Purification and Properties of a Nuclear Protein Kinase associated with Ribonucleic Acid Polymerase I

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A cyclic AMP-dependent nuclear protein kinase was found to be closely associated with rat liver nucleolar RNA polymerase I throughout most of its purification. This protein kinase was purified to near homogeneity. It exhibits a number of unusual catalytic properties, including the inability to utilize Mn^{2+} when RNA polymerase is the substrate and the ability to phosphorylate both acidic and basic substrates. Phosphorylation of RNA polymerase I by this protein kinase results in the formation of phosphoester bonds characteristic of phosphoserine and phosphothreonine. Radioautography of polyacrylamide-gel electrophoretograms of the phosphorylated RNA polymerase I revealed that the ^{32}P was located primarily on enzyme subunits SA_1 , SA_3 , SA_5 and SA_6 [nomenclature of Kedinger, Gissinger & Chambon (1974) *Eur. J. Biochem.* **44**, 421–436].

There have been many reports describing the effects of nuclear and cytoplasmic protein kinases on mammalian RNA polymerases (Hirsch & Martelo, 1976a; Martelo & Hirsch, 1974; Jungman *et al.*, 1974). Both stimulation of RNA synthesis and phosphorylation of RNA polymerases have been described. We have shown that covalent phosphorylation of RNA polymerase I occurred when intact rat liver nuclei were incubated with [γ - ^{32}P]ATP (Hirsch & Martelo, 1976b). This phosphorylation appears to be mediated by a nuclear protein kinase that co-purified closely with RNA polymerase I throughout most of its purification. This nuclear protein kinase, separated from RNA polymerase I by sucrose-density-gradient ultracentrifugation, exhibited several unusual catalytic properties that are the subject of the present paper.

Materials and Methods

Chemicals

[5,6- 3H]UTP (20 Ci/mmol) and [γ - ^{32}P]ATP (>10 Ci/mmol) were purchased from New England Nuclear Corp. (Boston, MA, U.S.A.). Unlabelled nucleotides were purchased from P-L Biochemicals (Milwaukee, WI, U.S.A.). Ultrapure enzyme-grade $(NH_4)_2SO_4$ and ribonuclease-free sucrose were products of Schwartz–Mann (Orangeburg, NY, U.S.A.). Calf

thymus DNA, phosvitin, phenylmethanesulphonyl fluoride and dibutyl cyclic AMP were products of Sigma Chemical Co. (St. Louis, MO, U.S.A.). The livers used as the source of nuclei for these experiments were obtained from female Sprague–Dawley rats.

Enzyme purification

Purification of rat liver RNA polymerase I was performed as previously described (Hirsch & Martelo, 1976b). Briefly, the procedure is as follows. The rat liver nuclear extract was prepared by the method of Schwartz *et al.* (1974). The extract was subjected to successive column chromatography on Sepharose 4B, DEAE-Sephadex, Sepharose 6B and phosphocellulose columns as described previously (Hirsch & Martelo, 1976b). Fractions from the phosphocellulose column containing RNA polymerase I activity also contained protein kinase activity, which was able to phosphorylate endogenous proteins. At this point it was not possible to determine whether RNA polymerase I itself or some other protein contaminant was the phosphate acceptor.

The RNA polymerase I/protein kinase mixture from the phosphocellulose column was precipitated with 2 vol. of satd. $(NH_4)_2SO_4$ and the precipitate was recovered by centrifugation (15000g, 20 min), suspended in 0.4 ml of 50 mM-Tris/HCl buffer, pH 7.9, 0.5 mM-EDTA, 1 mM-dithiothreitol, 1 mM-phenylmethanesulphonyl fluoride. Separation of RNA polymerase I from the nuclear protein kinase was carried out by ultracentrifugation in a linear 5–20% sucrose gradient containing 0.1 M- $(NH_4)_2SO_4$ and 0.1 mM-phenylmethanesulphonyl

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fluoride. Sucrose density centrifugation was for 20h at 140000g in the no. 40 rotor of a Beckman L-2 ultracentrifuge at 4°C.

Enzyme assays

The protein kinase was assayed with phosvitin, casein or RNA polymerase I as phosphate acceptors. The reaction mixture (0.2ml) contained: 50mM-Tris/HCl (pH 7.2), 0.1mM-ATP, 2mM-MgCl₂, 10mM-NaF, 1mM-dithiothreitol, 10mM-NaCl, 5 μ M- $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ and protein kinase (20 μ g/ml). When used as phosphate acceptors, the concentration in the reaction mixture of phosvitin, casein and histone was 2mg/ml. For RNA polymerase I the concentration was 20 μ g/ml. Samples were incubated for 20min at 37°C, and the ^{32}P radioactivity in trichloroacetic acid-precipitable protein was determined as previously described (Hirsch & Martelo, 1976b).

The RNA polymerase I activity was assayed in a reaction mixture (40 μ l) containing: 40mM-Tris/HCl, pH 7.9, 0.7mM-ATP, -GTP and -CTP, 2.5mM-MnCl₂, 4.5 μ M- $[\text{H}^3]\text{UTP}$ (1 μ Ci), 80mM-(NH₄)₂SO₄ and 2mg of heat-denatured calf thymus DNA. $[\text{H}^3]\text{UMP}$ incorporated into trichloroacetic acid-precipitable material was determined as previously described (Hirsch & Martelo, 1976b). RNA polymerase I activity was identified by lack of inhibition by α -amanitin at a concentration of 5 μ g/ml (Hirsch & Martelo, 1976b).

Gel electrophoresis and stability

Polyacrylamide-gel electrophoresis under non-denaturing and denaturing conditions was performed as previously described (Hirsch & Martelo, 1976b; Martelo *et al.*, 1974).

Radioautography of stained polyacrylamide gels in 0.1% sodium dodecyl sulphate was carried out by slicing gels with an Ames gel slicer, embedding them in a sheet of Whatman no. 3MM filter paper under heat and vacuum, and exposing to Kodak RP-X-Omat X-ray film as previously described (Hirsch & Martelo, 1976b; Martelo *et al.*, 1974).

The acid- and base-stability of the phosphoester bonds generated by the protein kinase was determined as previously described (Fischer *et al.*, 1959). Each assay contained 200 μ g of ^{32}P -labelled RNA polymerase I. This was prepared by incubating the RNA polymerase I from the phosphocellulose column (containing protein kinase) under conditions optimal for protein kinase activity. After separation of RNA polymerase I from the contaminating protein kinase, the ^{32}P -labelled RNA polymerase I was adjusted to 0.25M-HCl or 0.25M-NaOH.

The experiments and purification procedures described in the present paper were performed at least three times and all were highly reproducible.

Results

The separation of RNA polymerase I from the associated nuclear protein kinase in fractions from the phosphocellulose column is shown in Fig. 1. Although there was some overlap of both enzyme activities in fractions 18–22, fractions 14 and 30 contained RNA polymerase I activity and nuclear protein kinase activity exclusively.

Fig. 2 shows a 5%-(w/v)-polyacrylamide-gel electrophoretogram of a sample from fractions containing RNA polymerase I activity from the last column during the purification of RNA polymerase I. Two protein bands are easily seen, the top one corresponding to RNA polymerase I and the bottom one to the nuclear protein kinase. A diagram of the gel is also shown to facilitate identification of the protein bands. Protein kinase activity, but not RNA polymerase I, can be eluted from non-stained gels (results not shown). Thus it appears that the RNA polymerase I preparation from the phosphocellulose column contained a protein kinase as its only visible contaminant at this point in the purification.

Fig. 3 shows a 5%-polyacrylamide-gel electrophoretogram, and corresponding diagram, of the

Fig. 1. Separation of the protein kinase from rat liver RNA polymerase I

The RNA polymerase I/nuclear protein kinase mixture from the phosphocellulose column was prepared as described in the Materials and Methods section. It was then subjected to sucrose-density-gradient centrifugation on a 5–20% (w/v) linear sucrose gradient containing 0.1M-(NH₄)₂SO₄ and 0.1mM-phenylmethanesulphonyl fluoride. Fractions (0.2ml) were collected, and RNA polymerase (○) was assayed as described previously (Martelo & Hirsch, 1974). Protein kinase (●) was assayed in 10 μ l reaction mixtures as described in the Materials and Methods section.

Fig. 2. Polyacrylamide-gel electrophoresis of RNA polymerase I under non-denaturing conditions

A sample of 50 μ g of protein from the phosphocellulose column fraction was applied to the gel. Electrophoresis was for 6 h at 1 mA/gel at 4°C. The gel was stained with Coomassie Blue.

Fig. 3. Polyacrylamide-gel electrophoresis of purified nuclear protein kinase, after sucrose-density-gradient centrifugation, under non-denaturing conditions

A sample of 50 μ l of fraction 30 from the density gradient, as in Fig. 1, was applied to the gel. Electrophoresis and staining was performed as described in the legend to Fig. 2.

nuclear protein kinase (fraction 30 of the sucrose-density gradients).

Fig. 4(a) shows the time course of phosphorylation of RNA polymerase I by the associated protein kinase. Fractions from the phosphocellulose column giving only two protein bands in gel electrophoresis were used for these experiments. The decrease in incorporation after 10 min of incubation at 37°C probably represents a phosphatase activity, since it was not seen in the presence of 10 mM-NaF. Therefore NaF (10 mM) was included in the reaction mixture of experiments used in Figs. 4(b), 4(c) and 4(d). However, a protein band that could correspond to a phosphatase was not visible in the non-denaturing polyacrylamide gels. Linear kinetics were obtained by including cyclic AMP in the reaction mixture, but the nucleotide did not stimulate phosphorylation at concentrations ranging from 1 nM to 1 mM. The linear kinetics obtained by adding cyclic AMP may be explained by inhibition of a contaminating phosphatase by cyclic AMP (Chelala & Tarres, 1970), but further studies are required to prove this hypothesis.

Fig. 4(b) depicts the phosphorylation of proteins in the phosphocellulose fractions by nuclear protein kinase. The marked non-linearity of the curve suggested that more than one protein in the reaction mixture was a phosphate acceptor or the presence of one protein receptor with a concentration below the K_m .

Fig. 4(c) shows the phosphorylation of phosvitin by nuclear protein kinase purified through the sucrose-density step as a function of the bivalent-cation

concentration. The optimum concentration for Mg^{2+} , Mn^{2+} and Co^{2+} was 2 mM under these conditions; greater concentrations of Mn^{2+} and Co^{2+} inhibited phosvitin phosphorylation.

Fig. 4(d) depicts RNA polymerase I phosphorylation; identical results were obtained by using enzyme from the phosphocellulose fraction or sucrose-density-gradient fraction. The optimal concentration of Mg^{2+} and Co^{2+} was approx. 2 mM, but Mn^{2+} at all concentrations inhibited RNA polymerase phosphorylation.

The phosphorylation of other substrates by the kinase was also determined (results not shown). Along with RNA polymerase I and phosvitin, the nuclear protein kinase also phosphorylated histone (calf thymus type II) and casein. The substrates were phosphorylated in the order: phosvitin > polymerase > histone > casein.

Table 1 shows the acid- and base-stability of the phosphoester bonds in ^{32}P -labelled RNA polymerase I phosphorylated by the nuclear protein kinase after the sucrose-density-gradient step. Hydrolysis in NaOH was complete by 20 h at 37°C. This is characteristic of phosphoserine and phosphothreonine bonds (Fischer *et al.*, 1959). We have previously shown (Hirsch & Martelo, 1976b; Martelo *et al.*, 1974) that phosphoserine and phosphothreonine bonds in phosphorylated RNA polymerases are labile in 0.25 M-NaOH and stable in 0.25 M-HCl.

Fig. 4. Catalytic properties of the nuclear protein kinase

(a) Time course of phosphorylation of RNA polymerase I (20 µg/ml) by the associated protein kinase. ●, Control; ○, plus 1 µM-cyclic AMP; △, plus 10 mM-NaF; □, minus Mg^{2+} . (b) Protein kinase activity as a function of protein concentration. Phosphocellulose column fractions were used in this experiment. (c) Phosphorylation of phosphocellulose column fractions and (d) phosphorylation of RNA polymerase I (20 µg/ml) by nuclear protein kinase (20 µg/ml) as a function of cation concentration: ●, Mg^{2+} ; ○, Mn^{2+} ; △, Co^{2+} .

Table 1. Acid- and base-stability of phosphoester bonds in RNA polymerase I phosphorylated by the nuclear protein kinase

Samples (200 µg) of ^{32}P -labelled RNA polymerase I, labelled by the nuclear protein kinase from the sucrose-density step, were incubated at 37°C for the times indicated, in 1 ml reaction mixtures in either 0.25M-HCl or 0.25M-NaOH. The percentage hydrolysis was determined as described in the Materials and Methods section.

Time at 37°C (h)	Hydrolysis (%)	
	0.25M-HCl	0.25M-NaOH
0	0	0
3	0	50
6	0	70
9	0	80
20	5	95

Fig. 5 depicts a radioautograph of a 5% polyacrylamide gel of phosphorylated RNA polymerase I under denaturing conditions. For this experiment, the fractions from the phosphocellulose column

containing RNA polymerase I and nuclear protein kinase were incubated under conditions optimal for protein kinase activity, followed by filtration of the reaction mixture through a Sephadex G-100 column to remove unchanged ^{32}P , followed by sucrose-density-gradient centrifugation to separate protein kinase from RNA polymerase I activity (as shown in Fig. 1). The RNA polymerase fraction was then subjected to sodium dodecyl sulphate/polyacrylamide-gel electrophoresis, followed by the embedding of gel slices in Whatman paper and exposure to X-ray film as previously described (Hirsch & Martelo, 1976b; Martelo *et al.*, 1974). Fig. 5(a) depicts the sodium dodecyl sulphate/polyacrylamide-gel electrophoretogram of the phosphorylated polymerase. Fig. 5(b) shows a radioautograph of a slice of the same gel embedded in paper. Phosphorylation of subunits SA_1 , SA_3 , SA_5 and SA_6 predominates.

Discussion

The present results show that rat liver RNA polymerase I is closely associated with a cyclic AMP-independent nuclear protein kinase. The

Fig. 5. Polyacrylamide-gel electrophoresis of phosphorylated RNA polymerase I

The 5%-polyacrylamide gel containing 0.1% sodium dodecyl sulphate was prepared as described in the Materials and Methods section. Samples (20 μ g) of phosphorylated RNA polymerase I (after Sephadex G-100 column) were applied to the gel. Electrophoresis was for 8 h at 25°C at 3 mA/gel. Radioautography of a destained gel slice was performed as described in the Materials and Methods section. Exposure to the X-ray film was for 3 days at 25°C. Sodium dodecyl sulphate/polyacrylamide-gel electrophoretogram of the phosphorylated RNA polymerase I is shown in (a) (Coomassie Blue stain); radioautograph of non-denaturing gel of phosphorylated RNA polymerase I is shown in (b). Enzyme subunits were designated by the nomenclature of Keding *et al.* (1974).

protein kinase has been purified extensively. It phosphorylates not only RNA polymerase I but also phosvitin, casein and histone. The fact that it phosphorylates histone, a basic protein, is

noteworthy. The protein kinase was unable to utilize Mn^{2+} when phosphorylating RNA polymerase I, but utilized this cation when phosvitin was the substrate. This suggests that polymerase phosphorylation may be controlled by the types and concentration of bivalent cations in nuclei, analogous to the results of Kang *et al.* (1974). Inhibition of protein kinases by Mn^{2+} and nucleotides has also been reported previously (Gold & Segal, 1974).

Phosphorylation of RNA polymerase I by the closely associated protein kinase results in phosphoester bonds characteristic of phosphoserine and phosphothreonine. The protein kinase reported here remains closely associated with polymerase I throughout most of the purification steps. This suggests that the association of these two enzymes may have an important physiological role. Whether the phosphorylation of certain RNA polymerase I subunits observed is associated with a modification of its activity requires intensive investigation. Preliminary results from our laboratory suggest that a template-specific stimulation of RNA polymerase I activity results from this phosphorylation (J. Hirsch & O. J. Martelo, unpublished work).

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References

- Chelala, C. A. & Tarres, H. N. (1970) *Biochim. Biophys. Acta* **198**, 504–513
- Fischer, E. H., Graves, D. J., Crittenden, E. R. S. & Krebs, E. G. (1959) *J. Biol. Chem.* **234**, 1698–1704
- Gold, M. & Segal, I. (1974) *J. Biol. Chem.* **249**, 2417–2423
- Hirsch, J. & Martelo, O. J. (1976a) *Clin. Res.* **24**, 29a
- Hirsch, J. & Martelo, O. J. (1976b) *J. Biol. Chem.* **251**, 5408–5413
- Jungman, R. A., Hiestand, P. C. & Schweppe, J. S. (1974) *J. Biol. Chem.* **249**, 5444–5451
- Kang, Y. M., Olsen, M. & Busch, H. (1974) *J. Biol. Chem.* **249**, 5580–5585
- Keding, C., Gissinger, F. & Chambon, P. (1974) *Eur. J. Biochem.* **44**, 421–436
- Martelo, O. J. & Hirsch, J. (1974) *Biochem. Biophys. Res. Commun.* **58**, 1008–1015
- Martelo, O. J., Woo, S. L. C. & Davie, E. W. (1974) *J. Mol. Biol.* **87**, 685–696
- Schwartz, L. B., Sklar, V. E. F., Joehning, J. A., Weinman, R. & Raeder, R. G. (1974) *J. Biol. Chem.* **249**, 5889–5897