DNA PROTEIN INTERACTIONS IN CpG ISLETS AND DNase I-SENSITIVE CHROMATIN

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An important step in the activation of chromatin is a change in the character of DNA–protein interactions. Disturbance of the nucleosomal structure or weakening of the bond between DNA and histones is observed as a result of their modifications [3, 9, 10, 13]. Disturbance of DNA–protein interactions leads to a change in the degree of superspiralization of DNA, characteristic of transcribed chromatin [11]. Conversely, during inactivation of chromatin, nonhistone proteins, simplifying their structure, are additionally bound with it [2, 8], and the chromatin is organized into rosettes [1]. To fill in the details of the picture we have studied DNA–protein interactions in hypothetical active and inactive chromatin, separated on the base of differences in their sensitivity to DNase I [4] and restriction endonucleases [14], by the method of nucleoprotein-celite chromatography (NCC), by means of which it is possible to discover particular features of DNA–protein interactions not usually revealed by other methods [5-7].

EXPERIMENTAL METHOD

Experiments were carried out on cells of a transplantable Ehrlich's carcinoma [5] and of a culture of transformed Djungarian hamster 4/21 fibroblasts; conditions of passage and labeling of these cells were described in detail [7]. Nuclei were isolated by extracting the cells with 1% Triton X-100. Isolated nuclei of 4/21 cells were treated with DNase I in 25 mM Tris-HCl, pH 7.6, 5 mM $MgCl_2$.

The enzyme was used in doses of 20 or 200 μg to 1 ml of suspension and the DNA concentration was adjusted to 100 μg/ml. The reaction (30 min, 0°C) was stopped by addition of 6 mM EDTA To separate DNase-sensitive and resistant fractions, the isolated nuclei from Ehrlich's ascites carcinoma cells were suspended in 10 mM Tris-HCl pH 7 5, with 10 mM NaCl and 3 mM $MgCl_2$ to a concentration of 2.5 mg/ml as DNA. To 1 mg DNA was added 450 U of DNase I; the mixture was incubated at 37°C (10 min), cooled, and centrifuged for 10 min at 1000*g*. The supernatant contained DNase sensitive, the residue DNase-resistant chromatin. Active and inactive chromatin were separated on the basis of the presence of CpG-islets in the former, as described in [14]. Nuclei from Ehrlich's ascites carcinoma cells were isolated in a solution containing 10 mM triethanolamine (pH 7.9), 25 mM NaCl, 50 mM KCl, 0.1% Triton X-100, 1 mM dithiothreitol, 10 mM sodium butyrate, in a lightly ground Dounce homogenizer (30 frictions), and centrifuged for 10 min at 1000*g*. The isolated nuclei were washed in 50 mM Tris-HCl, pH 8, 100 mM NaCl, 8 mM $MgCl_2$, 0.1 mM EGTA, 10 mM sodium butyrate, and suspended in the same solution up to a concentration of 400 μg/ml as DNA. To obtain active chromatin the suspension was incubated for 1 h at 37°C with 800 U/ml of restriction endonuclease MspI, and to obtain inactive chromatin, with 300 U/ml of AluI. The reaction was stopped by addition of EDTA up to 10 mM and incubation for 20 min at room temperature The suspension was then recentrifuged, the supernatant kept, and the residue incubated for fuller extraction of the chromatin for 1 h at 4°C

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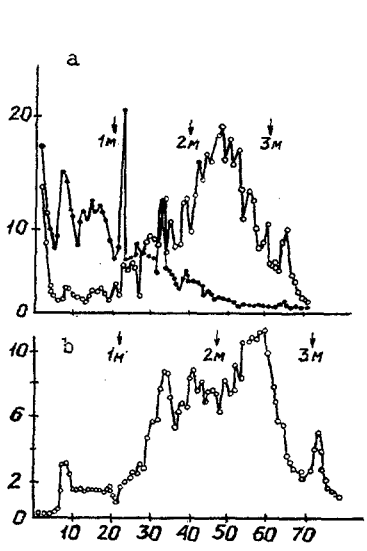

Fig. 1

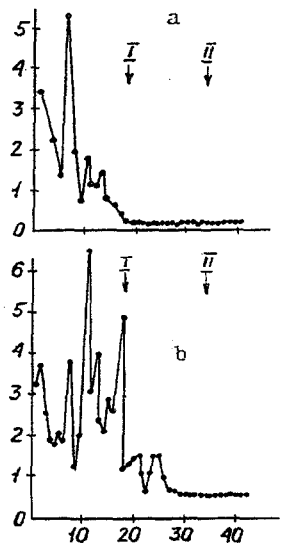

Fig. 2

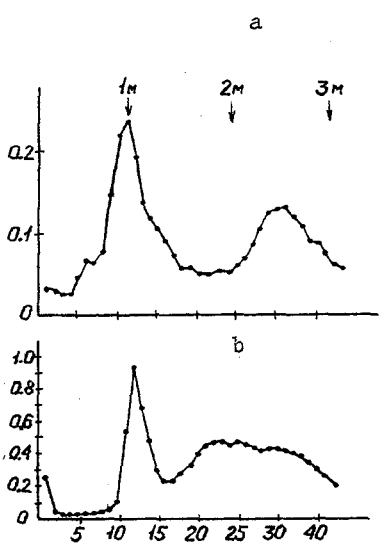

Fig. 3

Fig. 1. NCC chromatograms of DNA from isolated cell 4/21 nuclei after treatment with DNase I: a) proliferating cells: 1) 20 μg/ml enzyme, 30 min; 2) 200 μg/ml, 30 min; b) resting cells, 20 μg/ml, 30 min. Volume of NaCl gradient 200 ml. After completion columns were washed with 30 ml 4 M LiCl. 8 M urea. Arrows indicate NaCl concentrations. Abscissa, No of fractions; ordinate, radioactivity of ^{3}H-thymidine (in cpm $\times 10^{-3}$).

Fig 2. NCC chromatograms of chromatin from Ehrlich's ascites carcinoma cells, obtained by treatment of nuclei with DNase I: a) soluble chromatin, b) insoluble chromatin. Arrows indicate: I) end of NaCl gradient (60 ml), II) end of LiCl-urea gradient and beginning of temperature gradient. Remainder of legend as to Fig. 1.

Fig. 3. NCC chromatograms of chromatin solubilized after treatment of isolated nuclei of Ehrlich's ascites carcinoma cells with restriction endonucleases: a) MspI, b) AluI. Volume of NaCl gradient 100 ml, chromatography stopped after its completion. Abscissa, Nos. of fractions; ordinate, optical density at 260 nm (relative units).

in 10 mM triethanolamine buffer, pH 7.0, 75 mM KCl, 0.15 mM spermine, 0.5 mM spermidine, 1 mM EDTA, 0.5 mM EGTA, 10 mM sodium butyrate. After incubation the suspension was centrifuged, and supernatants from the 1st and 2nd centrifugations were pooled and used for NCC chromatography, which was carried out as described previously [5, 7]. The chromatin preparations were adsorbed on celite, and DNA freed from the complex with protein by NaCl (0-3 M), LiCl-urea, and temperature gradients. In most experiments only an NaCl gradient was used. In individual experiments DNA was precipitated from the chromatographic fractions with 2 volumes of ethanol and subjected to agarose gel electrophoresis [5].

EXPERIMENTAL RESULTS

Since it was shown previously that DNA–protein bonds in chromatin, unconnected with the nuclear matrix, dissociate completely during NCC chromatography in an NaCl gradient [5], in the present investigation a simplified version of NCC-chromatography was used, consisting of an NaCl gradient only. To increase the resolving power of the method the volume of the gradient was increased. In the first stage of the work it was necessary to make sure that previous results were reproducible with the new modification of the method NCC chromatograms of cell 4/21 nuclei after treatment with 20 and 200 μg/ml DNase I for 30 min at 0°C are shown in Fig 1a. Clearly with mild treatment (DNA passed into the acid-soluble fraction) most DNA was eluted after 2 M NaCl. As was assumed, the DNA was eluted almost entirely in the NaCl gradient, and washing the column with 4 M LiCl, 8 M urea led to elu-

tion of only a small DNA fraction, which remained bound to the nuclear matrix. With more rigorous treatment most DNA was eluted up to 1 M NaCl.

Changes in DNA–protein bonds, accompanying transition of the cells into a resting state, also were reflected in the NCC chromatograms. The chromatograms given in Fig. 1a were obtained on exponentially growing cells (2 days after seeding). The NCC chromatogram of DNA from resting cell nuclei, treated with 20 μg/ml DNase I is shown in Fig. 1b. Besides the peak between 2 and 3 M NaCl, obtained by this treatment of proliferating cells, there was also an extra peak between 1 and 2 M NaCl. This heterogeneity with respect to strength of the DNA–protein bond also was observed in chromatin in resting cells, spontaneously detached from the matrix [7].

The discrete nature of the DNA fractions in the NaCl gradient will be noted: peaks eluted by $\approx$1, 2, and 3 M NaCl can be distinguished. The discrete fractions differed also in preparations of soluble and insoluble chromatin after digestion with DNase I (Fig. 2). The peak at 1 M predominated in soluble chromatin, peaks at 2 and 3 M NaCl in insoluble chromatin. However, the differences found can only be due to greater fragmentation of the soluble chromatin. As will be clear from Fig. 1a, increased fragmentation facilitates the discovery of the fraction in the 1 M NaCl region. Although we showed previously that the size of the chromatin particles does not finally determine the strength of DNA–protein interactions [6], this statement requires further proof. We therefore used another approach to obtain active and inactive chromatin, namely their different sensitivity to restriction endonucleases attacking nonmethylated CpG-islets [14]. Restriction endonuclease MspI (CCGG) introduces cuts mainly at sites located in promoter regions of active genes, whereas AluI (AGCT) has no restriction sites in these regions. Thus chromatin, solubilized after digestion with MspI, is rich in transcribed sequences after digestion with MspI, but with untranscribed after digestion with AluI [14]. When this method was used to obtain it, the active chromatin was not fragmented to a greater degree than inactive chromatin. NCC chromatograms of chromatin obtained after treatment with MspI (Fig. 3a) and AluI (Fig. 3b) are given in Fig. 3. As a rule the peak at 1 M NaCl predominated in active chromatin, whereas a fraction eluted by 2 M NaCl predominated in inactive chromatin. Electrophoresis of DNA precipitated from the chromatographic fractions showed that during hydrolysis by MspI, a high-molecular-weight DNA ($\approx$11,000 b.p.) predominated in all fractions of the chromatogram, but oligonucleosomes after hydrolysis with AluI, in both cases, however, some enrichment of the first fractions of the gradient by smaller fragments, and of the last fractions by larger fragments was nevertheless observed (photographs of the gels are not reproduced here). Homogeneity of the chromatin fragments with respect to size also was observed after digestion by other restriction endonucleases [12].

The results indicate the presence of a distribution of particles by strength of DNA–protein interactions specific for active and inactive chromatin. Active chromatin is characterized by fractions eluted at 1 and 3 M NaCl, inactive chromatin by one eluted at 2 M NaCl. The results obtained with restriction endonucleases reproduce very well the data obtained by NCC chromatography of chromatin separated into active and inactive fractions by agarose electrophoresis [6].

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POSTINTOXICATION HYPOTHERMIA AND FREE-RADICAL LIPID OXIDATION IN THE BRAIN AND HEART OF RATS POISONED WITH ORGANOPHOSPHORUS CHOLINESTERASE INHIBITORS

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The aim of this investigation was to study the role of lipid peroxidation (LPO) in the realization of the toxic effects of organophosphorus compounds (OPC).

EXPERIMENTAL METHOD

Experiments were carried out on rats poisoned experimentally with two cholinesterase inhibitors: carbophos (malathion, M) and armin (A), The toxic compounds were given to experimental Wistar rats weighing initially 200-220 g, in a dose of LD_{50} (260 and 1 mg/kg respectively), Healthy animals constituted the control group. The animals were under observation for 30 days after poisoning. Biochemical tests were carried out on the cerebral hemispheres and myocardium Lipids were extracted from homogenates of rat organs during the period from 2 to 30 days after poisoning [4]. Conjugated dienes (CD) were determined spectrophotometrically [5]. Schiff's bases (SB) were determined by a spectrofluorometric method [6]. The temperature in the rats' esophagus was measured by a TPÉM-I electrothermometer. The results were subjected to statistical analysis by Student's t test.

EXPERIMENTAL RESULTS

The experimental results show activation of LPO in the rats' organs in the postintoxication period of poisoning by M and A. This is confirmed by an increase in the content of CD and SB in the cerebral hemispheres and myocardium (Tables 1 and 2). Maximal accumulation of CD in the brain of rats poisoned by M was observed 2, 14, and 21 days after poisoning. At the same time, the maximal increase in CD and SB levels in the brain and myocardium of the rats after poisoning with A was observed after 14 days (Table 2).

Malathion caused hypothermia, as shown by lowering of the esophageal temperature of the poisoned rats. Potentiation of the hypothermic reaction was noted after 14 and 21 days of the experiment (Table 1).

Thus the times of maximal accumulation of CD and SB in the organs of the poisoned rats and the severity of the hypothermic reaction coincided. Armin also evokes a hypothermic reaction, as shown by lowering of the esophageal temperature of the poisoned rats. The maximal fall of temperature was observed after 14 days of the postintoxication period (Table 2). Thus on a model of armin, just as with the action of M, a similar agreement was observed

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