CHROMATIN SUB-STRUCTURE.

THE DIGESTION OF CHROMATIN DNA AT REGULARLY SPACED SITES BY A NUCLEAR DEOXYRIBONUCLEASE.

Dean R. Hewish and Leigh A. Burgoyne

School of Biological Sciences, Flinders University of South Australia, Bedford Park, South Aust. 5042.

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SUMMARY : Evidence is presented that indicates a regular sub-structure in nuclear nucleoprotein with regularly distributed sites that are specifically susceptible to the cellular Ca-Mg endonuclease.

Recent work on the structure of the mammalian nuclear nucleoprotein complex (1-4) has shown that the accessibility of the nuclear DNA to deoxyribonuclease action is severely limited by the chromosomal proteins and few regions of uncomplexed DNA exist. In particular, specific histones seem to be important in limiting the sites of action of deoxyribonuclease (2). The possibility exists, therefore, that any periodicity of regularity of positioning of the histones on the nuclear DNA will be reflected in the spectrum of molecular weights produced in the DNA after deoxyribonuclease digestion.

Previous work in this laboratory (5-7) has shown the presence of an endo-deoxyribonuclease in isolated mammalian nuclei which digests the nuclear DNA *in vitro* when the nuclei are incubated in the presence of calcium ions and magnesium ions. This endonuclease, therefore, provides an excellent opportunity to study the structure of the nuclear chromatin in the whole isolated nuclei. This nuclease seems to be unique, in that it can attack nuclear DNA *in situ* very readily whereas the other free cellular nucleases in rat liver do not attack nuclear DNA *in situ* very readily at all (7).

In this report, studies on the structure of the nuclear chromatin utilising this nuclear system are described.

Materials and Methods - Nuclei were isolated from normal livers from male hooded wistar rats (3-4 months old) by the method previously described (5,6), with minor modifications (8). This method is based on buffer A* and uses polyamines as stabilizing agents instead of divalent metals.

Incubations were carried out in 0.34 M sucrose buffer A* with 1 mM EDTA, 0.2 mM EGTA**, 10 mM $MgCl_2$ and 1 mM $CaCl_2$. Incubations were carried out in 0.5 ml lots at 37^{o}.

Reactions were terminated by the addition of 4 ml of 0.02 M phosphate-K, 5 mM EDTA, then 0.5 ml of 10% Na-dodecyl sulphate. The samples were sheared by vortex mixer, made to 1.0 M NaCl and phenol extracted twice. The aqueous phase was dialysed overnight against 0.02 M tris-HCl, pH 7.4, 1 mM EDTA.

Samples were concentrated by dialysis against a concentrated solution of polyethylene glycol before electrophoresis.

Polyacrylamide electrophoresis was carried out by the method of Loening (9) with 2.5% acrylamide gels in siliconised glass tubes.

Gels were stained for 1 hour at 4^{o} in a solution of 20 µg/ml ethidium bromide in 10% acetic acid. Destaining is not necessary as the fluorescence of the bound dye can be observed by illuminating with U.V. U.V. at 354 mµ was suitable as an exciting wavelength.

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*Buffer A - 60 mM KCl, 15 mM NaCl, 0.15 mM spermine, 0.5 mM spermidine, 15 mM 2-mercaptoethanol, 15 mM tris adjusted to pH 7.4 with HCl.

**EGTA - Ethylene glycol-bis-(2-amino ethyl ether)-N,N'-tetra acetic acid.

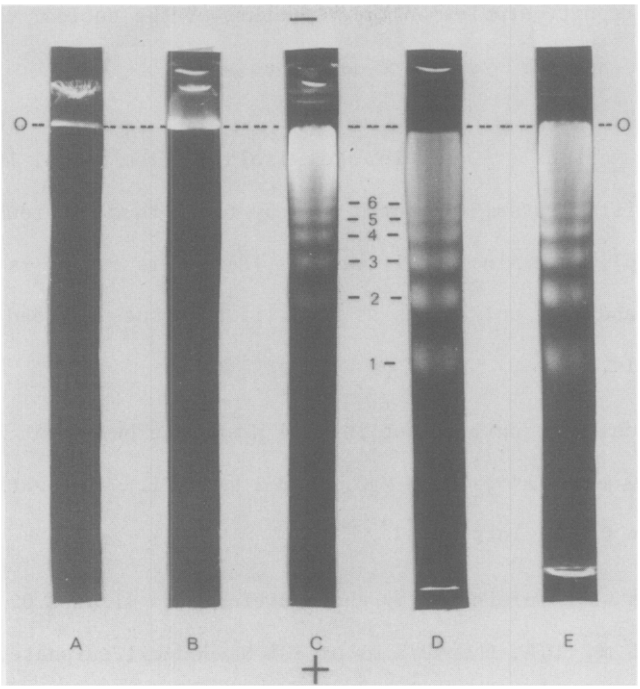

Figure 1. Band patterns obtained by the action of Ca-Mg endonuclease on nuclear DNA in situ.

Polyacrylamide gel electrophoresis of nuclear DNA digest by the Ca-Mg endonuclease. Rat liver nuclei were isolated and incubated in the presence of 1 mM $CaCl_2$ and 10 mM $MgCl_2$ as described in Methods, for (a) 0, (b) 20, (c) 40, (d) 60 and (e) 80 min. Under these conditions the Ca-Mg endonuclease in the nuclei attacked the nuclear DNA producing a limit pattern of fragments. DNA was isolated from the nuclei as described in Methods. Electrophoresis was carried out in 2.5% acrylamide gels for 1.5 hours at 5 mA per gel by the method of Loening (9). Gels were stained after electrophoresis with ethidium bromide as in Methods.

Results - Rat liver nuclear DNA was degraded in situ by the endogenous Ca-Mg endonuclease. The DNA was then purified and run on acrylamide gels. It gave a series of bands of increasing molecular weight (Fig. 1).

As degradation progressed, the quantity of DNA in the bands representing the lower molecular weight species at first increased but there appeared to be a limit pattern that was reached relatively quickly.

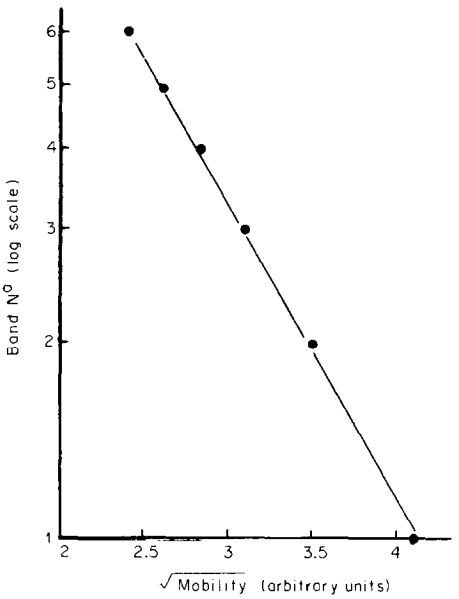

Figure 2. Evidence for a regular series of size-classes of fragments.

Analysis of the results in Figure 1. The relative mobilities of the DNA species observed in gels (c) and (d) of Fig. 1 were calculated from their distances from the origin of the gel. The DNA species are numbered 1-6 as shown in Fig. 1.

After this pattern of bands had been produced no new bands appeared on further incubation. Moreover, further incubation did not appear to significantly change the relative intensities of the bands although very prolonged incubation did seem to cause some widening of the bands.

When the bands were eluted from the gel and rerun they moved with their original mobility, indicating that the species of high molecular weight are not loose concatenates of the smaller species.

The square root of the mobility of the DNA molecules is proportional to the logarithm of their molecular weight in acrylamide gels of the type used here (10). When the relative mobilities of the bands observed were plotted in this manner they were found to lie on a straight line (Fig. 2) indicating that their molecular weights were multiples of the molecular weight of the smallest species.

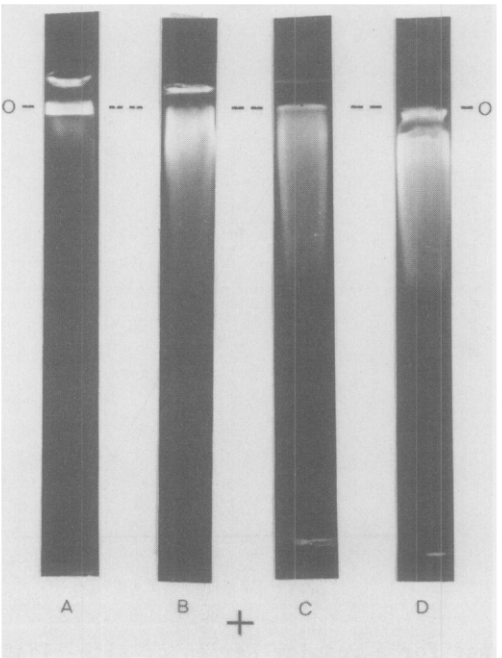

Figure 3. Failure to detect bands when crude Ca-Mg endonuclease digests free DNA.

Digestion of calf thymus DNA by Ca-Mg endonuclease extracted from rat liver nuclei. Ca-Mg endonuclease was extracted from rat liver nuclei as in Results (7). 0.1 ml of the extract was incubated with 0.4 ml of calf thymus DNA ($O.D._{260\ nm}$ = 70) in the presence of $CaCl_2$ and $MgCl_2$ as in Methods for (a) 0, (b) 45, (c) 90 and (d) 120 min. at 37°. After incubation the DNA was purified by phenol extraction and subjected to polyacrylamide gel electrophoresis as in Fig. 1.

The Ca-Mg endonuclease responsible for the degradation of the nuclear DNA was obtained as a crude extract by extracting with 0.14 M KCl, 0.1 M K-phosphate, pH 7.4 (7) and dialysing into 0.02 M tris, 5 mM $MgCl_2$, 20% ethylene glycol, 15 mM 2-mercaptoethanol, pH 7.4 with HCl (7). This crude enzyme was then used to degrade calf thymus DNA and rat liver DNA and the products of the degradation studied as the degradation progressed. The products always showed a continuous range of molecular weights and no discrete bands were ever observed (Fig. 3).

Pancreatic DNAase-I digested purified calf thymus DNA in a

similar manner, and also did not produce discrete bands in its product during the course of digestion.

When pancreatic DNAase-I was allowed to act on isolated nuclei in the absence of calcium and presence of EGTA (to prevent Ca-Mg endonuclease action) it degraded the nuclear DNA to a continuous range of molecular weights which did not show any discrete bands.

Discussion - When the chromatin of nuclei isolated by the buffer A technique (5,6) is digested by the nuclear Ca-Mg endonuclease a relatively stable limit digestion product is formed. This limit product appears to have a regular series of molecular weights. The regular series appears to be a series of multiples of the smallest size unit.

Series similar to these have been observed previously, although in a different context by Williamson (10). He reported similar molecular weight series in newly labelled DNA found in the cytoplasm of necrotic tissue cultures and discussed their possible origin.

As the Ca-Mg endonuclease does not appear to produce discrete bands while digesting naked DNA it seems most likely that the regularity of the digestion products produced from DNA digested in nuclei is a reflection of regularity in the distribution of protein on the DNA.

Pancreatic DNAase-I did not produce discrete size-classes of DNA fragments with nuclei. Thus the Ca-Mg endonuclease appears to be an unusually specific probe for some feature of chromatin structure.

These results have many potential explanations. However, one obvious and relatively simple explanation is as follows. It is proposed that chromatin has some simple, basic, repeating substructure with a repetitive spacing of sites that are potentially accessible

to the Ca-Mg endonuclease. However, some of these sites are sterically blocked by superstructure proteins. Thus the Ca-Mg endonuclease would produce fragments which were all equal to, or greater than, the basic substructure repeat distance and the larger ones would be all multiples of the smallest. The smallest would represent the substructure repeat distance.

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