FORMATION OF A STABLE DNA – PROTEIN BOND BY THE ACTION OF A BIFUNCTIONAL ALKYLATING MUTAGEN (EMBICHIN-HN2) ON CHROMATIN

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By the use of various methods of deproteinization of chromatin (gel-filtration, ultracentrifugation, chromatography on calcium hydroxyapatite) the authors showed that by interaction between the chromatin and a bifunctional alkylating agent (embichin-HN2) a DNA–protein complex is formed.

Key words: thymus chromatin; DNA and its bond with protein; alkylating mutagens (embichin).

By the action of bifunctional nitrogen mustards on chromatin in vivo and in vitro indirect evidence has been obtained that the action of these mutagens on the genetic apparatus of the cell may be connected with the formation of DNA–protein crosslinkages in the composition of the chromatin [2, 4].

In this investigation DNA obtained after dissociation of chromatin preparations in solutions of high ionic strength or in the presence of a high concentration of anionic detergents, followed by separation of the dissociated components of the chromatin by gel-filtration, ultracentrifugation, or fractionation on a column with calcium hydroxyapatite, was analyzed. The question of the causes of the transition of the DNA into interphase after treatment of the chromatin with embichin also was investigated.

EXPERIMENTAL METHOD

Chromatin was isolated from calf thymus by method of Shaw et al. [7]. Treatment of the chromatin with HN2 (methyl-bis-2-chloroethylamine) and HN1 (β-chloroethylamine) was carried out in a solution of 2.5×10^{-3} M $NaHCO_3$ in which the final concentration of the agent was 10^{-3} M and the ratio of mutagen to nucleotide 1 : 3. The incubation time was 30 min at 37°C. Unreacted agent was removed by dialysis in the cold.

Chromatin preparations were dissociated in one or other system of solvents. For chloroform deproteinization solutions of chromatin in 2 M NaCl were mixed with an equal volume of a mixture of chloroform and butanol (3:1), shaken vigorously (30 min), and centrifuged at 8000 g for 20 min. Gel-filtration was carried out in columns (2×40 cm) packed with Sepharose-4B and equilibrated with 3 M NaCl. From 4 to 5 ml chromatin in 3 M NaCl, with a DNA concentration of 250–300 μg/ml, was applied. The rate of elution was 0.8–1.2 ml/h. Ultracentrifugation was carried out in the Spinco L centrifuge at 150,000 g for 14 h. Adsorption chromatography was carried out on calcium hydroxyapatite (HAP), synthesized by Bernardi's method [3], with the use of a stepwise elution gradient as described by MacGillivray et al. [6]. The rate of elution was 4–6 ml/h. DNA eluted by phosphate buffer in a concentration of 0.5 M was collected for analysis. The chromatin preparations after dialysis were treated with a solution of pronase (1 mg/ml in 10^{-3} M Tris-HCl buffer, pH 7.5) to deproteinize the DNA. The enzyme concentration in the chromatin was 100 μg /ml. Incubation continued for 2 h at 37°C. The DNA concentration was determined by Spirin's method [1]. The protein concentration was estimated by Lowry's method [5].

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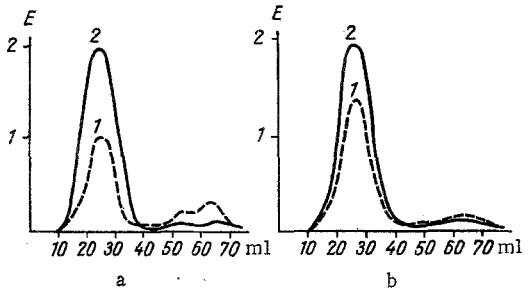

Fig. 1. Gel-filtration of the aqueous phase after chloroform deproteinization through Sepharose-4B: a) chromatin; b) chromatin + 10^{-3} M HN2; 1) E_{230nm}; 2) E_{260nm}.

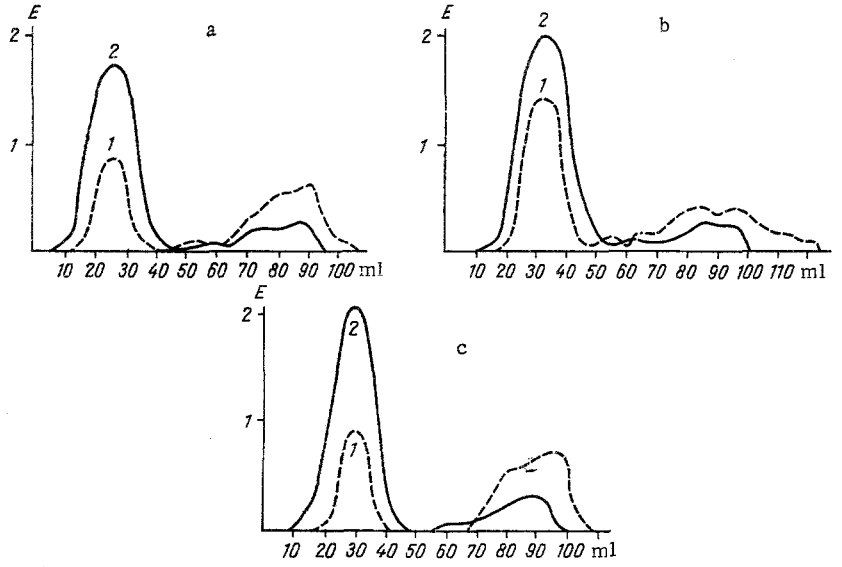

Fig. 2. Gel-filtration of chromatin preparations in 3 M NaCl through Sepharose-4B; a) chromatin; b) chromatin + 10^{-3} M HN2; c) chromatin + +10^{-3} M HN1; 1) E_{230nm}; 2) E_{260nm}.

EXPERIMENTAL RESULTS

As was shown previously [2], treatment of ascites tumor cells and chromatin with the preparation HN2 causes 60% more protein to change into interphase than in the control. However, this does not answer the question whether the DNA draws into interphase the protein firmly bound with it and whether the DNA left in the aqueous phase is free from protein. To study why the action of HN2 on the chromatin causes the DNA to pass into the interphase, calf thymus chromatin was treated with pronase immediately before the addition of chloroform. Pronase treatment completely abolished the effect of increased passage of DNA into the interphase. Consequently, the passage of DNA into the interphase is connected with strengthening of the DNA–protein bond. Gel-filtration of DNA of the aqueous phase (Fig. 1) showed that this DNA is also enriched with protein firmly bound with DNA. The fact that the aqueous phase contains DNA firmly bound with protein points to a redistribution of the DNA–protein complex formed by the action of bifunctional alkylating agents between the aqueous phase and the interphase. Treatment with HN1, incidentally, did not lead to any significant passage of DNA into the interphase.

To rule out any possible effect of chloroform or phenol on the formation of the firm DNA–protein bond and also to determine the quantity of protein bound with DNA, other methods of deproteinization, more adequate in the writers' view, were used. Control chromatin preparations and others treated with HN2 or HN1 were dissociated in solutions of high ionic strength or in the presence of sodium dodecylsulfate, after which fractionation was carried out by gel-filtration, ultracentrifugation, or adsorption chromatography.

As Fig. 2 shows, chromatin treated in vitro with 10^{-3} M HN2 was characterized during gel filtration by an increased protein content in the DNA peak. The E_{260}/E_{230} ratio, which was 2.35 for the control preparations, fell to 1.85. The preparation HN1 had no significant effect on the character of the elution curve of the dissociated chromatin after gel-filtration.

Since the increase in the protein content in DNA obtained by this method was not necessarily entirely due to the formation of a firm DNA–protein bond under the influence of the HN2 but could also have been due to aggregation of the proteins themselves through the action of this agent, chromatin was dissociated in other systems of solvents (2 M NaCl; 2 M NaCl + 5 M urea; 1% sodium dodecylsulfate solution) and then the DNA and protein were separated by ultracentrifugation. To test whether firm bonds could be formed between DNA and protein in chromatin by the action of HN2 the method of adsorption chromatography on HAP also was used; this method differs in principle from gel-filtration and centrifugation methods, which are based on the principle of fractionation of the chromatin components by molecular weight. The results showed that methods of dissociation based on rupture of ionic, hydrophobic, and hydrogen bonds under conditions preventing protein aggregation, and the various types of subsequent fractionation had virtually no effect on the quantity of firmly bound protein in the chromatin (3-5% in the control, 15-17% in the experiment).

The experiments thus showed that the action of the bifunctional alkylating mutagen HN2 on chromatin in fact leads to the formation of stable DNA–protein complexes, evidently covalent in nature.

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