study of nucleocytoplasmic controls operating at the post-transcriptional nuclear level, it was of particular interest to extend the assay to the study of controls operating at the transcripitonal level by providing all four nucleoside triphosphates for RNA synthesis. The inclusion of cytosol in any cell-free system seems most essential if the system is to have any physiological significance (i.e. if nucleocytoplasmic controls are to remain operative). Further, it would seem essential that any system designed to study RNA synthesis in isolated nuclei should also exhibit normal RNA processing and transport, since the processes of nRNA transcription, processing and transport must be directly or indirectly coupled.

After an initial rapid rate of incorporation of label from UTP during the first 5min, the rate of RNA synthesis in this system falls to approx. 30% of the initial rate, but continues in a linear fashion for periods up to at least 1 h. It is proposed that the initial burst of incorporation represents chain completion, whereas the second phase represents both chain initiation and completion. The synthesis of RNA in this system exhibits a time- and temperature-dependence, is linearly proportional to the concentration of nuclei, requires the presence of all four nucleoside triphosphates and synthesizes both rRNA and poly(A)-containing α-amanitin-sensitive mRNA, both of high molecular weight.

As previously shown for RNA processing and transport (Schumm & Webb, 1973; Schumm *et al.*, 1973*a*,*b*; Schumm & Webb, 1974*a*,*b*; Yu *et al.*, 1972), RNA synthesis is also modulated by proteins in the cytosol. However, the latter process does not show that absolute dependence characteristic of the post-transcriptional processes, possibly because many of the regulatory macromolecules which influence transcription remain tightly bound to the DNA during the isolation of the nuclei. The regulatory macromolecules which modulate RNA synthesis appear to bear a positive charge, to be relatively heat-stable and to exhibit a molecular weight of over 50000. As such, the components do not belong to the class of acidic nuclear proteins, but rather are basic in character. Relatively heat-stable factors which are non-dialysable, not adsorbed to DEAE-cellulose and which stimulate unfractionated nRNA polymerases or RNA polymerase I from nucleoli, have been

isolated previously from cell extracts (Higashinakagawa *et al.*, 1972; Stein & Hausen, 1970). However, these factors appear to be somewhat different from the thermolabile components, which adsorb to DEAE-cellulose and which enhance rRNA processing and transport (J. Racevskis & T. E. Webb, unpublished work).

The synthesis of RNA molecules with sedimentation coefficients up to 80S have been observed in a cell-free system consisting of isolated nucleoli (Grummt & Lindigkeit, 1973). Their results suggest that either the nucleoli had lost some factors which *in vivo* cause RNA polymerase to terminate the RNA chains at the end of the 45S transcription unit, or that this reflects a normal transcription mechanism. Although the synthesis of transcripts with sedimentation coefficients greater than 45S were not observed in the nuclear system described in the present paper, it is clear that the significance of these differences will require further study.

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