EFFECTS OF NUCLEAR PROTEINS ON THE ACTIVITY OF SOYBEAN DNA POLYMERASE

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Summary: The effects of nuclear proteins on DNA synthesis were investigated before and after incubation with radioactive ATP and a crude preparation of nuclear protein kinase. After partial purification by DEAE-cellulose chromatography, the major protein fractions were added separately to DNA polymerase assays. One of the seven protein fractions inhibited DNA synthesis by 50%, whereas three other fractions stimulated DNA polymerase activity 3 to 4-fold. After incubation with ATP, one fraction became inhibitory, and the three stimulatory fractions, which had high levels of radioactivity, were more effective. This stimulation of DNA polymerase activity was proportional to added nuclear protein and was maximum at 6 µg/20 µg DNA.

Nuclear proteins, especially phosphoproteins, function in gene expression by binding to DNA and regulating the synthesis of specific RNA's during growth and development (1-3). Studies which relate to this hypothesis include the phosphorylation of mammalian nuclear proteins by protein kinases (4-6). Since plant tissues have the capacity to phosphorylate proteins (7-9) including nuclear proteins (10,11), it is possible that plants may also regulate gene expression by use of protein kinases and phosphoproteins. Since DNA synthesis is a normal prerequisite of cell replication, an investigation of the effects of nuclear proteins and phosphorylation on DNA replication was undertaken. These studies report the effects of nuclear proteins on the activity of partially purified DNA polymerase from soybean hypocotyls and indicate that nuclear proteins and protein phosphorylation may regulate DNA synthesis.

MATERIALS AND METHODS

Preparation of nuclear fractions. A nuclear fraction was prepared by a modification of the method of Chen and coworkers (12). Four

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day old etiolated soybean (Glycine max var. Harosoy) hypocotyls (150 g) were minced in buffer (1:2, w/v) containing Tris-HCl, 50 mM, pH 7.5; KCl, 10 mM; $MgCl_2$ 10 mM; 2-mercaptoethanol, 10 mM; sucrose, 1.2M; and glycerol, 30% (v/v). Minced hypocotyls were homogenized with a Polytron PT 10 at setting 2.5 for 1 min or until the tissue was well macerated. The homogenate was filtered through Miracloth and centrifuged at 5,000 x g for 20 min. The upper layer of the pellet (crude nuclear fraction) was scraped from the underlying starch and served as the source of nuclear proteins and DNA polymerase.

Preparation of nuclear proteins. Nuclear proteins were solubilized from the nuclear pellet by suspension in 4 ml of distilled water and homogenization with the Polytron for 1 min. After centrifugation at 44,000 x g for 30 min (SW-50L rotor), the nuclear proteins in the supernatant were separated and partially purified by DEAE-cellulose chromatography.

Preparation of DNA polymerase. DNA polymerase activity was solubilized according to Dunham and Cherry (13). Following solubilization with ammonium sulfate, the enzyme preparation was desalted by passage through a Sephadex G-50 column, and further purified on DEAE-cellulose.

Enzyme assays. Protein kinase activity was assayed according to the method of Labrie and coworkers (14). The reaction mixture contained sodium acetate, 0.1 M, pH 6.5; $MgCl_2$, 0.01M; ATP, 20 nmoles; and [γ-^{32}P]ATP, 1-3 x 10^6 cpm (specific radioactivity 50-200 Ci/mmole) in a total volume of 0.2 ml. Reactions, 5 min at 30°, were stopped with 0.4 ml of 1.0 N NaOH followed by 1.5 ml of 10% trichloroacetic acid in 0.1 M H_3PO_4, and 0.1 ml of 1% bovine serum albumin as carrier. DNA polymerase assays were performed as described by Dunham and Cherry (13) and based on the

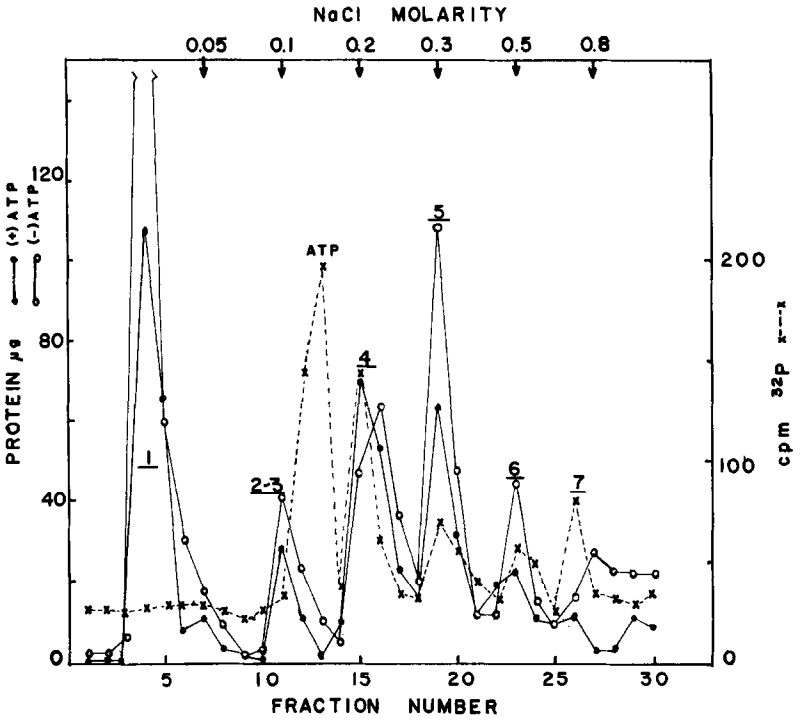

Fig. 1: DEAE cellulose chromatography of proteins from nuclear fraction before and after incubation with [γ-^{32}P]ATP. Nuclear pellets from 180 g of hypocotyls were suspended in 4 mls of distilled water and homogenized with the Polytron (1 min, setting 2.5), then centrifuged at 20,000 rpm (SW50L rotor) for 30 min. The supernatant was divided into two 2 ml aliquots, one was held on ice with no treatment. The other aliquot was incubated with 0.6 µmoles ATP + approximately 7.5 x 10^8 cpm [γ-^{32}P]ATP for 15 min at 30° for phosphorylation of nuclear proteins with endogenous protein kinase. Each aliquot was then separately placed on DEAE cellulose column (0.6 x 13 cm) equilibrated with buffer (KH_2PO_4, 5mM; EDTA, 2mM; 2-mercaptoethanol 5mM; pH 6.8). NaCl solutions in buffer (12 ml each), as a step gradient, were added for the elution of adsorbed proteins in 3 ml fractions. Protein concentrations from radioactive [(+)ATP] and nonradioactive [(-)ATP] fractions were determined by the Lowry method (15) as µg/0.5 ml of each 3 ml fraction. Fractions (approximately 2.5 mls) from the radioactive column were precipitated and washed with 10% TCA in 0.1 M H_3PO_4 and 0.1 N NaOH as for the typical protein kinase assay.

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incorporation of [^{3}H]TMP into trichloroacetic acid-precipitable material. Protein concentrations were determined by the Lowry method (15).

RESULTS

Seven major protein fractions were recovered from DEAE-cellulose chromatography of nuclear proteins (Fig. 1). Incubation with the crude protein kinase preparation did not significantly alter the elution profile of these protein fractions. Separation of proteins in peaks 2 and 3 was not complete and they were treated as a single fraction in these experiments. Proteins of peaks 4-7 were phosphorylated during the incubation with [^{32}P]ATP, whereas proteins of peaks 1 and 2-3 showed little if any incorporation of radioactivity. The peak of radioactivity designated ATP indicates elution of [^{32}P]-ATP. Protein kinase activity was associated with all fractions except peak 2-3, with the major kinase activity eluting with peak 4 (manuscript in preparation). These results indicate protein kinase activity associated with the nuclear fraction and that some, but not all, nuclear proteins were phosphorylated by incubation with ATP.

Assays of the desalted DNA polymerase preparation indicated a greater than 80% recovery of the activity in the nuclear fraction. Additional purification (500-fold over activity in the crude nuclear fraction) was achieved by use of a step gradient of 0.2 and 0.4 M KCl on a DEAE-cellulose column. The protein peak which elutes at 0.4 M KCl had a specific activity of 100-140 pmoles TMP incorporated/mg protein/30 min and had characteristics of the large molecular weight enzyme DNA polymerase-α, (16,17).

Following partial purification and removal of ATP and [^{32}P]-ATP on DEAE-cellulose (Fig. 1), the protein fractions were assayed for their effect on DNA polymerase activity. Peak 1, which had little effect on polymerase activity, became more inhibitory after incubation with ATP (Table 1). Peak 2-3, however, became less inhibitory after phosphorylation. The addition of the proteins of peaks 4,

Table 1. Effects of nuclear proteins on DNA polymerase activity.

Additions to DNA polymerase assay*	Specific Activity (pmoles TMP incorporated/mg/30 min.)	% Control
None	135.8	100
DEAE-cellulose fraction (-[γ-^{32}P]ATP)		
1	81.7	60.2
2-3	58.8	43.3
4	162.4	119.6
5	256.6	189.0
6	448.0	329.9
DEAE-cellulose fraction (+[γ-^{32}P]ATP)		
1	47.6	35.0
2-3	73.8	54.3
4	180.2	132.7
5	284.2	209.2
6	517.5	381.0

*Nuclear protein fractions (0.05 ml) which either have been incubated with [γ-^{32}P]ATP or have not been incubated (-[γ-^{32}P]ATP), were preincubated for 30 min at 37°C with DNA polymerase assay components. Following preincubation, DNA polymerase assays were initiated by addition of the polymerase (see Materials and Methods).

and especially 5 and 6 to the polymerase assay resulted in a 2-3-fold stimulation of DNA synthesis. Although DNA polymerase activity is present in fractions 5 and 6 (46 and 38% of control, respectively) combination with the partially purified enzyme should result in additive activity, assuming no interaction between proteins in these fractions and the enzyme. The observed stimulation, therefore, appears to result from protein-protein and/or protein-nucleic acid interactions in the assay. This increase of DNA polymerase activity

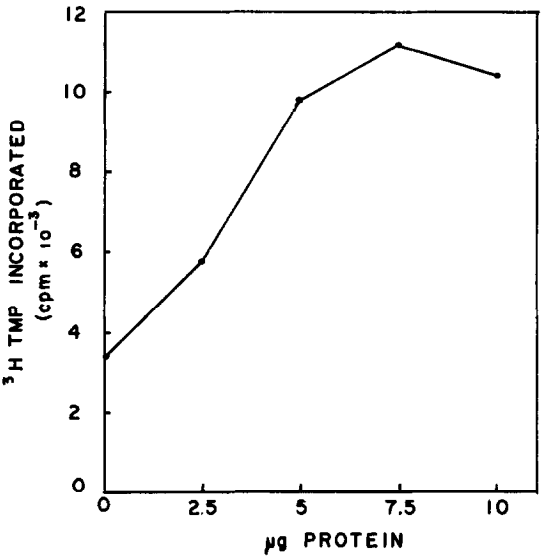

Fig. 2: DNA polymerase activity with added nuclear protein (DEAE-cellulose, fraction 6). DNA polymerase assays were as described in the text and in Table 1.

by the nuclear proteins in peak 6, for example, was proportional to nuclear protein concentration and saturable at approximately 6 µg of protein (Fig. 2). Small amounts of nuclear proteins, therefore, have significant and, perhaps, specific effects on the synthesis of DNA.

DISCUSSION

Several studies have shown that nuclear proteins function in the regulation of transcription, but little information is available regarding the control of DNA replication. Data presented above indicate that nuclear proteins affect the activity of DNA polymerase and thus are implicated as possible regulators of DNA replication. Protein kinases may also be involved in regulation since phosphoproteins as well as one or more kinases are associated with the nuclear fraction.

Although the kinase activities and nuclear proteins are not,

as yet, characterized and identified, the proteins separated on DEAE-cellulose do have different characteristics. The proteins elute from the column at different salt concentrations and differ in their ability to serve as a substrate for phosphorylation by protein kinases. In addition, the proteins exhibit different effects on DNA polymerase activity. The large increase in DNA polymerase activity in the presence of nuclear proteins is not a result of the presence of NaCl in the elution buffer. Although NaCl enhances DNA polymerase activity by 80% (18), the enhancement reported here is three times this value.

Although the results do not yet enable an identification of a specific protein kinase-nuclear protein control mechanism for DNA synthesis, the phosphoprotein control of transcription is a model useful for comparisons. Phosphorylation and dephosphorylation of nonhistone proteins alter the rate of RNA synthesis as well as the pattern of gene transcription (2, 19). In addition, the RNA polymerase molecule is a possible substrate for nuclear protein kinases (20-21). Although little information is available from plant systems, a protein kinase with a high specificity for histone H1 has been isolated from soybean hypocotyls (22). It is notable that in neuroblastoma cells the phosphorylation of histone H1 appears to be coupled to cell proliferation (23). In addition, certain plant growth regulators (cytokinins) have been shown to regulate nuclear protein phosphorylation (24). The regulation of DNA synthesis by nuclear phosphoproteins, therefore, may be an important mechanism in plant growth and development.

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REFERENCES

1. Allfrey, V.G. (1974) In: Acidic proteins of the nucleus, eds. Cameron, I.L., and Jeter, J.R., pp. 1-27, Academic Press, New York.
2. Stein, G.S., Spelsberg, T.C., and Kleinsmith, L.J. (1974) Science 183:817-824.
3. Bluthmann, H., Mrozek, S., and Gierer, A. (1975) Eur. J. Biochem. 58:315-326.
4. Cohen, M.E., and Kleinsmith, L.J. (1976) Biochim. Biophys. Acta 435:159-166.
5. Thomson, J.A., Brade, W.P., Chiu, J., Hnilica, L.S., and McGill, M. (1976) Can. J. Biochem. 54:86-92.
6. Chuang, D.M., Hollenbeck, R., and Costa, E. (1976) Science 193:60-62.
7. Keates, R.A.B. (1973) Biochem. Biophys. Res. Commun. 54:655-661.
8. Polya, G.M., and Sia, J.P.H. (1976) Plant Science Letters 7:43-50.
9. Ralph, R.K., Bullivant, S., and Wojcik, S.J. (1976) Biochim. Biophys. Acta 421:319-327.
10. van Loon, L.C., Trewavas, A., and Chapman, K.S.R. (1975) Plant Physiol. 55:288-292.
11. Chapman, K.S.R., Trewavas, A., and van Loon, L.C. (1975) Plant Physiol. 55:293-296.
12. Chen, Y., Lin, C., Chang, H., Guilfoyle, T.J., and Key, J.L. (1975) Plant Physiol. 56:78-82.
13. Dunham, V.L., and Cherry, J.H. (1973) Biochem. Biophys. Res. Commun. 54:403-410.
14. Labrie, F., Lemaire, S., and Courte, C. (1971) J. Biol. Chem. 246:7293-7301.
15. Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. (1951) J. Biol. Chem. 193:265-275.
16. Tymonko, J., and Dunham, V.L. (1976) Plant Physiol. 57:14.
17. Tymonko, J., and Dunham, V.L. (1977) Physiol. Plant. 38: In press.
18. Chang, L.M.S. (1973) J. Biol. Chem. 248:3789-3795.
19. Kleinsmith, L.J., Stein, J., and Stein, G. (1976) Proc. Nat. Acad. Sci. USA 73:1174-1178.
20. Zillig, W., Fujiki, H., Blum, W., Janekovic, D., Schweiger, M., Rahmsdorf, H., Ponta, H., and Hirsch-Kauffmann, M. (1975) Proc. Nat. Acad. Sci. USA 72:2506-2510.
21. Bell, G.I., Valenzuela, P., and Rutter, W.J. (1976) Nature 261:429-431.
22. Lin, P.P.C., and Key, J.L. (1976) Biochem. Biophys. Res. Commun. 73:396-403.
23. Lazo, J.S., Prasad, K.N., and Ruddon, R.W. (1976) Exp. Cell Res. 100:41-46.