NONHISTONE CHROMATIN PROTEIN PHOSPHORYLATION DURING AZO-DYE CARCINOGENESIS

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1. Introduction

The nonhistone proteins of chromatin are believed to participate in the regulation of gene activity [1–3]. The phosphorylated chromatin proteins are of a particular interest since they display properties compatible with the functions of gene activator or repressor proteins [4, 5]. Since the neoplastic disease most likely reflects altered biochemical control mechanisms and abnormal gene expression, knowledge of the effects of chemical carcinogens on the chromatin components in affected tissues may contribute to the eventual control of neoplasia. We report here that the activity of protein phosphokinase enzymes in the rat liver cytoplasm and chromatin increases after the administration of an azo-dye carcinogen, *N,N*-dimethyl-*p*-(*m*-tolylazo) aniline (3′-MDAB). Furthermore, the phosphorylation of several nonhistone proteins increases in the liver chromatin of rats fed the azo-dye containing diet. At the same time, the ability of isolated liver chromatin to template for the *in vitro* RNA synthesis is considerably higher than that of the controls.

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2. Methods

Male Fisher rats (200–250 g) were fed Wayne Laboratory Meal (Allied Mills, Inc., Chicago, Ill.) soaked with 10% (w/w) of corn oil (Mazola) as the basic diet. The diet of the experimental group was supplemented with 0.06% of *N,N*-dimethyl-*p*-(*m*-tolylazo) aniline (3′-MDAB), purchased from Eastman Kodak Organic Chemicals, Rochester, N.Y. [6]. The azo-dye was dissolved in corn oil prior to its soaking into the commercial feed pellets.

The livers of 3–4 rats in each group were homogenized in 10 vol of 0.32 M sucrose containing 5 mM $MgCl_2$ and centrifuged at 100 *g* for 10 min. The supernatant, after recentrifugation at 105 000 *g* for 1 hr, was used to determine the activity of cytoplasmic phosphokinase. The sediment was rehomogenized in 10 vol of 2.2 M sucrose containing 5 mM $MgCl_2$ and centrifuged at 100 000 *g* for 1 hr. The pelleted nuclei were used for preparation of chromatin following a previously described procedure [7]. All experimental points were repeated twice.

The phosphoprotein kinase activity was measured according to Kamiyama and Dastugue [8] using casein as a protein substrate. After their incubation with [γ-^{32}P]ATP, chromatin samples were solubilized in a solution containing 1% sodium dodecyl sulfate and 1% 2-mercaptoethanol in 8 M urea–0.01 M sodium phos-

Table 1
Template activities of rat liver chromatin from control and 3′-MDAB fed rats.

Samples	Specific activity (cpm/μg DNA)	Increase (%)
Control	251	–
Days on 3′-MDAB		
2	277	10.3
8	294	17.2
15	309	23.2
22	436	73.6
28	449	78.8
36	527	110.0

The reaction mixture for the *in vitro* RNA synthesis consisted of (in one assay): 0.2 μmoles ATP, 0.2 μmoles GTP, 0.2 μmoles CTP, 0.05 μmoles [^{3}H]UTP, 10 μmoles Tris-HCl, pH 8.0, 30 μmoles KCl, 0.025 μmoles dithiothreitol, 0.025 μmoles EDTA and 0.625 μmoles $MnCl_2$. The chromatin DNA concentration in each assay was 25 μg together with 15 units of *E. coli* polymerase (specific activity 600–700 units/mg protein). The total volume of each reaction mixture was adjusted with distilled water to 0.25 ml. The assays were incubated at 37°C for 10 min and the reaction was terminated by adding 1 ml of cold 10% trichloroacetic acid (TCA). The precipitated RNA was collected on filter paper (Whatman 3 MM) discs, washed with TCA and sodium pyrophosphate solutions, dried with absolute ethanol and counted in a toluene based scintillation counting liquid.

Table 2
Specific activity of phosphoproteins in liver chromatin of 3′-MDAB rats.

Sample	^{32}P-(pmoles/mg/min)	Increase (%)
Control:	19.0	–
Days on 3′-MDAB		
8	19.2	1.0
15	21.7	14.2
22	22.5	18.4
28	23.6	24.2
36	24.0	26.3

The control and 3′-MDAB liver chromatin samples were incubated with [γ-^{32}P]ATP as described in table 3. Phosphorylated chromatin proteins were extracted with 1% sodium dodecyl sulfate – 1% 2-mercaptoethanol in 50 mM Tris-HCl buffer, pH 8.0 as described in the Methods. After centrifugation, dialysis, and lyophilization, protein aliquots were dissolved in 1% sodium dodecyl sulfate – 5 M urea solution and the radioactivity of samples was determined in the Packard Scintillation Spectrometer. The amount of proteins was measured by the method of Lowry et al. [12].

phate buffer, pH 7.0 and centrifuged at 135 000 *g* for 24 hr [9]. The supernatant containing histones and nonhistone proteins was dialyzed against deionized water and lyophilized. Dry protein aliquots were dissolved in 0.1 M sodium phosphate buffer containing 0.1% sodium dodecyl sulfate, 0.1% 2-mercaptoethanol and 8 M urea, pH 8.0 and electrophoresed in 10% polyacrylamide gels containing 0.1% of sodium dodecyl sulfate [7]. The gels were stained with Coomassie Brilliant Blue and scanned at 600 nm in a Gilford spectrophotometer with a linear transport and scanning attachment. After scanning, the gels were cut into 1.0 mm thick slices, dissolved in 30% H_2O_2 and their radioactivity was determined in a liquid scintillation spectrometer [10].

The capacity of individual chromatin samples was determined with the aid of exogenous RNA polymerase isolated from *E. coli* according to Burgess [11]. Conditions for the RNA polymerase assay were essentially identical to those described by this author. The Lowry et al. [12] protein assay was used to determine the protein concentration and the DNA was measured by the Burton procedure [13].

3. Results and discussion

As can be seen in table 1, the capacity of chromatin to template for RNA *in vitro* rose considerably in livers of rats fed the 3′-MDAB diet. After 36 days, it reached more than twice the control values. This increase was accompanied by a similar, although not as dramatic, increase in the incorporation of [γ-^{32}P]-ATP into the chromatin proteins (table 2) which in turn reflected the progressive activation of chromatin-bound and of the cytoplasmic phosphoprotein kinase (table 3). These results are consistent with the report of Granner [14] who found the protein kinase activity in HTC hepatoma cells to be several times higher than in normal liver.

To determine which proteins are affected by the increased phosphorylation, ^{32}P activities of electrophoretically separated chromatin proteins (histones and nonhistones) were compared. After inspecting the photometric scans (fig. 1A) it can be concluded that there are no marked qualitative differences between the protein patterns of chromatins isolated

Table 3
Cytoplasmic and chromatin bound phosphoprotein kinase activities in control and 3′-MDAB fed rat livers.

	Cytoplasmic Experiment 1		Experiment 2	
	^{32}P(pmole/mg protein)	(%)	^{32}P(pmole/mg protein)	(%)
Control: Days on 3′-MDAB	21.0	(100.0)	22.2	(100.0)
2	32.4	(154.2)	31.4	(141.4)
8	35.7	(170.0)	38.3	(172.5)
15	45.1	(214.7)	49.8	(224.3)
22	52.4	(249.5)	63.3	(285.1)
28	57.8	(275.2)	76.7	(345.4)
36	78.2	(372.3)	104.7	(471.6)
	Chromatin bound			
	Experiment 1		Experiment 2	
	^{32}P(nmole/mg DNA)	(%)	^{32}P(nmole/mg DNA)	(%)
Control: Days on 3′-MDAB	6.1	(100.0)	6.3	(100.0)
2	6.5	(106.5)	6.4	(101.5)
8	6.2	(101.6)	6.0	(95.2)
15	6.8	(111.4)	6.2	(98.4)
22	7.7	(126.2)	6.8	(107.9)
28	11.1	(181.9)	11.8	(187.3)
36	10.3	(168.8)	10.3	(163.4)

The reaction mixture contained 20 μmoles of Tris-HCl, pH 7.5, 4 nmoles of [γ-^{32}P]ATP (0.23 Ci/mmole), 5 μmoles of $MgCl_2$, 25 μmoles of NaCl and 0.3 mg protein of cytoplasmic preparations or 25 μg DNA of chromatin preparations. The total volume was 0.25 ml [8]. The incubation was carried out at 37°C for 10 min and was terminated by addition of 10% trichloroacetic acid (TCA). The precipitate was washed with 5% TCA 3 times and dissolved in NCS (Amersham-Searle) solubilizer. The radioactivity was determined in the Packard Scintillation Spectrometer.

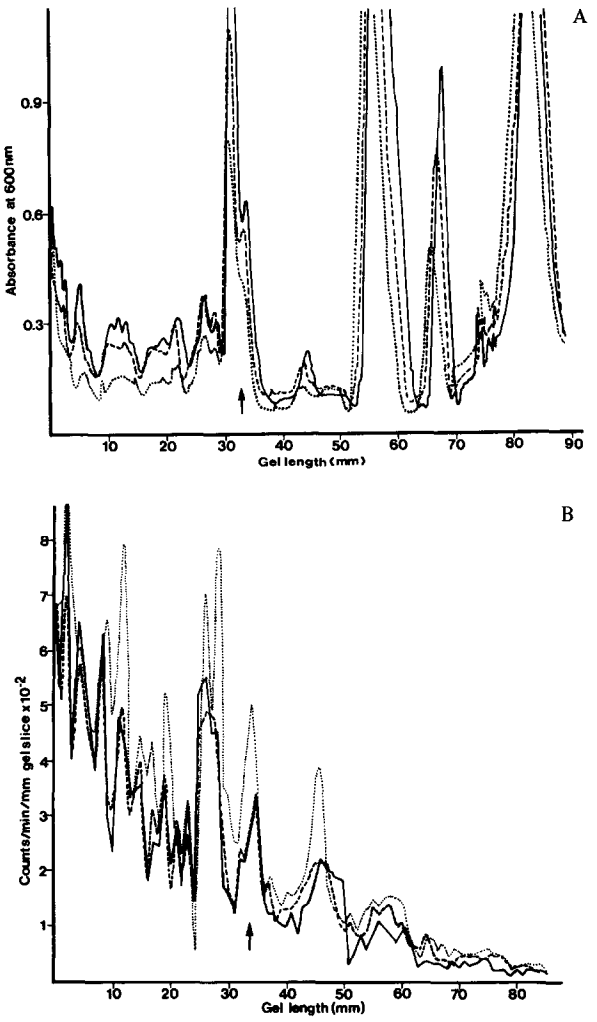

Fig. 1. A) Optical scans of polyacrylamide gel electrophoreograms of liver chromatin proteins from normal and 3′-MDAB fed rats. B) Distribution of ^{32}P radioactivity in 1 mm slices of polyacrylamide gels shown in fig. 1A. (——) Control rats; (------) rats on 3′-MDAB diet for 15 days; (·····) rats on 3′-MDAB diet for 28 days. The position of very lysine rich KAP (F1 or I) histones is indicated by an arrow.

from control and 3′-MDAB fed animals. This is in good agreement with the findings of Gronow [15] who reported essentially identical Coomassie Blue-stained polyacrylamide gel protein patterns between normal and nitrosamine treated rat livers. However, when he compared the distribution of thiol groups in the electrophoresed proteins, a marked increase in the thiol content was evident in animals fed with the carcinogen. A similar conclusion can be extended to the comparison of ^{32}P radioactivity patterns shown in fig. 1B. Here, the exposure of rats to the carcinogenic diet for 28 days produced a selective change in the phosphorylation pattern of chromosomal proteins in liver. While the overwhelming presence of histones

can be noticed in the optical scans (fig. 1A), they are, with the exception of the KAP (F1 or I) fraction, only marginally phosphorylated. Indeed, all the histones account for less than 10% of the ^{32}P incorporation into chromatin proteins. It is of interest that among the approx. 7 protein bands which phosphorylation increased during the first 28 days of 3′-MDAB diet, one belongs to the KAP (F1 or I) histones. It was reported in the literature that increased phosphorylation of the lysine-rich KAP (F1 or I) histones can be correlated with cellular proliferation [16].

Although many biochemical changes produced in livers of experimental animals fed with carcinogenic diets may be irrelevant in the sequence of events leading to the malignant transformation, changes in the phosphorylation of chromatin proteins from various tissues were correlated by several investigators to the modifications of genetic template restriction [17–20]. Our data are in accord with this information and the observed functional modification of chromatin accompanied by changes in the phosphorylation of its proteins and by the increased activity of the phosphokinase enzymes may represent one of the early steps necessary for the establishment of neoplastic growth.

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