CONDITIONS OF INITIAL ENDONUCLEASE HYDROLYSIS OF CHROMATIN AND CHARACTERISTICS OF ITS PRODUCTS

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Initial hydrolysis of chromatin by endogenous nucleases (up to 1-4% with respect to the acid-soluble fraction) is used to obtain its template-active fragments [6]. The arrangement of nuclease-sensitive zones of chromatin is evidently determined by the topology of these enzymes provided that its structure is as close to native as possible. The obtaining of chromatin or cell nuclei with the highest degree of polymerization of DNA and the presence of accurate methods of quantitative estimation of the degree of hydrolysis of chromatin are of great importance in this case. The writers showed previously [7] that the use of solutions with pH 8.5, containing Ca^{++} ions and not containing Mg^{++} ions, to isolate cell nuclei enables high-polymer chromatin to be obtained. If the conditions for inhibition of nuclear Ca^{++}, Mg^{++}-dependent endonuclease are not observed, the resulting preparations contain hydrolysis products of chromatin: oligonucleosomes, including mononucleosomes [3], and a background acid-soluble fraction of the order of several percent of total DNA. The term "initial autohydrolysis" is used to describe subsequent cleavage of chromatin, and the acid-soluble fraction of unincubated samples is taken to be zero.

To determine those endonucleases whose maximal action is manifested in the initial stages of autohydrolysis, it was decided to compare the initial and subsequent stages of hydrolysis of chromatin as reflected in the cleavage products. Histamine was used as nonspecific regulator of the intensity of chromatin autohydrolysis. Amines are known to regulate processes of repair of irradiated DNA [1, 2] and to activate the template activity of chromatin, i.e., they participate in processes involving the endogenous nucleases of chromatin.

EXPERIMENTAL METHOD

Noninbred male albino rats weighing 150-180 g were used. Cell nuclei were isolated from liver tissue homogenate by a modified method in [8]: All buffer solutions had pH 8.5 at 4°C and contained 0.1 mM phenylmethylsulfonyl fluoride (PMSF) and 10 mM $CaCl_2$. Chromatin was obtained from freshly isolated nuclei by the method in [10]. The final solution of chromatin contained 0.2 mM EDTA.

Autohydrolysis of DNA in isolated cell nuclei was carried out in 50 mM Tris-HCl buffer, pH 7.4, at 37°C, containing 0.25 M sucrose, 5 mM $MgCl_2$, 2 mM $CaCl_2$, and 0.1 mM PMSF. Chromatin was hydrolyzed in buffer of the same composition, but without the addition of sucrose. Each sample contained 1 mg/ml DNA in the composition of the nuclei or chromatin. The samples were incubated at 37°C. The reaction was stopped by cooling the samples in an ice bath, after which 0.5 M EDTA was added up to a concentration of 25 mM. To study the effect of histamine on autohydrolysis it was added to the samples in the form of a 100-fold solution up to a final concentration of 10^{-5} M. The degree of cleavage of chromatin was estimated from the increase in acid-soluble and solubilized fraction. The acid-soluble fraction was obtained by precipitating high-polymer DNA in the samples with an equal volume of 1 N $HClO_4$ at 2°C, followed by centrifugation at 10,000g for 15 min. Solubilized DNA was separated at the stages of hydrolysis by the method in [3]. The suspension of incubated and cooled nuclei was centrifuged at 1000g for 5 min at 2°C. The residue was suspended in 10 mM Tris-

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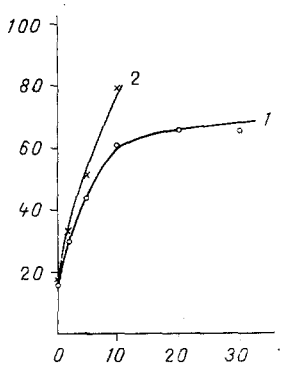

Fig. 1

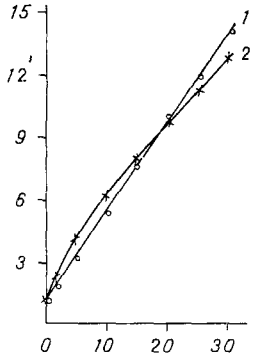

Fig. 2

Fig. 1. Determination of intensity of endonuclease hydrolysis of chromatin in rat liver cell nuclei by solubilization method. Abscissa, incubation time (in min); ordinate, DNA content in solubilized fraction (in % of total). 1) Without addition of histamine, 2) in presence of 10^{-5} M histamine.

Fig. 2. Determination of intensity of endonuclease hydrolysis of chromatin in rat liver cell nuclei according to acid-soluble fraction. Abscissa, incubation time (in min); ordinate, quantity of acid-soluble DNA (in % of total). Remainder of legend as to Fig. 1.

HCl buffer, pH 8.5, with 0.2 mM $CaCl_2$ in volume equal to the original volume of the sample. The samples were incubated on ice for 10 min and centrifuged at 780g for 20 min. The solubilized fraction (S fraction) was the term applied to pooled samples after the first and second centrifugations, whereas the nonsolubilized fraction (P fraction) was the nuclear residue after the second centrifugation. Chloroform extraction of DNA, measurement of its concentration, electrophoresis in 1.5% agarose, densitometry of the gels, and determination of the mean weighted molecular weight of DNA were carried out as described previously [7].

EXPERIMENTAL RESULTS

Addition of 10 mM $CaCl_2$ to the buffer during isolation of the cell nuclei yielded preparations containing not more than 0.9% in the acid-soluble fraction. Under these circumstances the fraction not solubilized by buffer with low ionic strength, i.e., bound with the nuclear matrix, was 84% of total nuclear DNA. The acid-soluble fraction contained only mononucleotides and short oligonucleotides [5]. The method based on fractionation of large DNA fragments, namely the solubilization method [3], was considered more adequate for the study of limited autohydrolysis.

It will be clear from Fig. 1 that the quantity of DNA freed from binding with the matrix during autohydrolysis (S fraction) increased during the first 10 min of incubation from 16 to 61%, i.e., by 45% of total DNA. Meanwhile (Fig. 2) the acid-soluble fraction changed from 0.9 to 5.3% — by 4.4% of total DNA, i.e., 10 times less than the solubilized fraction. The solubilization method is not only highly sensitive. It also enabled the rapid hydrolysis (initial autohydrolysis) phase, up to 10 min of incubation, to be separated sufficiently clearly from the slow hydrolysis phase — after 10 min. The inflection on the solubilization curve occurred at the time when 4-5% of DNA was present in the acid-soluble fraction. The quantity of acid-soluble product continued to increase in a straight line in this case. According to the results of electrophoresis, the mean weighted molecular weight of the hydrolysis products was reduced.

After hydrolysis for 30 min the size of the DNA molecules of the P fraction was 400-500 base pairs, and the P fraction itself amounted to 30% of the total DNA.

If the chromatin loop is taken to measure on average 68,000 base pairs [4], the quantity of DNA attached at this stage to the matrix is 0.6-0.7%. Consequently, most DNA of the P fraction is bound with the matrix, not on a "skeleton" of high-polymer DNA, but through DNA-protein interactions. According to data in the literature [9], an increase in ionic strength of the eluting buffer significantly increases the yield of solubilized fraction, and this can be explained by removal of these stabilizing proteins.

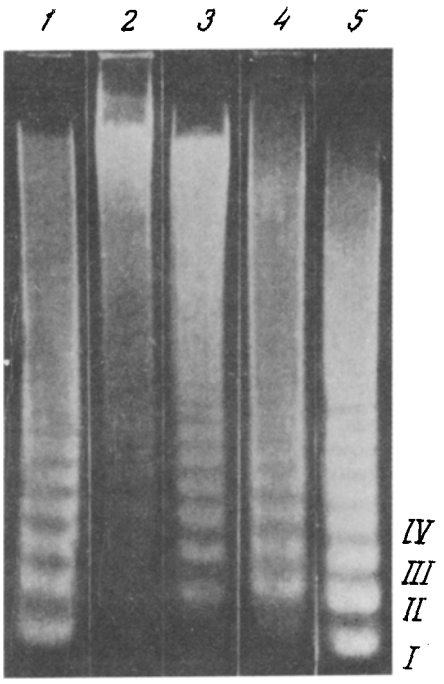

Fig. 3. Electrophoresis of DNA of solubilized (S) and nonsolubilized (P) chromatin fractions from nuclei in 1.5% agarose during initial autohydrolysis. 1) Unfractionated DNA from nuclei incubated for 15 min; 2, 3) DNA of S and P fractions respectively, incubation for 2 min; 4, 5) DNA of S and P fractions respectively, incubation for 10 min. Roman numerals denote DNA of oligomers of nucleosomes.

The results of electrophoresis of DNA of the S and P fractions showed (Fig. 3) that incubation of the nuclei for 2 min leads to removal of a complete set of oligomers of nucleosomes, with the exception of mononucleosomes. Endonucleases of chromatin, acting at this stage, evidently cannot remove the terminal nucleosome from oligonucleosomes. The beginning of removal of the first mononucleosomes usually coincided with the stage of change in velocity of removal of the solubilized fraction at the 10th minute of incubation (Fig. 3). The mechanism of endonuclease hydrolysis of chromatin evidently changed from "large-cleaving" to "small-cleaving," possibly attributable to the action of different fractions of nuclear endonucleases.

The presence of histamine in a concentration of 10^{-5} M activated autohydrolysis, as could be determined both as solubilization and as the acid-soluble fraction (Figs. 1 and 2). In the latter case, the maximal activating effect was observed at the 4-6% level and it continued until the stage of 9% of DNA in the acid-soluble fraction. Maximal activation was 20% of the original level. Further hydrolysis of chromatin in the nuclei was inhibited by the presence of histamine. The change of effects was determined only by the stage of hydrolysis of chromatin. For instance, addition of histamine during incubation up to 9% in the acid-soluble fraction led after 5 min to activation of autohydrolysis by 10-15% of the control level, but after 9% led to inhibition of autohydrolysis. If isolated chromatin was used, the dependence was similar: activation up to 9-10%, inhibition thereafter. It can thus be tentatively suggested that histamine may be an intracellular regulator of the intensity of endonuclease hydrolysis of chromatin.

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PROTEIN, RNA, AND DNA SYNTHESIS IN CULTURES OF SKIN FIBROBLASTS FROM HEALTHY SUBJECTS AND PATIENTS WITH RHEUMATIC DISEASES

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KEY WORDS: DNA; RNA; protein; skin fibroblasts; rheumatoid arthritis, systemic scleroderma.

Skin fibroblasts from patients with rheumatic fever, rheumatoid arthritis (RA) and systemic scleroderma (SS) possess enhanced functional activity, manifested as increased production of collagen, proteoglycans, and glycoproteins [1, 2, 4, 5]. A considerable increase in the rate of collagen biosynthesis by fibroblasts persisted during long-term cell culture, indicating a lasting disturbance of regulation of this process, possibly at the genome level, in patients with SS [5].

To study the mechanism of the lasting disturbance of fibroblast function, protein, RNA, and DNA synthesis was investigated in skin fibroblasts from patients with RA and SS.

EXPERIMENTAL METHOD

Strains of skin fibroblasts grown in the Laboratory of Biochemistry of Connective Tissue, Institute of Rheumatology, Academy of Medical Sciences of the USSR, were used. Cultures were tested at the third and fourth passages in the stationary phase of growth. The culture medium contained 50% of Eagle's medium, 30% of albumin hydrolysate, and 20% of bovine serum. The labeled precursors used to analyze synthesis of protein, RNA, and DNA were ^{14}C-protein hydrolysate (Czechoslovakia), [^{14}C]uridine (Czechoslovakia), and [^{14}C]thymidine (USSR) respectively. The labeling time was 4 h for the study of protein synthesis, and 2 h for the study of RNA and DNA synthesis. To investigate the stimulating effect of bovine embryonic serum (from the N. F. Gamaleya Institute of Microbiology and Epidemiology, Academy of Medical Sciences of the USSR) 5% serum was added to Eagle's medium and stimulation was determined by measuring incorporation of [^{14}C]proline (Czechoslovakia) into fibroblast proteins. The labeling time was 2 h. When actinomycin D (from "Reanal," Hungary) was used in these experiments it was either preincubated with the cells for 1 h or added to the Eagle's medium along with the [^{14}C]proline. To determine the rate of fission of prelabeled RNA after incubation of the fibroblasts for 1 h with [^{14}C]uridine, the cells were rinsed twice with Earle's medium and then incubated in growth medium (medium 199 with 20% bovine serum) with the addition of 50 μg/ml of uridine (from "Reanal"). All experiments with labeled precursors ended with a single washing of the cells with ice-cold Earle's medium.

During analysis of stability of fast-labeled RNA tests were carried out to discover whether all measurable radioactivity belonged to RNA molecules. For this purpose the fibroblasts were hydrolyzed in 0.3N KOH for 1 h at 37°C, after which they were precipitated with N $HClO_4$ and the precipitate applied to millipore filters (Synpor, No. 2, Czechoslovakia). The amount of radioactivity remaining in the cells was about 5% of its amount in unhydrolyzed

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