THE SUBUNIT STRUCTURE OF SEA URCHIN SPERM CHROMATIN: A KINETIC APPROACH

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1. Introduction

Many reports have appeared showing that digestion of chromatin DNA by endonucleases results in formation of fragments, apparently multiples of a unit length. Hewish and Burgoyne [1] initially reported the formation of these fragments in experiments of autodigestion of rat liver nuclei. The evidence for an oligomeric structure of the histones [2] led to the proposal of a repeating unit for the chromatin structure consisting of eight histones and 200 base pairs of DNA [3]. DNA fragments of about 200 base pairs in length have been obtained by adding micrococcal nuclease to rat liver nuclei [4], to mouse liver nuclei [5] and to developing trout testis nuclei [6]. In experiments with chromatin from duck reticulocyte nuclei [7], with chromatin from pea seedlings [8] and with chromatin from yeast [9], the monomer DNA has been found to be about 130 base pairs in length. Recently it has been shown that digestion of nuclei or of native chromatin from rat liver gives similar results [10]. At variance with these results are those on trout sperm chromatin that show no fragmentation pattern upon digestion [6]. We wish to compare here the DNA fragmentation pattern, obtained by digestion of the chromatin of nuclei of sea urchin sperms with different DNases, and the time course of the digestions. The electrophoretic size distributions of the DNA fragments observed at different digestion times with micrococcal nuclease suggest that sperm chromatin is homogeneously structured as a highly regular series of histone oligomers along the DNA molecule.

The DNA length, protected by each oligomer, depends on the percent DNA digested and ranges from about 160 base pairs, when about 3% DNA is digested, to about 110 base pairs as a limiting value. A value of 200 base pairs for the monomeric unit can be obtained only by extrapolation of the migration pattern of the longer fragments. This suggests that there is a minimum DNA length, smaller than the 200 base pairs of the complete unit, able to bind histone oligomers, and which is protected from enzyme hydrolysis.

Similar analyses with DNase I and II, give different fragmentation patterns, though these enzymes digest chromatin as efficiently as micrococcal nuclease.

2. Materials and methods

2.1. *Preparation of sea urchin sperm heads*

Paracentrotus lividus sea urchins were collected from the Bay of Naples. The animals were opened and sperms obtained by shaking the gonads in filtered sea water. The sperms were pelleted by low speed centrifugation, washed three times with filtered sea water and then filtered through a nylon net. Sperm heads were prepared according to Mizuno et al. [11] with minor modifications. Sperms were washed three times with 4.25% KCl and then once in a buffer containing 0.25 M sucrose, 0.05 M Tris, 0.005 M $MgCl_2$ (pH 7.5) and 1% Triton. The same buffer without Triton was used to wash out the detergent. Sperm heads were isolated by centrifuging the suspension at 14 000 *g* for 10 min through a solution containing 2 M sucrose, 0.05 M Tris, 0.005 M $MgCl_2$ (pH 7.5). The purified sperm heads, collected as a pellet, were used within 24 hr of their preparation.

2.2. *Digestion of chromatin in the sperm heads*

All the digestions were carried out at 30°C. For

DNase I (Worthington) digestion, sperm heads were suspended in the following buffer: 5 mM Na phosphate, 3 mM $MgCl_2$, 0.25 M sucrose (pH 6.7). For DNase II (Sigma) digestion, the buffer was 0.25 M sucrose, 0.25 M Na acetate, 1 mM EDTA (pH 5.5). Digestions with micrococcal nuclease (Worthington) were carried out according to Noll [4]. The DNA concentrations of the sperm head suspensions were between 0.3 and 0.45 mg/ml as determined by the Diphenylamine reaction [12] and by spectrophotometric measurements on the acidic supernatant after PCA hydrolysis of sperm heads at 75°C for 45 min [13]. For the preparation of fragments, samples were removed from the incubation mixture at the specified times and the reaction was stopped by addition of 1 part of a solution containing 20% SDS to 9 parts of sample. For the kinetics of DNA digestion, samples were removed from the incubation mixture and diluted in two volume of ice cold 0.5 M PCA; the precipitates were centrifuged and the optical density determined on the supernatants at 260 nm. DNA in the supernatants and pellets was also determined with the Diphenylamine reaction of Burton [12].

2.3. *Preparation and analysis of the DNA fragments*

Samples in 2% SDS were made 1 M in $NaClO_4$, and the solutions were mixed with an equal volume of chloroform–isoamylic alcohol (24 : 1, v : v) and shaken at about 130 cpm for 30 min at 4°C. The mixtures were centrifuged at 3000 *g* for 15 min to separate the two phases and the interphase was extracted a second time. The chloroform–isoamylic alcohol extractions of the aqueous phase were repeated until no more interphase appeared. The aqueous phase was then precipitated overnight at –20°C after addition of two volumes of ethanol. DNA from DNase treated chromatin was collected by centrifugation. The DNA was suspended in 20 mM Tris (pH 7.4) and incubated with 25 μg/ml of RNase for 2 hr at 37°C, then treated for the same length of time with 25 μg/ml of preincubated pronase. The solution was then extracted with chloroform–isoamylic alcohol and the DNA was precipitated overnight as described above. DNA samples were then dissolved in a small volume of 20 mM Tris, 1 mM EDTA (pH 7.4) and stored at 4°C. Samples digested with DNase I were also dissolved in 100% formamide. Electrophoreses of the DNA fragments were carried out according to Loening [14] in 2.5% acrylamide gels. Electrophoreses in denaturing conditions were carried out in 100% formamide according to Stoynov, Pinder and Gratzer [15]. The gels were scanned in a Gilford Spectrophotometer at 260 nm or at 545 nm after staining with 'Stains all' (Eastman Kodak) to locate positions of the DNA bands. Gels in formamide were washed in distilled water before scanning at 260 nm. The lengths of the DNA fragments were determined by comparing their migration distances with those of fragments of λ DNA obtained by digestion with *B. subtilis* restriction nuclease.

3. Results and discussion

In fig.1 are reported typical time courses of the hydrolysis of DNA in sperm heads treated with nucleases. Micrococcal nuclease and DNase I were used at concentrations that solubilized similar amounts of DNA; DNase II was used at a much lower concentration to determine whether there were effects depending on the enzyme concentration. Fig.2 shows gel scans of the DNA fragments remaining after different digestion times with micrococcal nuclease. At the shortest incubation time, when only about 3% DNA is digested into soluble fragments (see fig.1), more than 50% of the chromatin DNA has already been cut into pieces that show a regular pattern of migration.

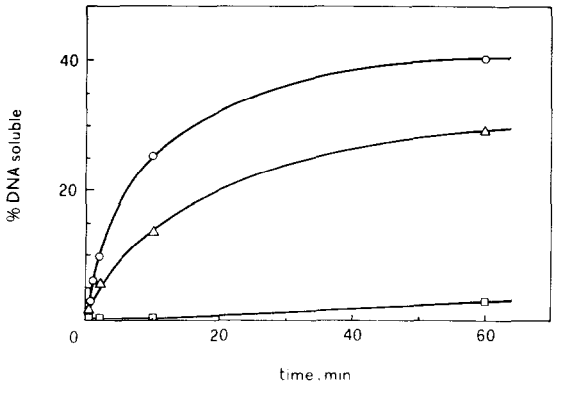

Fig.1. Digestion kinetics of sperm heads chromatin with (○——○) DNase I, 23 U/ml; (△——△) micrococcal nuclease, 600 U/ml; (□——□) DNase II, 32 U/ml. Conditions for digestions as described in Materials and methods.

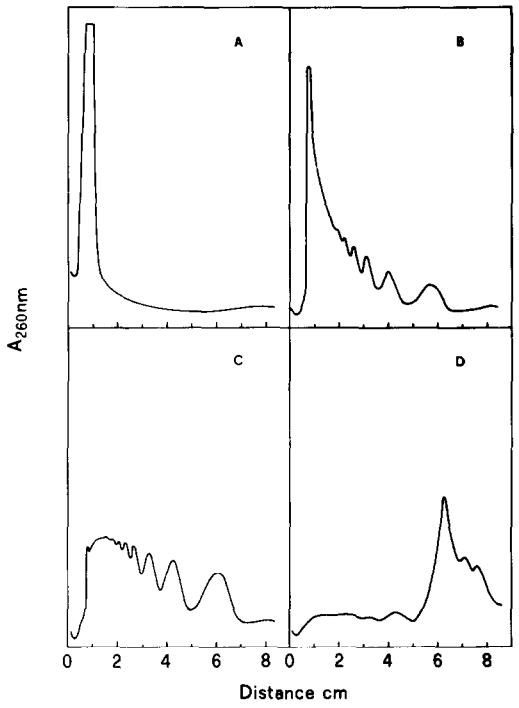

Fig.2. Densitograms of electrophoretic separations on 2.5% polyacrylamide gel of DNAs prepared from untreated sperm heads (A) and from sperm heads digested with 600 U/ml of micrococcal nuclease for the following lengths of time: (B) 30 sec, (C) 1 min and (D) 60 min. Electrophoresis was carried out for 2 hr at 5 mA per gel at room temperature.

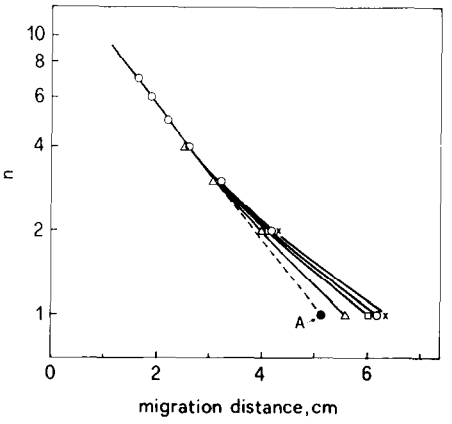

Fig.3. Log plots of band number versus distance migrated on the gel. Digestion times: (△——△) 30 sec; (□——□) 1 min; (○——○) 2 min; (X——X) from 10 min to 1 hr. For points corresponding to n values greater than 2, not all symbols are shown because they overlap. The two minor bands shown in fig. 2D, are not considered in this plot. Point A is discussed in the text.

When the percent DNA soluble in PCA increases, the amount of larger fragments decreases and when about 25% DNA is soluble the remaining 75% DNA migrates essentially in one band. After 1 hr of digestion (fig.2D), when about 40% DNA is soluble in PCA, two additional bands are observed ahead of the main band. Similar bands were also reported by Axel et al. for duck reticulocyte [7]. When the log of the band number, starting with the fastest moving one, is plotted against the distance migrated in the acrylamide gel, ignoring the two shoulders appearing at 1 hr (see fig.3), the points fall almost on a line for the shortest incubation time. At longer incubation times, bands 1 and 2 show an increase in mobility up to 10 min, but remain constant thereafter. The lengths corresponding to the different positions of the fastest moving band range from about 160 base pairs, at short incubation time, to a limit of about 110 base pairs, at long incubation time. The two shoulders observed ahead of the main band (fig.2D) correspond to about 60 and 50 base pairs in length. A unit of about 200 base pairs was determined from the migration distance corresponding to point A (see fig.3). This point is the intersection of the horizontal line (n = 1) with the limiting tangent (dotted line) of the curve at the highest n values. These data indicate that part of the 200 base pairs of the complete unit can be digested by nucleases without complete destabilization of the unit. There is, however, a minimum DNA fragment, about 110–130 base pairs in length able to resist nuclease attack. The two shoulders, observed at long incubation times, point to a specific and slower cleavage step leading to the fast and complete hydrolysis of the resistant unit. These two smaller fragments may bind much more weakly the histone oligomer, and consequently, may dissociate and be rapidly hydrolyzed. Indeed no fragments smaller than about 50 base pairs were ever apparent.

In the conditions of fig.1, a pattern similar to that observed at 30 sec of digestion with micrococcal nuclease is observed at 1 hr of digestion with DNase II, when again about 3% DNA is soluble in PCA. This pattern, however, remains essentially the same when

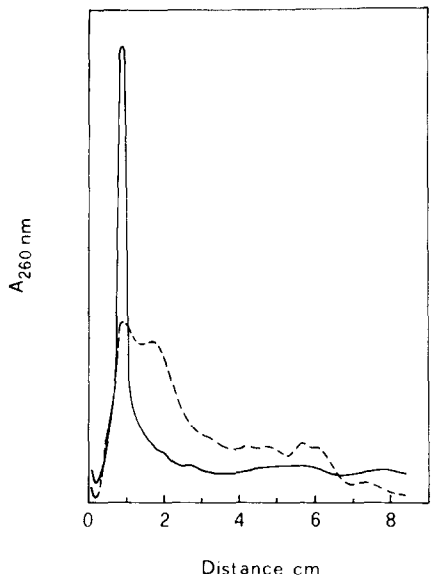

Fig.4. Densitograms of electrophoretic separations of DNA prepared from sperm heads digested with 23 U/ml of DNase I for 1 hr: (——) 2.5% polyacrylamide gel; (– – –) in dissociating conditions, on a 4% polyacrylamide gel in 98% formamide. Electrophoresis in formamide was carried out for 6 hr at 4 mA per gel at room temperature.

a higher percentage of DNA is hydrolyzed, indicating that the monomers have a lower stability with DNase II than with micrococcal nuclease. The digestion with DNase I shows a completely different size distribution pattern. As shown in fig.1, DNA is digested as efficiently with DNase I as with micrococcal nuclease, but as shown in fig.4, the size distribution of the DNA fragments is almost exclusively in the high molecular weight range. The results demonstrate that sperm chromatin has little or no regularly spaced regions available for digestion for DNase I.

In conclusion, it is evident that sea urchin sperm chromatin is readily available for digestion with all nucleases, and the tight packing of the chromatin in the sperm head does not appear to restrict substantially the action of the enzymes. A model of regularly spaced histone oligomers on the DNA molecule is in perfect agreement with the micrococcal and DNase II data. It is relevant to point out that the size distribution observed during the hydrolysis with these enzymes is a function of the nuclease type and of the percent of DNA hydrolyzed. The highly ordered structure that sperm head chromatin shows when digested with micrococcal nuclease or DNase II cannot be correlated with any special function since this chromatin is not a good template for RNA or DNA synthesis [16]. It seems plausible, instead, to associate this organization with a resting chromosome structure.

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