Incorporation of Ribonucleic Acid *in vitro* into Dense Ribonucleoprotein-Like Materials by Isolated Rat Liver Nuclei

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A cell-free system of isolated rat liver nuclei is described which permits an active incorporation of newly synthesized RNA into 'dense' ribonucleoprotein-like materials. The reaction is stimulated with increasing amounts of cytosol protein isolated from rat liver. This indicates that cytosol protein plays an important role in the formation of such materials.

Several studies suggest that cytoplasm contains factors which *in vitro* stimulate RNA synthesis by nuclei under conditions of low ionic strength (Stein & Hausen, 1970; Wu & Zubay, 1974; McNamara *et al.*, 1975; Bastian, 1977). These systems may be appropriate to study nucleo-cytoplasmic controls operating at a post-transcriptional nuclear level. However, the stimulatory effect of cytosol factors on RNA synthesis still remains obscure. In a previous paper, we have demonstrated the incorporation *in vitro* of synthesized RNA into 30S ribonucleoprotein-like materials by isolated rat liver nuclei (Suzuka & Umemura, 1975). The reaction required at least two subcellular components, one of them being present in the cytosol fraction and the other in the nuclear-sap fraction. As an extension of that work, the present paper describes the role of cytosol protein in RNA synthesis by isolated rat liver nuclei. It was found that inclusion of cytosol protein in a nuclear RNA-synthesizing system resulted in an appreciable incorporation of synthesized RNA into a dense ribonucleoprotein-like material which penetrated a 2M-sucrose layer on density-gradient centrifugation.

Materials and Methods

Materials

The following materials were obtained as indicated: *Escherichia coli* RNA polymerase (EC 2.7.7.6) from Miles Laboratories (Kankakee, IL, U.S.A.); creatine kinase (EC 2.7.3.2), tRNA from *E. coli* and calf thymus DNA from Boehringer (Mannheim, West Germany); DNAase I (EC 3.1.4.5) and RNAase (EC 2.7.7.16) from bovine pancreas from Worthington Biochemical Corp. (Freehold, NJ, U.S.A.);

proteinase type IV from *Streptomyces griseus* from Sigma Chemical Co. (St. Louis, MO, U.S.A.); [³H]UTP (23 Ci/mmol) from The Radiochemical Centre (Amersham, Bucks., U.K.); Triton X-100 from Beckman (Palo Alto, CA, U.S.A.).

Preparation of nuclei

Male Wistar rats weighing 150–200 g were obtained commercially. All procedures were carried out at 0–4°C. The livers were homogenized in 2.0 vol. of 0.25M-sucrose in buffer A [50mM-triethanolamine/HCl/25mM-KCl/5mM-MgCl₂, pH 7.6] in a Potter-Elvehjem homogenizer with a Teflon pestle. The homogenate was filtered through two layers of cheesecloth and centrifuged for 10 min at 3°C and 1000g. The crude nuclear pellet was suspended in the same sucrose solution. Nuclei were further purified as described by Blobel & Potter (1966). Isolated nuclei were suspended in buffer B [50mM-triethanolamine / HCl / 4mM-MgCl₂ / 1mM-dithiothreitol/20% (v/v) glycerol, pH 8.0] adjusted to a concentration at 200 *A*₂₆₀ units of nuclear DNA/ml, and stored at –70°C until used.

Preparation of cytosol protein

Cytosol protein (1.25g of protein), which was obtained from the liver as described previously (Suzuka & Umemura, 1975), was further fractionated by chromatography on a column (2.5 cm × 18 cm) of DEAE-cellulose (Whatman DE 52). The column was exhaustively washed with 0.15M-KCl in buffer C [20mM-Tris/HCl/1mM-dithiothreitol/0.2mM-EDTA/10% (v/v) glycerol, pH 7.6], which removes much of the protein. All of the factor which stimulates the formation of dense ribonucleoprotein-like materials was then eluted with 0.35M-KCl in buffer C, but together with an RNAase inhibitor activity. To eliminate this inhibitor, the eluted fraction was fractionated by adding 100%-saturated

Abbreviations used: DNAase, deoxyribonuclease; RNAase, ribonuclease; SDS, sodium dodecyl sulphate.

(NH₄)₂SO₄ solution containing 0.1 mM-EDTA and 20 mM-Tris/HCl, pH 7.6, to give a 37% saturation of (NH₄)₂SO₄ (Gagnon & Lamirande, 1973). The precipitate (about 35 mg of protein) was dissolved in 3 ml of buffer C containing 40 mM-KCl, then dialysed overnight against 2 litres of the same buffer at 4°C and adjusted to a concentration of 10 mg of protein/ml. This fraction, which contained a negligible activity for inhibiting RNAase action (Fig. 4), was used as cytosol protein. Protein was determined by the method of Lowry *et al.* (1951), with crystalline bovine serum albumin as standard.

Reaction system for the formation of dense ribonucleoprotein-like materials and sucrose gradient analysis

The reaction for the incorporation of [³H]UMP into dense ribonucleoprotein-like materials was carried out in two steps as described previously (Suzuka & Umemura, 1975) but with the following modifications. The reaction mixture for the first step (RNA synthesis) contained the following, in a final volume of 50 µl: 40 mM-triethanolamine/HCl, pH 8.0, 80 mM-KCl, 2 mM-MnCl₂, 4 mM-MgCl₂, 3 mM-dithiothreitol, 10 mM-phosphocreatine, 2 mM-ATP, 1 mM-CTP and 1 mM-GTP. In addition it contained 5 µg of creatine kinase, 8 µg of cytosol protein, 0.6 unit (1 unit = 1 nmol of [³H]AMP incorporated/10 min at 37°C) of *E. coli* RNA polymerase, 1 *A*₂₆₀ unit of nuclei and 2 µCi of 0.1 mM-[³H]UTP. The mixture was incubated for 30 min at 32°C. For the second step, 50 µl of a solution containing 7.5 mM-triethanolamine/HCl, pH 8.0, 140 mM-NaCl, 5 mM-MgCl₂, 3.5 mM-dithiothreitol, 15% (v/v) glycerol and 20 µg of DNAase I was added to 50 µl of the mixture of the first step and the incubation continued for 20 min at 23°C. At the end of the reaction, 0.3 ml of 0.8 M-NaCl containing 18 mM-triethanolamine/HCl, pH 7.6, and 4.5 mM-MgCl₂ was added to the mixture at 0°C to give a final concentration of 0.6 M-NaCl. The mixture was immediately layered on 4 ml of a discontinuous sucrose gradient, which consisted of 1 ml each of 2.3 M- and 2 M-sucrose and 2 ml of 1 M-sucrose dissolved in buffer A. The tube was centrifuged at 84000g for 30 min in a Beckman SW 50.1 rotor at 3°C. After the centrifugation, six-drop fractions were collected from the bottom of the tube. Each fraction was precipitated with cold 10% (w/v) trichloroacetic acid containing 1% (w/v) Na₄P₂O₇. Each precipitate was collected on a Whatman GF/C glass filter and counted for radioactivity in toluene containing 0.5% (w/v) 2,5-diphenyloxazole in an Intertechnique liquid-scintillation spectrometer.

Reaction system for the formation of 30S ribonucleoprotein-like materials and sucrose gradient analysis

The incorporation of [³H]UMP into 30S ribonucleoprotein-like materials was carried out as described above, except that the mixture of the second

step contained nuclear sap fraction, which was isolated from 5 *A*₂₆₀ units of nuclei as described previously (Suzuka & Umemura, 1975). After the incubation, the mixture was centrifuged in 8 ml of 0.34–1.0 M-sucrose linear gradient in buffer A made on top of 2 ml of 2.3 M-sucrose cushion at 27000g for 18 h in a Beckman SW 41 rotor at 3°C. Six-drop fractions were collected from the bottom of the tube and the radioactivity was measured as described above.

CsCl-density-gradient analysis of the reaction mixture

For the analysis on CsCl density gradients, at the end of the incubation the mixture of the second step was centrifuged at 57000g for 15 min at 3°C. The pellet was suspended in 0.1 ml of 0.14 M-NaCl and mixed with 0.7 ml of 4.6% (v/v) formaldehyde in a buffer containing 10 mM-triethanolamine/HCl, pH 7.6, 1 mM-MgCl₂ and 0.14 M-NaCl at 0°C (Spirin, 1969). After 2 h, the mixture was dialysed against the same buffer containing 2% (v/v) formaldehyde for 20 h at 0°C. The fixed sample was layered on 4 ml of preformed CsCl gradient of densities of 1.3–1.6 g/cm³ prepared in a buffer containing 20 mM-triethanolamine/HCl, pH 7.6, and 2 mM-MgCl₂. After the sedimentation at 92700g for 46 h in a Beckman SW 65 rotor at 5°C, eight-drop fractions were collected from the bottom of the gradient and the radioactivity was measured as described above.

Assay for RNAase-inhibitory activity of cytosol protein

This was done by a method based on that of Shortman (1962). The reaction mixture contained, in a volume of 0.24 ml: 20 mM-Tris/HCl, pH 7.8, 40 mM-KCl, 1 mM-dithiothreitol and 0.2 mM-EDTA. In addition it contained 5.8 *A*₂₆₀ units of *E. coli* tRNA, 40 ng of RNAase and 200 µg of cytosol protein. The incubation was performed at 32°C. Samples (50 µl) of the reaction mixture were taken at various time intervals, then acidified with 1 ml of cold 4% (v/v) HClO₄, and 10 µl of 6 M-KOH was added to the mixture. The precipitate was discarded after 10 min spinning at 2000g, and the *A*₂₆₀ of the supernatant was taken as a measure of the RNA degradation.

Results and Discussion

Incorporation of synthesized RNA into dense materials in vitro

In the experiment shown in Fig. 1(a), the incorporation of [³H]UMP into dense materials was carried out in two steps; in the first step, RNA synthesis was performed in the presence of cytosol protein. In the second step, the mixture was treated with DNAase and a high concentration of NaCl was added to solubilize residual chromatin. Subsequently, the mixture was layered on the discontinuous sucrose gradients, which were centri-

Fig. 1. Effect of cytosol protein on the incorporation of synthesized RNA into dense ribonucleoprotein-like materials. The reaction and analysis were carried out as described in the Materials and Methods section. (a) Complete system. (b) Cytosol protein was omitted from the reaction mixture. (c) Cytosol protein was treated at 80°C for 3 min. (d) The reaction was done as in (a) except that cytosol protein was added at the end of the first step.

Fig. 2. Sedimentation profile of RNA synthesized in isolated nuclei

[³H]RNA was isolated from the reaction mixture by phenol deproteinization (Steele *et al.*, 1965) and

fuged and analysed for the distribution of radioactivity. It is clear that appreciable amounts of synthesized RNA sedimented at the position of the 2M-sucrose layer, where dense materials would collect. About 65% of the radioactivity incorporated into acid-insoluble form was located in the zone of 2M-sucrose. In contrast, as shown in Fig. 1(b), the omission of cytosol protein markedly diminished the incorporation of RNA into the dense materials. After heat treatment, this protein lost its ability to incorporate RNA into the materials (Fig. 1c). In the experiment shown in Fig. 1(d), RNA synthesis was done without cytosol protein, which was then added at the second step. This system decreased the incorporation of RNA to the same value as when the cytosol protein was omitted altogether. Thus the stimulatory effect of cytosol protein could not be demonstrated 30 min after the onset of RNA synthesis. These results showed that, under the conditions where the reaction system

layered over 4.7 ml of 10–30% (w/v) linear sucrose gradient in 10 mM-Tris/HCl (pH 8.0)/100 mM-NaCl/0.1 mM-EDTA/0.2% (w/v) SDS. The tube was centrifuged for 2.5 h at 171 000g in a Beckman SW 65 rotor at 23°C. After the sedimentation, three-drop fractions were collected from the bottom to the top and the radioactivity was measured as described in the Materials and Methods section. The arrows indicate the position of the peaks when rRNA and tRNA from rat liver ran on an identical gradient.

contained cytosol protein, an appreciable amount of synthesized RNA existed in the form of the 'dense' materials. Therefore, in addition to an apparent stimulation of nuclear transcription by the presence of cytosol protein (Stein & Hausen, 1970; Wu & Zubay, 1974; McNamara *et al.*, 1975; Bastian, 1977), cytosol protein which displays the activity in the described system seems to have an important function in the active incorporation of RNA into the dense materials. RNA synthesized

under the conditions described distributed heterogeneously in the sucrose gradient with *s* values ranging from 5 to 20S (Fig. 2).

Some properties of cytosol protein

A cell-free system for nuclear-directed transcription is capable of a relatively high and prolonged synthesis in the presence of cytoplasm (Wu & Zubay, 1974). In the experiment shown in Fig. 3, RNA synthesis and the incorporation of RNA into the dense materials were studied with various amounts of cytosol protein. Both reactions were stimulating by increasing amounts of cytosol protein, reaching a plateau at about $3\mu\text{g}$ of protein per reaction tube. It should be pointed out that at this concentration RNA synthesis was approximately doubled, whereas about a 4-fold stimulation of RNA incorporation into the dense materials

Fig. 3. Effect of concentration of cytosol protein on RNA synthesis and the formation of dense ribonucleoprotein-like materials

The reaction and analysis were carried out as described in the Materials and Methods section, except that cytosol protein was added as indicated in the Figure. ○, Total amounts of synthesized RNA; ●, amounts of RNA incorporated into dense ribonucleoprotein-like materials. For point Δ, nuclei ($1 A_{260}$ unit) were preincubated with $8\mu\text{g}$ of cytosol protein in 0.3 ml of buffer B at 32°C for 10 min, then centrifuged and suspended in the first-step mixture from which cytosol protein was omitted.

Fig. 4. Effect of cytosol protein on RNAase activity. The assay for RNAase activity was performed as described in the Materials and Methods section. ○, Cytosol protein absent; ●, cytosol protein present; Δ, the reaction mixture contained RNAase inhibitor, which was isolated from post-ribosomal supernatant of rat liver by the procedure of Shortman (1962).

was observed. A possible explanation is that this non-parallelism would indicate at least two effects of cytosol protein, one of them possibly on RNA synthesis and the other on the formation of the dense materials. Additional evidence is, however, necessary to elucidate these effects by further purification of cytosol protein. In the experiment shown by Δ in Fig. 3, nuclei were preincubated with cytosol protein before RNA synthesis, then sedimented for removal of this protein, and the reaction was initiated without the protein. Although an appreciable decrease occurred, this system partially retained the ability to make the dense materials, despite the removal of a major portion of cytosol protein. This may reflect a high affinity of cytosol protein for nuclei or nuclear components.

Since cytosol fraction has an active RNAase-inhibitory action (Shortman, 1962), a possibility existed that the stimulatory effect was caused by inhibition of latent RNAase in the preparations. As shown in Fig. 4, cytosol protein isolated under the conditions described had very little effect in inhibiting RNAase action, suggesting that the reason for the effects of cytosol protein was not the inhibition of RNAase.

Some characteristics of dense materials carrying synthesized RNA

The dense materials carrying [^3H]RNA were susceptible to degradation by proteinase and RNAase (results not shown). To confirm further the characteristics of the materials, the reaction mixture was fixed with formaldehyde and examined by equilibrium density-gradient centrifugation in CsCl. Fig. 5 shows the presence of radioactive peaks with buoyant densities of 1.356 g/cm³ and 1.371 g/cm³. According to the estimation of protein content from the empirical formula [protein % = 1.85 - (ρ /0.006); Spirin, 1969], the major peak contained more than 80% protein. These characteristics seem to be similar to those of nuclear ribonucleoprotein particles (Samarina *et al.*, 1968) or ribonucleoprotein network complexes (Faiferman & Pogo, 1975). Thus the 'dense' materials will be referred to as a dense or large ribonucleoprotein-like material. There was no appreciable change in sedimentation behaviour of the dense ribonucleoprotein-like materials after treatment with Triton X-100, which dissolves lipids (results not shown). This suggests that the materials are not directly associated with nuclear membranes.

Lack of formation of dense ribonucleoprotein-like materials by a purified DNA-dependent RNA-synthesizing system

A possibility existed that the effect of cytosol protein was due to its binding to synthesized RNA. To examine this possibility, nuclei were replaced

Fig. 5. CsCl-density-gradient analysis of the reaction mixture

The reaction and CsCl gradient centrifugation were as described in the Materials and Methods section. ○, Radioactivity; —, density.

with purified DNA as template and RNA synthesis proceeded with cytosol protein. In spite of an active RNA synthesis, this system had no ability to make the dense ribonucleoprotein-like materials. This indicates that the stimulatory effect of cytosol protein is not due to an artificial binding between the protein and RNA. In view of the current notion that the nuclear protein matrix participates in the transcription and transport of RNA (Busch & Smetana, 1970; Berezney & Coffey, 1976), this inability appears to reflect the structural requirement of the protein matrix in the nucleus to form the dense ribonucleoprotein-like material.

Effect of nuclear-sap fraction on the formation of the dense ribonucleoprotein-like materials

In our previous paper (Suzuka & Umemura, 1975), the addition of nuclear-sap fraction to nuclear RNA-synthesizing system containing cytosol protein produced an incorporation of [^3H]UMP into 30S ribonucleoprotein-like materials. Fig. 6 shows the sedimentation behaviour of the reaction mixture when nuclear-sap fraction was added 30 min after the onset of RNA synthesis and the second step continued. When nuclear-sap fraction was present, there was a marked incorporation of RNA at the position to which 30S particles would sediment, whereas no such incorporation in the control system was observed. This observation confirms our previous report (Suzuka & Umemura, 1975). In addition,

Fig. 6. Effect of nuclear-sap fraction on the formation of dense ribonucleoprotein-like materials

The reaction and analysis were done as described in the Materials and Methods section. The arrows indicate the position of the peaks when rRNA from rat liver ran on an identical gradient. ○, Control system; ●, nuclear-sap fraction added.

compared with the control, the presence of nuclear-sap fraction appreciably decreased the radioactivity at the position where the dense materials would sediment. This decrease may indicate that the nuclear-sap fraction caused a release of a 'light' material from the dense ribonucleoprotein-like materials.

The present study was an attempt to elucidate the question of the role of cytosol protein on RNA synthesis in a nuclear system. From the present findings, one can conclude that when RNA synthesis proceeded with cytosol protein, an appreciable amount of synthesized RNA was present as a 'dense' ribonucleoprotein-like material. An interesting point of this system is that cytosol protein stimulated the formation of the dense ribonucleoprotein-like material as well as RNA synthesis. This conclusion may support the concept that the movement of cytoplasmic proteins into nuclei induces enlargement and initiation of DNA and RNA synthesis (Merriam, 1969). It appears that cytosol proteins in conjunction with the protein matrix of nuclei are involved in the regulation and/or modulation of nucleo-cytoplasmic communication (Berezney & Coffey, 1976; Bastian, 1977). In this respect, the present system may be a useful system to study a linkage between nuclei and cytoplasm in the processes of RNA transcription, processing and transport.

References

- Bastian, C. (1977) *Biochem. Biophys. Res. Commun.* **74**, 1109-1115
- Berezney, R. & Coffey, D. S. (1976) *Adv. Enzyme Regul.* **14**, 63-100
- Blobel, G. & Potter, V. R. (1966) *Science* **154**, 1162-1165
- Busch, H. & Smetana, K. (1970) in *The Nucleus* (Busch, H. & Smetana, K., eds.), pp. 361-379, Academic Press, New York
- Faiferman, I. & Pogo, A. O. (1975) *Biochemistry* **14**, 3808-3816
- Gagnon, G. & de Lamirande, G. (1973) *Biochem. Biophys. Res. Commun.* **51**, 580-586
- Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951) *J. Biol. Chem.* **193**, 265-275
- McNamara, D. J., Racevskis, J., Schumm, D. E. & Webb, T. E. (1975) *Biochem. J.* **147**, 193-197
- Merriam, R. W. (1969) *J. Cell Sci.* **5**, 333-349
- Samarina, O. P., Lukanidin, F. M., Molner, J. & Georgiev, G. P. (1968) *J. Mol. Biol.* **33**, 251-263
- Shortman, K. (1962) *Biochim. Biophys. Acta* **55**, 88-96
- Spirin, A. S. (1969) *Eur. J. Biochem.* **10**, 20-35
- Steele, W. J., Okamura, N. & Busch, H. (1965) *J. Biol. Chem.* **240**, 1742-1749
- Stein, H. & Hausen, P. (1970) *Eur. J. Biochem.* **14**, 270-277
- Suzuka, I. & Umemura, Y. (1975) *FEBS Lett.* **58**, 130-133
- Wu, G.-J. & Zubay, G. (1974) *Proc. Natl. Acad. Sci. U.S.A.* **71**, 1803-1807