PHOSPHORYLATION OF NUCLEAR MATRIX PROTEINS IN ISOLATED REGENERATING RAT LIVER NUCLEI

Steven L. Allen, Ronald Berezney* and Donald S. Coffey

Department of Pharmacology and Experimental Therapeutics,
The James Buchanan Brady Laboratory
for Reproductive Biology, and the Oncology Center
The Johns Hopkins University School of Medicine
Baltimore, Maryland 21205

Received December 13,1976

Summary. The phosphorylation of nuclear matrix proteins from normal and regenerating rat liver nuclei was examined using an in vitro system of isolated nuclei and γ-^{32}P-ATP. Phosphorylation of the nuclear matrix proteins was 2-3 fold higher than that of the total nuclear proteins in normal nuclei. The level of phosphorylation of the matrix proteins was enhanced an additional three fold at a period in liver regeneration (12 hours) just preceding the onset of DNA synthesis.

Recently, we reported the isolation of a nuclear protein matrix from rat liver nuclei (1). The nuclear matrix consists largely of acidic nonhistone proteins with 3 main polypeptide fractions in the molecular weight range of 60,000-70,000 daltons and represents less than 10% of the total nuclear proteins. Upon investigating the intranuclear site of DNA replication in regenerating liver, we reported that over 90% of newly synthesized DNA is initially associated with the nuclear matrix (2). This suggests that this residual nuclear structural framework may be involved in the regulation of nuclear functions such as DNA synthesis. Specific modifications of the nuclear matrix proteins may be involved in these processes. In this regard, we have determined the ability of these nuclear matrix proteins to undergo phosphorylation in vitro in the presence of γ-^{32}P-ATP.

*Present address: Division of Cell and Molecular Biology, State University of New York, Buffalo, New York 14214.

ISSN 0006-291X

Much interest in recent years has been focused on the possible role of the phosphorylation of acidic nonhistone proteins in the regulation of nuclear function (3,4,5).

MATERIALS AND METHODS

Male Sprague-Dawley rats (400-450 g) underwent partial hepatectomy by surgical removal of 2/3 of the liver following the methods of Higgins and Anderson (6). Animals were maintained in a controlled light environment and surgery was carried out under ether anesthesia between 8-10 a.m. Liver nuclei were isolated through high molarity sucrose by the procedure of Berezney and Coffey (1).

The isolated nuclei were phosphorylated by incubation with γ-^{32}P-ATP utilizing a modification of the method of Johnson et al. (7). Incubations were carried out at 37° C for 5 minutes in 6 ml containing: 3×10^{-8} moles γ-^{32}P-ATP (50μCi); 5 mM $MgCl_2$; 115 mM NaCl; 30 mM Tris-HCl, pH 7.4 with the addition of nuclei equivalent to 60 mg of total nuclear protein. The reaction was terminated by the addition of 6 ml of the reaction buffer at 4°C containing 6 mM ATP. The ^{32}P labeled nuclei were isolated and washed 3 times by centrifugation at 780 x g for 15 minutes.

The labeled nuclei were then processed for the isolation of the nuclear matrix proteins as described by Berezney and Coffey (1). The final step for the removal of tightly bound RNA and DNA employing nuclease digestion was omitted since SDS-acrylamide gel electrophoresis demonstrated no measurable difference in the polypeptide pattern before or after nuclease action. The nuclear pellet was subjected to the following extractions which were carried out at 4°C in 10 ml solutions containing 10 mM Tris buffer pH 7.4: (A) Three extractions with 0.2 mM $MgCl_2$ for 10 min. followed by centrifugation at 780 x g for 30 min; (B) Three extractions with 2 M NaCl containing 0.2 mM $MgCl_2$ for 10 min. followed by centrifugation at 780 x g for 60 min.; (C) 1% Triton X-100 in 5 mM $MgCl_2$ for 10 min. followed by centrifugation at 780 x g for 30 min. and two washes with 0.25 M sucrose, 0.05 mM Tris, pH 7.4 containing 5 mM $MgCl_2$.

Protein bound phosphate was isolated by modification of the procedures of Kleinsmith et al. (8). Removal of the nuclear lipid was not necessary since initial experiments revealed that extraction with chloroform:methanol (2:1; v:v) did not remove ^{32}P-containing material from the pellet. Nucleic acids were removed by the method of Munro and Fleck (9). The fractions were precipitated with 0.2 N PCA on ice for 10 minutes. After centrifuging at 27,000 x g for 10 minutes the pellets were washed two additional times in 0.2 N PCA and then resuspended in 0.8 N PCA and heated at 70°C for 20 minutes. The samples were cooled on ice, centrifuged at 27,000 x g, and dried with 10 ml of cold ether. To release protein bound phosphate, the pellets were heated at 100°C for 15 minutes in 0.5 ml of 1 N NaOH, cooled and then 0.49 ml of 70% perchloric acid was added to yield a final concentration of 0.2 N PCA. The suspensions were centrifuged at 27,000 x g for 10 minutes and the supernatants analyzed for inorganic phosphate by the method of Chen et al. (10). Radioactivity was determined by liquid scintillation counting in 10 ml of Hydromix (Yorktown Research Inc.). Protein was determined by the Lowry assay (11).

SDS (sodium dodecyl sulfate) acrylamide gel electrophoresis was performed according to the procedure of Weber and Osborn (12) and consisted of 10% acrylamide gels in 0.1% SDS, 0.05 M Tris, pH 7.4 which were run at 4 ma per gel with a solution of 0.1% SDS, 0.05 M Tris, pH 7.4 in both lower and upper chambers.

TABLE I

PHOSPHORYLATION OF TOTAL NUCLEAR AND NUCLEAR MATRIX PROTEINS

	Percent of Total Nuclear Protein	Total Phosphate in Protein	Specific Activity of Protein	Specific Activity of Phosphate
	(%)	(mg PO_4/mg Protein) x 10^5	(μμmole $^{32}PO_4$/mg Protein)	(mg $^{32}PO_4$/mg PO_4) x 10^4
Normal Liver Nuclei				
Total Nuclear Protein	100	73 ± 5	4.01 ± 0.41 (A)	1.60 ± 0.18 (C)
Nuclear Matrix Proteins	3.57 ± 0.97	70 ± 24	8.46 ± 1.07 (B)	5.15 ± 1.39 (D)
Regenerating Liver Nuclei (12 hours)				
Total Nuclear Protein	100	68 ± 9	8.20 ± 1.13 (E)	4.25 ± 1.12 (G)
Nuclear Matrix Proteins	5.73 ± 1.84	67 ± 6	30.94 ± 9.40 (F)	13.81 ± 3.66 (H)

Averages of five individual experiments involving pooled liver from 4 rats per determination.

± Standard error of the mean

Significance of differences between groups: A vs B < 0.005, A vs E < 0.005, E vs F < 0.05, C vs G < 0.05, C vs D < 0.025, B vs F < 0.05, G vs H < 0.05, D vs H < 0.10

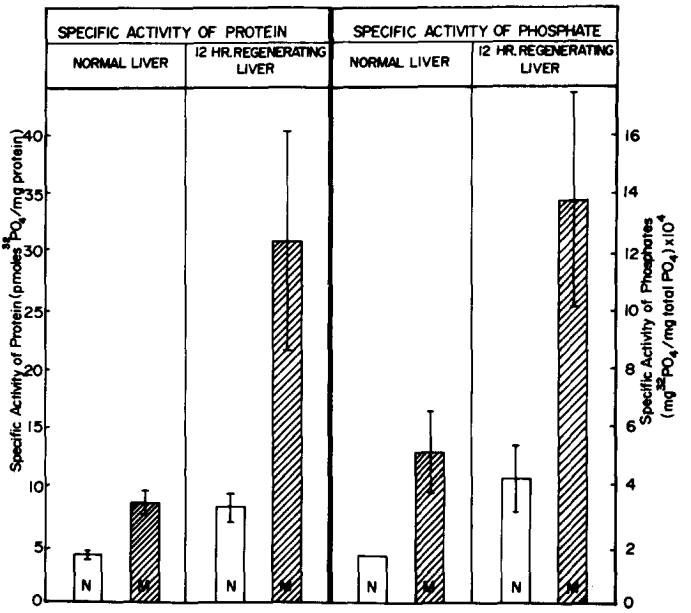

FIGURE 1. γ-^{32}P-ATP phosphorylation of isolated nuclei and the resulting specific activity of the total nuclear proteins (N) and the isolated nuclear matrix proteins (M). Data are presented for normal liver nuclei and nuclei isolated 12 hours following partial hepatectomy.

Samples for electrophoresis were dissolved as lyophylized powders in 5% SDS, 0.05 M Tris, pH 7.4 containing 2% β-mercaptoethanol by heating at 70°C for 20 min. followed by incubation at room temperature for three hours.

RESULTS AND DISCUSSION

In vitro phosphorylation by γ-^{32}P-ATP of the total nuclear protein in 12 hour regenerating rat liver nuclei was 2-3 fold higher than that observed with normal rat liver nuclei. This difference was significant and was observed if the specific activity was expressed either as incorporation per unit amount of protein or per unit phosphate (Table I, Fig. 1). After *in vitro* phosphorylation of the isolated nuclei, nuclear matrix proteins were isolated from both normal and regenerating liver nuclei. The recovered matrix proteins represent approximately 5% of the total nuclear proteins and contained essentially the same amount of phosphate residues per mg of protein as was observed in the total nuclear proteins. The matrix proteins, however, were differentially phosphory-

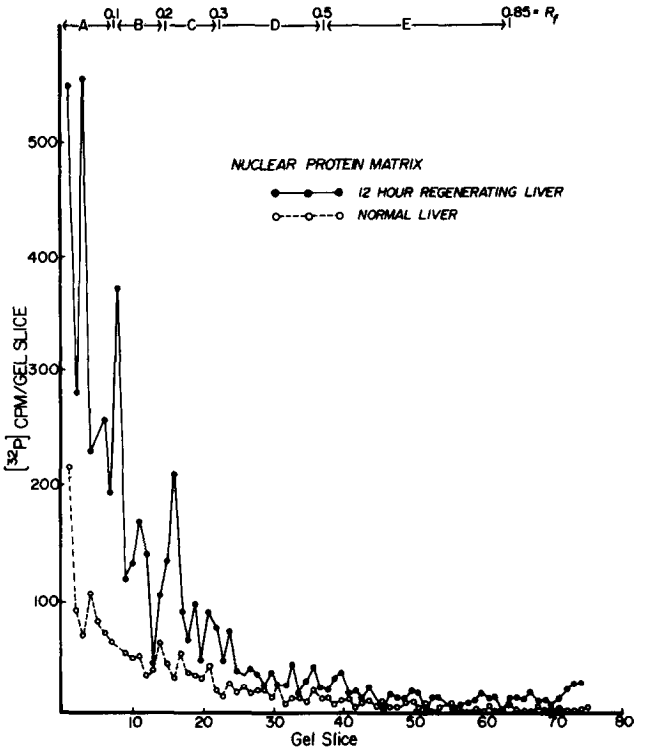

FIGURE 2. Radioactivity associated with the nuclear matrix proteins isolated from normal livers and 12 hours following partial hepatectomy. SDS acrylamide gel electrophoresis was performed on 100 μg of protein according to Berezney and Coffey (4).

lated in both normal and regenerating liver nuclei by a factor which was 2-3 fold higher than total nuclear proteins. Phosphorylation of the nuclear matrix protein was markedly increased during liver regeneration (Fig. 1).

SDS acrylamide gel electrophoresis of the solubilized nuclear matrix proteins was performed as previously reported (1); comparable amounts (100 μg) of protein from both normal and regenerating nuclear matrix were resolved on each gel (Fig. 2). Although the phosphate labeled proteins appeared in the R_f zones A-C, which corresponded to the major high molecular weight proteins previously identified in the nuclear protein matrix (1), the resolution was not sufficient to definitively identify the specific nuclear matrix polypeptides phosphorylated.

A time study of the onset of phosphorylation of the nuclear protein in regenerating liver demonstrated a maximum level of phosphorylation at 12 hours after partial hepatectomy. This preceded the onset of DNA synthesis which occurs 14-16 hours after partial hepatectomy. It is important to note that the phosphorylation by ATP was performed *in vitro* and that the extent of dephosphorylation was not measured in these experiments.

Since newly replicated DNA *in vivo* is associated with the isolated nuclear matrix in regenerating rat liver (2), it is conceivable that the phosphorylation of specific proteins of the nuclear matrix preceding this event could be of importance in the regulation of DNA synthesis in the nucleus. We have recently reviewed the isolation, structure and functions of the nuclear protein matrix (13).

ACKNOWLEDGEMENTS

This work was supported by the National Cancer Institute, Grant CA13745.

REFERENCES

1. Berezney, R. and Coffey D.S. (1974) Biochem. Biophys. Res. Comm. 60, 1410-1417.
2. Berezney, R. and Coffey, D.S. (1975) Science 189, 291-293.
3. Kleinsmith, L. (1975) J. Cell Physiol. 85, 459-476.
4. Stein, G.S., Spelsberg, T.C. and Kleinsmith, L. (1974) Science 183, 817-824.
5. Allfrey, V.G., Inoue, A., Kain, J., Johnson, E.M. and Vidali, G. (1974) Cold Spring Harbor Symp. Quant. Biol. 38, 785-801.
6. Higgins, G.M. and Anderson, R.M. (1931) Arch. Pathol. 12, 186-202.
7. Johnson, E.M., Vidali, G., Littan, V.C., and Allfrey, V.G. (1973) J. Biol. Chem. 248, 7595-7600.
8. Kleinsmith, L., Allfrey, V.G. and Mirsky, A.E. (1966) Proc. Natl. Acad. Sci. 55, 1182-1189.
9. Munro, H.N. and Fleck, A. (1965) Meth. Biochem. Anal. 14, 113-176.
10. Chen, P.S., Toribara, T.Y. and Warner, H. (1956) Anal. Chem. 28, 1756-1758.
11. Lowry, O.H., Rosenbrough, N.J., Farr, A.L., and Randall, R.J. (1951) J. Biol. Chem. 193, 265-275.
12. Weber, R. and Osborn, M. (1969) J. Biol. Chem. 244, 4406-4412.
13. Berezney, R. and Coffey, D.S. (1976) Adv. Enzyme Reg. 14, 63-100.