SELF REDIGESTION OF NATIVE CHROMATIN

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SUMMARY: Chromatin prepared by micrococcal nuclease digestion of nuclei and fractionated by sucrose density gradient ultracentrifugation self-redigests to a higher percentage of monomer DNA at a rate which depends on the enzyme concentration in the initial digestion. Thus, micrococcal nuclease becomes part of the native chromatin structure. A degree of caution should therefore be exercised when handling material prepared by this technique.

INTRODUCTION

Micrococcal nuclease digestion of rat liver nuclei produces DNA fragments which size on gel electrophoresis as multiples of a unit size (1) in a manner previously shown for an endogenous nuclease (2). Evidently, native histone-DNA interactions stabilize a conformation which permits DNA hydrolysis only at evenly spaced intervals. Brief, *in situ* digestion with micrococcal nuclease has been used to prepare chromatin which has never been precipitated or sheared, and which therefore retains a high percentage of these discrete nuclease sensitive sites (native chromatin: 3).

The distribution of DNA in the gel electrophoresis band pattern reflects the extent of digestion, determined by the product of enzyme concentration and incubation time. Background staining material between bands remains low as the percentage of monomer DNA increases at the expense of higher oligomers. Redigestion of native chromatin with added nuclease also yields an increased percentage of monomer without deterioration of the band pattern. Micrococcal nuclease digestion of nuclei and chromatin is an unusual tool for biochemical research: the same procedure has been used to give structural information, to prepare chromatin and to assess whether or not a chromatin sample retains its native conformation.

We describe here the self-redigestion of rat liver native chromatin.

When mixed with redigestion buffer (3) and incubated at 37°C without added nuclease, samples show an increased percentage of monomer DNA. The rate of digestion depends on the enzyme concentration in the original digestion. Chromatin fractionated by sucrose density gradient ultracentrifugation self-redigests at a rate which also depends on the original nuclease concentration. We conclude that the micrococcal nuclease used to prepare chromatin must be the agent responsible for self-redigestion, and that it remains tightly bound to chromatin.

METHODS

Fresh rat liver nuclei were prepared (4), washed and resuspended to A_{260} = 30 in digestion buffer (0.34 M sucrose, buffer A, 1 mM $CaCl_2$) and preincubated five minutes at 37°C. Samples were then digested with I) 75 U/ml and II) 15 U/ml of micrococcal nuclease, and III) without added enzyme. Aliquots were taken from I and II after 2, 5, 10, and 25 minutes of digestion and from III after 25 minutes incubation, and deproteinized for gel electrophoresis.

DNA was deproteinized in a 2.5 ml aqueous phase containing 1% SDS, 1 M NaCl with an equal volume of phenol-chloroform-isoamyl alcohol 50:48:2, then with chloroform-isoamyl alcohol 24:1, and precipitated overnight at -20°C with two volumes of absolute ethanol. Precipitates were recovered by centrifugation at 7500 x g and redissolved in 200 µl electrophoresis buffer (40 mM Tris Acetate pH 7.2, 20 mM Na Acetate, 1 mM EDTA). 5 µg of DNA was run with bromphenol blue and sucrose on each channel of a 3% acrylamide, 0.5% agarose slab gel until the dye was 1 cm from the bottom. An SV40-Hae III digest was used as a marker. The gel was stained with ethidium bromide at 1 µg/ml for two hours and photographed using a long wave UV lamp, orange filter, and Polaroid PN film (Figure 1).

We prepared native chromatin (3) by removing the bulk of the nuclei from I after two minutes and from II after ten minutes of digestion. After dialysis against 0.25 mM EDTA, samples were cleared of residual nuclear debris by a thirty minute ultracentrifugation at 75,000 x g. The supernatants, containing over 50% of the original nuclear A_{260}, were stored frozen at -20°C for up to several weeks.

The two chromatin samples were further fractionated by sucrose density gradient ultracentrifugation. Ten A_{260} units of I and II were layered on linear 12 ml 7.5-30% sucrose gradients containing 0.25 mM EDTA. Gradients were spun 19 hours at 75,000 x g. A high molecular weight chromatin sample was pooled from each gradient (Figure 1B; I-0 minutes and II-0 minutes) and incubated at 37°C at the same concentration in redigestion buffer (5 mM Tris Acetate, pH 7.8, 20 mM NH_4 Acetate, 0.4 mM $CaCl_2$, 0.2 mM EDTA). Samples for gel electrophoresis were taken 0, 5, 10, and 25 minutes after initiating self-redigestion (Figure 1B).

We have estimated digestion and self-redigestion rates by determining percentage monomer from a microdensitiometer trace of each channel of the negatives in Figure 1, plotting percentage monomer versus incubation time (Figure 2), and obtaining the slope (change in percentage monomer per

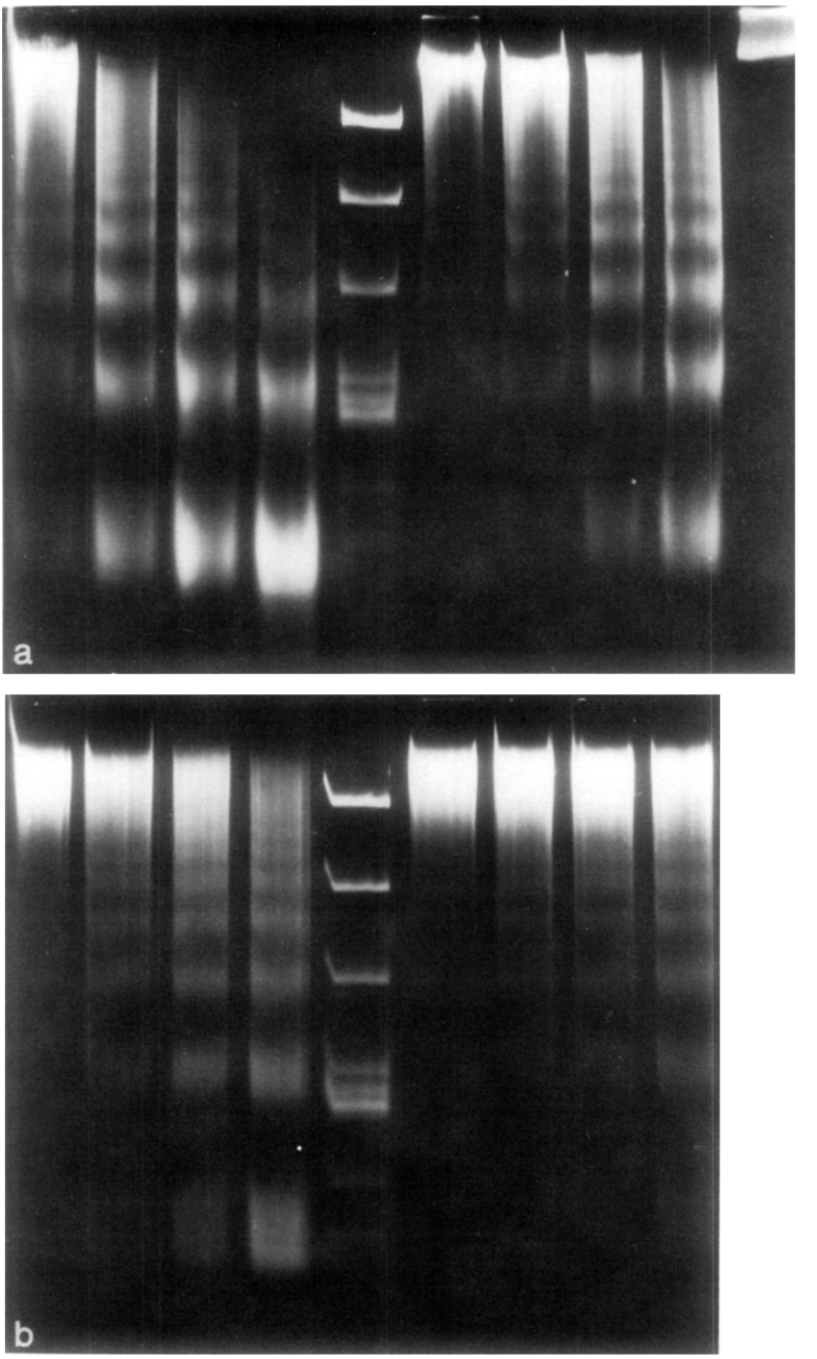

Figure 1. Gel electrophoresis of DNA. A) Digestion of nuclei. From left: I) 75 U/ml nuclease for 2, 5, 10, 25 minutes; SV40-Hae III: II) 15 U/ml for 2, 5, 10, 25 minutes; III) nuclei incubated 25 minutes without added nuclease. B) Self-redigestion of sucrose gradient fractions. From left: I) 0, 5, 10, 25 minutes; SV40-Hae III; II) 0, 5, 10, 25 minutes.

minute). Using percentage monomer allows us to compensate for variations in the total staining material in different channels. Areas under the scans were measured by planimetry using a horizontal base line, including a variable amount of submonomer staining material which increases with digestion time (Figure 1) and appears to be derived mainly from monomers which have been further degraded (3). The monomer value is the area enclosed between the trace and baseline, bounded by the minimum between monomer and dimer and the bottom of the gel; total staining material is simply the area enclosed between the entire trace and baseline.

RESULTS AND DISCUSSION

The distribution of DNA sizes is similar in channels I-2 minutes and II-10 minutes and in channels I-5 minutes and II-25 minutes (Figure IA). Native chromatin samples I and II, prepared after 2 and 10 minute incubations, respectively, have been digested to the same extent by different enzyme concentrations. As in the initial digestion, self-redigestion of these samples proceeds roughly in proportion to the original enzyme concentration: compare channels I-5 minutes and II-25 minutes in Figure 1B. No DNA in sample III, incubated as a control without added nuclease, runs faster than the limiting molecular weight (rightmost channel, Figure 1A). Thus, endogenous nuclease (2) did not contribute to digestion of samples I and II.

Figure 2A shows that the rates of digestion in samples I and II differ by a factor of four. Self-redigestion rates shown in Figure 2B differ by the same factor. The five-fold difference in enzyme concentrations is preserved, to within experimental error, in the ratio of rates of digestion and self-redigestion. Enzyme used in the initial digestion must therefore remain tightly bound to chromatin during subsequent fractionation procedures.

Comparisons between the rates of initial digestion and self-redigestion cannot be made at this time for a number of reasons, including differences in the buffers and chromatin concentrations used, and possible kinetic differences. Furthermore, the slopes of curves in Figure 2 should be converted to genuine velocities by multiplying the change in percentage monomer per minute by the A_{260} or substrate concentrations to yield the change in monomer concentration per minute. These considerations warrant further investigation; they do not alter conclusions drawn from comparisons

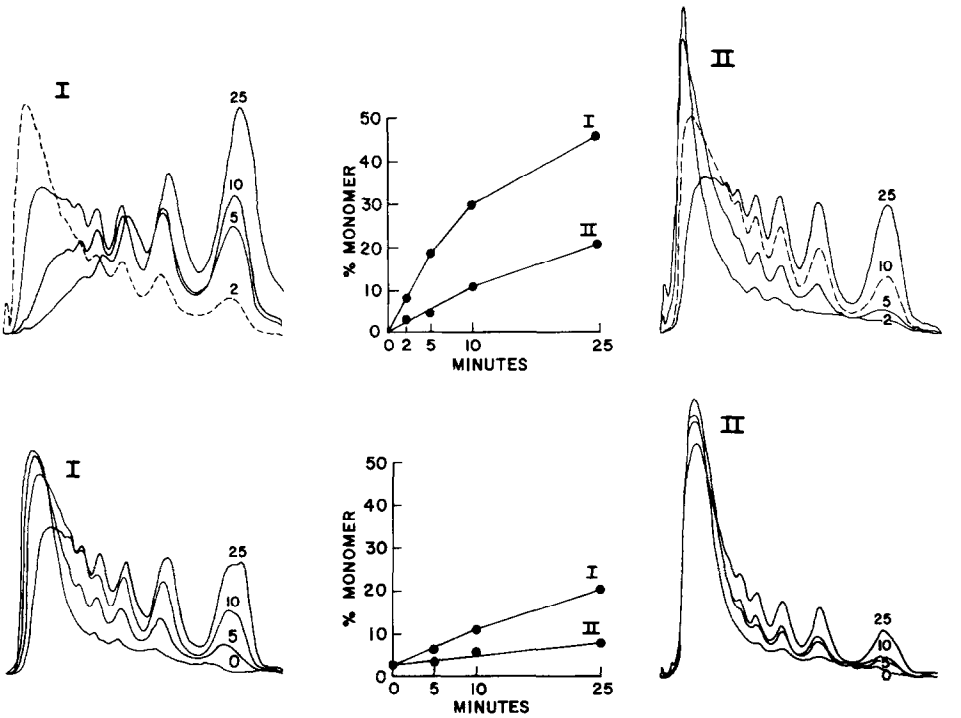

Figure 2. Superimposed microdensitometer traces of the channels in Figure 1 and plots of the rate of A) digestion and B) Self-redigestion of native chromatin from samples I and II. Samples used in B were obtained by sucrose density gradient ultracentrifugation of chromatin whose DNA size distribution is represented by the dashed traces in A.

between the behavior of samples I and II under the same incubation conditions.

Micrococcal nuclease used to prepare native chromatin becomes a part of the chromatin structure. A degree of caution should therefore be exercised in handling material prepared by this technique. The molecular weight of native chromatin remains essentially unchanged when it is dialyzed against 0.25 mM EDTA at 4°C for several hours, or stored frozen at -20°C for several weeks. Self-redigestion could be a complicating factor, however, in experiments with native chromatin incubated at or above room temperature.

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