Stimulation *in vitro* of a heparin-resistant RNA polymerase I transcription complex

Mari K. HADDOX* and Diane Haddock RUSSELL†

Department of Pharmacology, University of Arizona Health Sciences Center, Tucson, AZ 85724, U.S.A.

(Received 13 February 1981/Accepted 6 April 1981)

A soluble factor partially purified from calf liver increases transcription by RNA polymerase I in isolated nuclei. Addition of the factor to reactions which have reached a plateau owing to the inability to reinitiate on the endogenous chromatin template restores the initial rate of synthesis and stimulates an increased accumulation of RNA product. The RNA synthesis stimulated by factor addition is identical with that initiated *in vivo* in that it is resistant to heparin disruption.

The synthesis of RNA by RNA polymerase I is subject to rapid regulation, which is believed to be imposed at the initiation of transcription rather than at other stages of the polymerization reaction (Cedar & Felsenfeld, 1973). Inhibitor studies have shown that RNA synthesis is closely linked to continuous protein synthesis (Willems *et al.*, 1969; Craig & Perry, 1970; Muramatsu *et al.*, 1970; Yu & Feigelson, 1971; Franze-Fernández & Fontanive-Sengüesa, 1973; Gross & Pogo, 1974). A rapid decrease in rRNA synthesis follows the addition of protein-synthesis inhibitors to intact cells as the result of a decreased number of RNA polymerase I molecules engaged on the ribosomal cistrons (Lampert & Feigelson, 1974; Chesterton *et al.*, 1975). These findings have suggested that transcription by RNA polymerase I is regulated by a short-lived protein which serves to initiate the enzyme on the chromatin template. Several factors have been isolated that have stimulatory effects on the ability of purified RNA polymerase I to transcribe DNA (Rutter *et al.*, 1974; Chambon, 1975; Roeder, 1976); however, none of these factors appears to function as the putative labile initiation factor.

Previous studies in our laboratory (Manen & Russell, 1977a,b) have indicated that partially purified preparations of ornithine decarboxylase from rat liver are highly enriched in a factor which stimulates RNA polymerase I activity. In the present studies, we have utilized the polyanion heparin, an inhibitor of non-specific initiation (Ferencz & Seifart, 1975), to characterize a similar component isolated from calf liver (Haddox & Russell, 1978).

* Present address: University of Texas Medical School, Division of Endocrinology, P.O. Box 20708, Houston, TX 77025, U.S.A.

† To whom reprint requests should be addressed.

The transcription complex between the endogenous RNA polymerase I and chromatin promoted in isolated nuclei by this factor is identical with that formed *in vivo* in that it is resistant to heparin disruption.

Methods

Nuclei were isolated from calf liver by a modification (Goldberg *et al.*, 1977) of the methods of Blobel & Potter (1966) and Higashinakagawa *et al.* (1972). RNA polymerase activity was assayed (Goldberg *et al.*, 1977) at 30°C after preincubation of the nuclei for 2 min at 30°C with 50 mM-Tris/HCl, pH 8.4, in the presence or absence of the indicated concentrations of α -amanitin, heparin and/or stimulatory factor. The factor was purified from the liver of a Holstein calf (6 weeks old, male, 50 kg) injected intraperitoneally with 3-isobutyl-1-methylxanthine (10 mg/kg) 4 h before being killed. This methylxanthine derivative results in a 50–100-fold induction of ornithine decarboxylase within 4 h. Frozen liver (150 g) was powdered under liquid N₂, homogenized in 3 vol. of purification buffer (20 mM-sodium/potassium phosphate, pH 7.2, 0.1 mM-EDTA, 0.1 mM-dithiothreitol, 5 mM-NaF, 0.5 mM-phenylmethanesulphonyl fluoride, 0.1 mM-pyridoxal phosphate) and centrifuged at 40 000 g for 30 min. The supernatant fluid was mixed with 40 ml of aminohexyl-Sepharose (Pharmacia, equilibrated with purification buffer) for 30 min at 4°C. The resin was washed with 4 vol. of purification buffer and layered on top of 20 ml of additional resin previously settled in a 2 cm $\times$ 30 cm column. The column was eluted with a linear gradient of 0.025–0.6 M-NaCl in purification buffer (total volume 400 ml). Fractions eluted between 0.2 and 0.28 M-NaCl were combined

and dialysed against 100 vol. of purification buffer for 6 h at 4°C. The dialysed material was incubated for 30 min with 50 μ M-cyclic AMP and then mixed with 20 ml of DEAE-Sephacel (Pharmacia, equilibrated with purification buffer containing 25 mM-NaCl) for 30 min at 4°C. The resin was washed with 4 vol. of equilibration buffer and layered on top of 10 ml of additional resin previously settled in a 1.5 cm $\times$ 30 cm column. The column was eluted with a linear gradient of 0.025–0.6 M-NaCl in purification buffer (total volume 200 ml). Fractions eluted between 0.175 and 0.25 M-NaCl were combined, dialysed against 100 vol. of purification buffer for 6 h at 4°C, and stored at –80°C. Assuming minimal losses during the initial purification step, the factor was purified 250-fold by this procedure to a specific activity such that 1 mg of the factor preparation stimulated the incorporation by endogenous nuclear polymerase of 1 pmol of [3 H]UTP (measured at 2 min). The factor preparation possessed no polymerase activity itself as measured with exogenous DNA template.

Results and discussion

Fig. 1(a) shows the time course of RNA synthesis in isolated nuclei. Calf liver nuclei were incubated in the presence of high α -amanitin concentration

(0.4 mg/ml) as a measure of RNA polymerase I activity, at low α -amanitin concentration (0.6 μ g/ml) as a measure of RNA polymerase I and III activities, and under control conditions as a measure of the total of all RNA polymerase activity. Since RNA polymerase I constituted over 80% of the synthetic activity detectable on incubation with low α -amanitin, the lower amount of this toxin was used in most of the subsequent studies. RNA synthesis catalysed by RNA polymerase I in isolated calf nuclei proceeds for only a few minutes and then reaches a plateau (Fig. 1a). The amount of UTP remaining available as substrate at the end of the incubation was measured to ensure that the halt in polymerase activity was not due to substrate limitation. Formic acid-terminated reaction mixtures were spotted on polyethyleneimine thin-layer sheets and developed in 0.5 M-LiCl in 2 M-formic acid to separate UTP from all other uridine nucleotides and nucleosides. Some 80% of the initial UTP remained at a time when the polymerase assay had reached the plateau, indicating that the cessation of synthesis was not the result of substrate limitation. Continued incubation for an additional 10 min after the addition of a 50-fold excess of unlabelled UTP showed no decrease in radioactivity incorporation, indicating that ribonuclease activity was not primarily responsible for the plateau. Furthermore, addition of

Fig. 1. Time-dependence of nuclear RNA synthesis

(a) Nuclei were assayed for RNA polymerase activity at 30°C for the times indicated after preincubation for 2 min at 30°C with 50 min-Tris/HCl, pH 8.4, in the absence (●) or presence of α -amanitin (0.6 μ g/ml, ○; 0.4 mg/ml, ■). (b) RNA synthesis was assayed for the times indicated in the presence of α -amanitin (0.6 μ g/ml) either after a 2 min preincubation with 1 ml of the calf liver factor (IF, ○) or purification buffer (●) or after the addition of 1 mg of the factor after the polymerase reaction has proceeded for 10 min (□). * $P < 0.05$ (Student's t test) for difference from control (●).

exogenous DNA after 10 min of incubation when the endogenous synthesis had reached the plateau resulted in renewed incorporation of [^3H]UTP into RNA, indicating that the polymerase itself was still active and competent to transcribe a DNA template (results not shown). Therefore it appears that in the isolated calf nuclear preparation, as has been previously reported for other eukaryotic nuclear and nucleolar preparations (Grummt & Lindigkeit, 1973; McReynolds & Penman, 1974; Ferencz & Siefert, 1975; Udvardy & Seifart, 1976), only those RNA chains that were initiated *in vivo* are transcribed. Reinitiation *in vitro* is absent or very limited, owing to the lack of the putative labile initiation factor.

A preparation of ornithine decarboxylase partially purified (1145 pmol/min per mg of protein) from calf liver by hydrophobic and anion-exchange chromatography was tested for the presence of a transcription-stimulatory factor similar to that previously reported for rat liver preparations (Manen & Russell, 1977a,b). As shown in Fig. 1(b), addition of the preparation at zero time to nuclei stimulates both the initial rate of RNA polymerase I activity and an increased formation of total RNA product, suggesting that the presence of the factor resulted in an increase in the number of RNA chains polymerized. This occurred in the presence of high concentrations of α -amanitin, which implicates only RNA

polymerase I activity. The stability of the increased RNA synthesized after the addition of the factor was examined by adding a 50-fold excess of unlabelled UTP 10 min after the initiation of the polymerase reaction. The factor-stimulated incorporation had a similar stability to the control incorporation during the 10 min chase with unlabelled UTP. Therefore the increase in the RNA synthesized after factor addition is not the result of apparent changes in the turnover rate of RNA. The enhanced transcription detectable after factor addition is similar to endogenous synthesis, since a plateau is reached within about 10 min of incubation. Thus the stimulatory property of the factor is labile. That the halt in synthesis seen after 10 min is the result of loss of initiating activity and not because of any loss of integrity of the endogenous RNA polymerase or chromatin template is confirmed by the finding that factor addition will reinitiate transcription. As shown in Fig. 1(b), the addition of the factor to the reaction at plateau restores the initial rate of synthesis and stimulates an increased accumulation of RNA product.

Total RNA polymerase I present in nuclear preparations can be assessed *in vitro* by incubation with exogenous substrate. Addition of poly[d(A-T)] to a nuclear incubation mixture results in a 250% increase in the rate of incorporation of UTP into RNA (Fig. 2a). The increased activity represents

Fig. 2. Heparin resistance of the enhanced RNA synthesis promoted by the calf liver factor

Nuclei were assayed for polymerase activity in the presence of α -amanitin (0.6 $\mu\text{g}/\text{ml}$) for the times indicated in the absence (a) or presence (b) of heparin (130 units/ml). Nuclei were preincubated for 2 min at 30°C with 1 ml of the calf liver factor (IF, O) or purification buffer (control, ●) before the initiation of the polymerase reaction by the addition of the nucleoside triphosphates containing poly[d(A-T)] (40 $\mu\text{g}/\text{ml}$) where indicated (■). Heparin was always added with the nucleoside triphosphates, * $P < 0.05$ for difference from control.

transcription of the synthetic template by the non-engaged polymerase molecules present in the nucleus (Ferencz & Seifart, 1975; Grummt *et al.*, 1976). This interaction of the polymerase with the added deoxynucleotide is non-specific and not similar to the transcription complexes formed *in vivo* in that it can be disrupted by heparin. Fig. 2(b) shows that there is no increase in RNA synthesis when poly[d(A-T)] is added to the nuclei in the presence of the polyanion. Heparin is a potent template competitor for RNA polymerase molecules which are attached to DNA non-specifically (Cox, 1973; Ferencz & Seifart, 1975; Udvardy & Seifart, 1976). It has been shown to inhibit RNA polymerase interaction with nuclear chromatin, DNA, isolated homologous chromatin and synthetic deoxynucleotide templates. Only enzyme actually present in active transcription complexes initiated *in vivo* and elongating these chains *in vitro* has been found to be fully resistant to heparin (Ferencz & Seifart, 1975). As shown in Fig. 2, the endogenous rRNA synthesis in calf liver nuclei is similar in the presence or absence of heparin. The increased RNA synthesis stimulated by the addition of the factor is identical with that initiated *in vivo* in that it occurs to the same extent in the presence or absence of the polyanion (Fig. 2).

These findings demonstrate that a factor that stimulates RNA polymerase I, similar to that previously isolated from rat liver (Manen & Russell, 1977a,b), is present in partially purified preparations of ornithine decarboxylase from bovine liver. The characteristics of the enhanced incorporation demonstrable after addition of the factor to nuclei suggest that the increased RNA synthesis is a result of increased interaction of RNA polymerase I with chromatin. Furthermore, the heparin-resistant nature of the factor-promoted stimulation suggests that the partially purified factor preparation may contain the labile protein believed to regulate rRNA synthesis *in vivo*.

This work was supported by U.S. Public Health Service Research Grant CA-14783 from the National Cancer Institute to D. H. R.

References

- Blobel, G. & Potter, V. R. (1966) *Science* **154**, 1662–1665
- Cedar, H. & Felsenfeld, G. (1973) *J. Mol. Biol.* **77**, 237–254
- Chambon, P. (1975) *Annu. Rev. Biochem.* **44**, 613–638
- Chesterton, D. J., Coupar, B. E. H., Butterworth, P. H. W., Buss, J. & Green, M. H. (1975) *Eur. J. Biochem.* **57**, 79–83
- Cox, R. (1973) *Eur. J. Biochem.* **39**, 49–61
- Craig, N. C. & Perry, R. P. (1970) *J. Cell Biol.* **45**, 554–564
- Ferencz, A. & Seifart, K. H. (1975) *Eur. J. Biochem.* **53**, 605–613
- Franze-Fernández, M. & Fontanive-Sengüesa, A. (1973) *Biochim. Biophys. Acta* **331**, 71–80
- Goldberg, M. I., Perriard, J. C. & Rutter, W. J. (1977) *Biochemistry* **16**, 1655–1665
- Gross, K. J. & Pogo, A. O. (1974) *J. Biol. Chem.* **249**, 568–576
- Grummt, I. & Lindigkeit, R. (1973) *Eur. J. Biochem.* **36**, 244–249
- Grummt, I., Smith, V. A. & Grummt, F. (1976) *Cell* **7**, 439–445
- Haddox, M. K. & Russell, D. H. (1978) *J. Supramol. Struct.* **52**, 317
- Higashinakagawa, T., Muramatsu, M. & Sugano, H. (1972) *Exp. Cell Res.* **71**, 65–74
- Lampert, A. & Feigelson, P. (1974) *Biochem. Biophys. Res. Commun.* **58**, 1030–1038
- Manen, C. A. & Russell, D. H. (1977a) *Science* **195**, 505–506
- Manen, C. A. & Russell, D. H. (1977b) *Biochem. Pharmacol.* **26**, 2379–2384
- McReynolds, L. & Penman, S. (1974) *Cell* **3**, 185–188
- Muramatsu, M., Shimada, N. & Higashinakagawa, T. J. (1970) *J. Mol. Biol.* **53**, 91–106
- Roeder, R. G. (1976) in *RNA Polymerase* (Losick, R. & Chamberlin, M., eds.), pp. 285–329, Cold Spring Harbour Laboratory, Cold Spring Harbor
- Rutter, W. J., Goldberg, M. E. & Perriard, J. C. (1974) in *Biochemistry of Cell Differentiation* (Paul, J., ed.), pp. 267–300, University Park Press, Baltimore
- Udvardy, A. & Seifart, K. H. (1976) *Eur. J. Biochem.* **62**, 353–363
- Willems, M., Penman, M. & Penman, S. (1969) *J. Cell Biol.* **41**, 177–187
- Yu, F. L. & Feigelson, P. (1971) *Proc. Natl. Acad. Sci. U.S.A.* **68**, 2177–2180