THE DINUCLEOSOME AS AN INITIAL PRODUCT OF CHROMATIN CLEAVAGE BY ENDOGENOUS ENDONUCLEASES

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UDC 612.014.24:576.315.42].015.1:577.152.277

KEY WORDS: dinucleosome; chromatin; endogeneous nucleases; initial endonucleolysis

The existence of dinucleosomes, or elementary supranucleosomal structures of chromatin, was discovered by the writers with the use of an endonuclease from *Brevibacterium ammoniagenes* [1]. The endonuclease produces cleavage of rat liver chromatin DNA by single-stranded breaks in each second linker, and this leads to a decrease in the effectiveness of action of endogenous nuclear nucleases [1, 3]. Meanwhile, other investigators demonstrated the dinucleosomal organization of chromatin from sea urchin sperm [6], rat liver [7], and chick erythrocytes [7, 10]. Unmodified [6, 10] or ferritin-bound pancreatic DNase I [7] was used as the probe. In all the investigations cited above exogenous endonucleases were used. Endogenous enzymes under these circumstances either were inhibited [7, 8], their effect weakened by the use of short-term hydrolysis by high concentrations of exogenous nuclease [6], or tissues in which endogenous activity was absent were used [4, 7, 8, 10].

The fact that dinucleosomes have been discovered is evidence that this particular structural element can be recognized not only by exogenous, but also by endogenous chromatin nucleases. As the writers showed previously, during initial autohydrolysis of chromatin cleavage of dinucleosomes does not take place: At a certain stage they are the end-product of autohydrolysis [2].

The aim of the present investigation was to determine, by a study of initial autohydrolysis products, the ability of endogenous nucleases to recognize the dinucleosomal level of chromatin organization and, in addition, to answer the question whether this recognition is connected with the existence of alternate long and short linkers in chromatin.

EXPERIMENTAL METHOD

Noninbred male albino rats weighing 180-200 g were used. Cell nuclei were isolated from liver tissue homogenate as described previously [1, 2] with the use of buffer solutions containing 1 mM phenylmethylsulfonyl fluoride (PMSF) and 10 mM $CaCl_2$ to inhibit proteolysis and nucleolysis, respectively, during isolation. Autohydrolysis of chromatin in isolated nuclei was carried out at 37°C in 50 mM Tris-HCl, pH 7.7, containing 0.25 M sucrose, 5 mM $MgCl_2$, 2 mM $CaCl_2$, and 0.1 mM PMSF. The reaction was stopped by cooling the samples in an ice bath, and by the addition of 0.5 M EDTA solution to a final concentration of 25 mM, after which the DNA was deproteinized with an equal volume of a mixture of chloroform and isoamyl alcohol (24:1).

To obtain a preparation of autohydrolyzed chromatin, nuclei after incubation were sedimented by centrifugation at 1000g for 10 min and resuspended in a buffer of 50 mM Tris-HCl, pH 7.7, 0.2 mM EDTA to a DNA concentration of 2 mg/ml. The resulting chromatin solution was used as the substrate for nuclease S1 and for exonucleases. During cleavage of autohydrolyzed chromatin by nuclease S1, 100-µl samples containing 40 µg DNA, 4 units nuclease (Biolar, USSR), 50 mM Na-acetate buffer, pH 5.4, and 1 mM $ZnCl_2$, were incubated for 30 min at 37°C, after which the DNA was deproteinized. During treatment of the chromatin with exonucleases, 0.4 unit of viper venom exonuclease (Worthington, USA) was added to each 100-µl sample, containing 40 µg DNA in buffer consisting of 50 mM Tris-HCl, pH 7.7, and 0.2 mM EDTA, the samples were incubated for 15 min at 37°C, after which 3 units of alkaline phosphatase from *Escherichia coli* (Sigma,

Institute of Medical Enzymology, Academy of Medical Sciences of the USSR, Moscow. (Presented by Academician of the Academy of Medical Sciences of the USSR, K. V. Sudakov.) Translated from Byulleten' Eksperimental'noi Biologii i Meditsiny, Vol. 101, No. 4, pp. 463-466, April, 1986. Original article submitted August 1, 1985.

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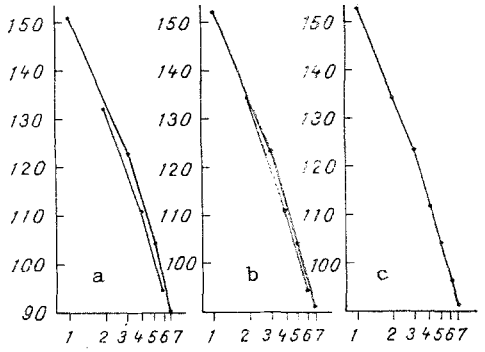

Fig. 1

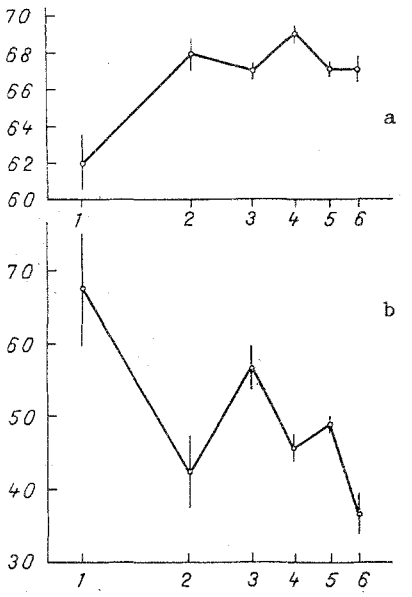

Fig. 2

Fig. 1. Changes in mobility of DNA of even and odd oligonucleosomes in agar gel during autohydrolysis of rat liver chromatin. Abscissa, No. of nucleosomal oligomer (1 — mononucleosome, 2 — dinucleosome, and so on); ordinate, distance on densitogram from beginning of gel to tip of peak of oligomer DNA (in mm). Duration of incubation: a) 5 min, b) 10 min, c) 20 min.

Fig. 2. Height and heterogeneity of peaks of DNA of nucleosomal oligomers of autohydrolyzed chromatin on a densitogram. Abscissa, No. of nucleosomal oligomer; ordinate: a) height of peak (in mm), b) heterogeneity of peak (in % deviation of length of DNA of oligomer from mean value).

USA) was added and incubation continued for a further 15 min, after which the samples were cooled on ice. Chromatin was sedimented by centrifugation at 100,000g for 20 min and dissolved in the original volume of 40 mM Na-acetate buffer, pH 5.4, and 5 mM $MgCl_2$. Exonuclease from bovine spleen (Worthington) was added to each sample in a dose of 0.4 unit, and the samples were then incubated at 37°C for 30 min, after which the DNA was deproteinized.

Electrophoresis of DNA was carried out in vertical blocks of 0.8% agarose; after staining with ethidium bromide the gels were photographed in UV light and the negatives scanned in a densitometer [1, 2]. The base lines in this case corresponded to regions of the stained gels not containing DNA. A digest of DNA of phage λ by restriction endonuclease Hind III was used as the molecular weight standard. Heterogeneity of DNA of the nucleosomal oligomers was determined in percentages of deviation of the length of the oligomer DNA at the point of intersection of the straight line continuing the high-molecular-weight shoulder of the peak with the base line from the length of DNA of that oligomer determined from the height of the peak. To obtain DNA of individual oligomers it was extracted from the gel by freezing [1], after which the DNA was deproteinized. The hyperchromic effect of the DNA on melting was determined in constant-temperature microcuvettes in a DU-8 spectrophotometer (Beckman, USA).

In agreement with data obtained previously [2], hydrolysis during the first 10 min of incubation, when the acid-soluble fraction contained 1-5% of DNA, was taken to be initial endonucleolysis of the chromatin in rat liver cell nuclei under conditions optimal for endogenous Ca,Mg-dependent endonuclease (2 mM $CaCl_2$, 5 mM $MgCl_2$, pH 7.4, at 37°C).

EXPERIMENTAL RESULTS

The mobility of DNA of nucleosomal oligomers of autohydrolyzed chromatin on electrophoresis in gel is known to be proportional to the logarithm of the quantity of mononucleosomes in the oligomer [9]. It will be clear from Fig. 1 that during initial endonucleolysis of chromatin the mobilities of the tips of the peaks of the oligonucleosomes were described by two nonintersecting curves, corresponding to oligomers with even and odd numbers of nucleosomes. The length of DNA of the mononucleosomes in the composition of the even oligomers was

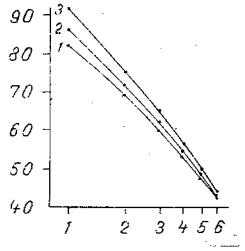

Fig. 3. Changes in electrophoretic mobility of DNA of oligonucleosomes from rat liver chromatin, autohydrolyzed for 20 min and treated with nucleases. Abscissa, No. of nucleosomal oligomer; ordinate, distance on densitogram from beginning of gel to tip of DNA peak of oligomer (in mm): 1) without nucleases, 2) action of nuclease S1, 3) combined action of snake venom and bovine splenic exonucleases.

greater than in the odd ones. After 5 min of autohydrolysis the difference was 24 ± 5 pairs of nucleotides, and after 20 min the difference in mobility of the DNA of the oligomers disappeared (Fig. 1). This means, first, that endogenous nucleases can recognize the dinucleosomal organization of chromatin, and this ability, moreover, is found only in the initial stages of hydrolysis, and second, that even oligonucleosomes, judging from the mobility of the tips of the peaks, arise only from certain particular regions of chromatin, and that the regions with dinucleosomal structure are distributed unevenly in chromatin.

Incubation of the nuclei for 10 min was accompanied by the appearance of higher peaks of the even oligonucleosomes, probably due to their less marked heterogeneity for size (Fig. 2a). The even oligonucleosomes were more homogeneous also after deep hydrolysis (incubation up to 40 min), which was used to obtain more reliable measurements of heterogeneity on account of the increased amplitudes of the peaks (Fig. 2b). These results also indicate the origin of even and odd oligomers of nucleosomes from different regions of chromatin.

If dinucleosome formation is connected with the difference in lengths of linker DNA within the dinucleosomes and between them, and if the chromatin, hydrolyzed by endonucleases, is treated with nuclease S1 or with exonucleases, an increase ought to be observed in the difference of mobility of the DNA of the even and odd oligonucleosomes. As will be clear from Fig. 3, degradation of chromatin, previously hydrolyzed for 20 min, by nuclease S1 led to an increase in mobility, i.e., to a reduction in the lengths of the DNA of all oligomers without exception. Mobility of DNA of the mononucleosomes was increased by the greatest degree, evidently because of its high content of single-stranded DNA. Determination of the hyperchromic effect on melting of the isolated DNase of the oligomers also showed that DNA of mononucleosomes is least native: The hyperchromic effect of DNA of mononucleosomes was 24%, dinucleosomes 34%, trinucleosomes 34%, tetranucleosomes 31%, pentanucleosomes 35%, and sextanucleosomes 38% (the error of measurement was ±2%). Combined hydrolysis by viper venom and bovine spleen exonucleases of the chromatin autohydrolysate likewise revealed no inequality of shortening of the DNA of the oligomers due to the different length of the terminal linkers of the even and odd oligonucleosomes (Fig. 3).

The following conclusions can thus be drawn. The orderly dinucleosomal structure is distributed irregularly in chromatin. It is not connected with the difference in length of the intra- and interdinucleosomal linkers. Endogenous nuclear nucleases can recognize the dinucleosomal level of chromatin structure. Under the conditions used the specificity of endogenous nucleases for the dinucleosomal structure was low, possibly due to the use of 50 mM Tris-HCl, whereas the formation of dinucleosomes was more marked at a lower ionic strength [14]. The second possible reason is that rat liver nuclear endonucleases have a molecular weight of not more than 35-40 kilodaltons (kD) [9, 11]. Consequently, their contact with the linker is spatially less restricted than in high-molecular-weight [1] or specially modified enzymes [7]. Recognition of dinucleosomes under these circumstances is possible only in tightly packed regions, as has been shown for DNase I [4, 6], whose molecular weight is 31 kD. Low-molecular-weight enzymes, such as staphylococcal nuclease, are in general unable to recognize and excise a dinucleosome [10].

Three main levels of structural organization are distinguished in eukaryote chromatin: the nucleosomal thread ("beads on a thread"), the 25 nm fibril, and the fibril loop, fixed in the matrix of the chromatid [5, 15]. The fibril is formed by spiralization of the nucleosomal thread. According to the "zig-zag" model of Worcel et al. [15], the fibril is a twisted band whose edges are formed by "cores" of nucleosomes, and the center by the linker DNA connecting them. The presence of zig-zag bends of DNA in regions of condensation on a histone octamer has been confirmed by electron-microscopic investigations of native [14, 15] and reconstructed [12] chromatin. The structural unit of the fibril packed in this way is thus a dinucleosome [15]. The second way of formation of dinucleosomes suggests the possibility that a histone octamer can slide along the DNA chain, with the formation of "compact oligomers," as has been demonstrated for chick erythrocyte chromatin, which is poor in histones H1 and H5, in the presence of 0.6 M NaCl [13]. In this case, the intra- and interdinucleosomal linkers must differ in length. Our own results show that the first mechanism of dinucleosome formation is more probable. Further investigations must be aimed at discovering the cause of recognition of the "zig-zag"; is it connected with screening of some linkers by nearby "cores" of neighboring nucleosomes during spiralization of the "zig-zag" [15], with the protrusion of each second linker under these circumstances, or with other factors.

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