Vol. 99, No. 3, 1981 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
April 15, 1981 Pages 893-899

CHROMATIN SUPERSTRUCTURE: THE NEXT LEVEL OF STRUCTURE ABOVE THE NUCLEOSOME HAS AN ALTERNATING CHARACTER. A TWO-NUCLEOSOME BASED SERIES IS GENERATED BY PROBES ARMED WITH DNAase-I ACTING ON ISOLATED NUCLEI.

L.A. Burgoyne and J.D. Skinner

School of Biological Sciences,
The Flinders University of South Australia,
Bedford Park, S.A. 5042.

Received February 9,1981

Summary: Intact nuclei were probed with DNAase-I-based probes and the resultant single strand pieces indicate that many or all of the nucleosomes exist in structures that cause every second nucleosome in a series to be resistant to attack. The effect is observed with chicken erythrocyte nuclei, rat liver nuclei and is weakly elicited by free DNAase-I. A method is described for the suppression of the intrinsic (Ca-Mg) endonuclease of rat liver nuclei.

Three major levels of coiling in interphase chromatin have been observed with chemical procedures. The coiling of the DNA around the nucleosome generates a 10 base-pair repeat (1, 2, 3, 10). The 200 base-pair repeat (4, 5) reflects the organisation of the histone into octomers (5) and very limited nucleolysis of chromatin indicate that there is some sort of organisation of nucleosomes into massive domains containing hundreds of nucleosomes (6). This paper addresses itself to the level of organisation between the nucleosome and the domains. The probes used in these experiments are all based on DNAase-I. This enzyme's effect on chromatin has been studied by many workers (e.g. 1, 2, 3, 10, 12), but in this report large probes armed with DNAase-I were used to attack chromatin. The attack pattern was then studied by sorting the denatured fragments on alkaline agarose gels.

Preparation of nuclei and suppression of resident hydrolases.

Phenyl methyl sulphonyl fluoride-treated nuclei were prepared from rat liver and chicken erythrocytes by the Tris buffer-A procedure (7, 8). Nuclei were finally dispersed and incubated in Hepes-buffered A. (60 mm KCL, 15 mm

0006-291X/81/070893-07$01.00/0

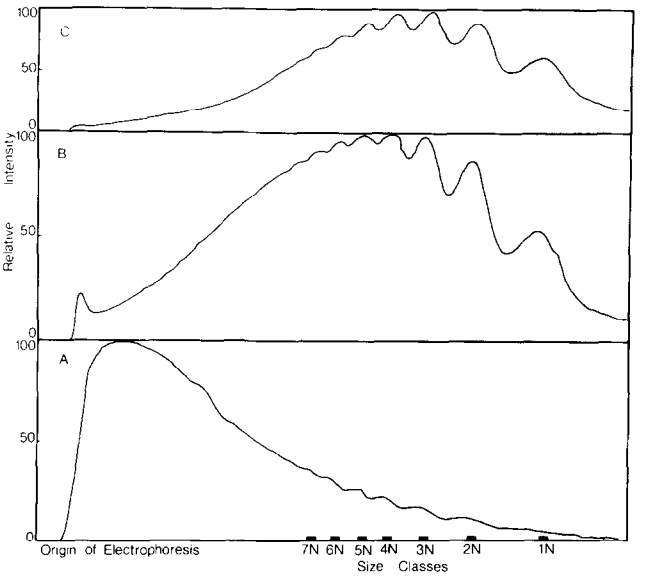

FIGURE 1 Suppression of the intrinsic Ca-Mg endonuclease of rat liver nuclei by reaction with dithiobisnitro benzoate.

Rat liver nuclei prepared and reacted with dithiobisnitro benzoate as described, and then incubated in Hepes-A-EDTA buffer 1 mm Ca^{++}, 5 mm Mg^{++} for 25 mins at 37°. Separation on the standard 1.5% Agarose alkaline gels at 10 MA per sq cm. Curve A - Dithiobisnitro benzoate treatment only. Curve B - As for A but plus 15 mm 2-mercapto ethanol. Curve C - No Dithiobisnitro benzoate treatment.

Hepes-acid, 0.15 mm spermine, 0.5 mm spermidine, 0.1 mm EDTA, 15 mm NaOH and HCl to pH 7.4. When present, 2-mercapto ethanol was 15 mm). Rat liver nuclei had their resident Ca-Mg endonuclease suppressed by centrifuging through a cushion of 0.8 M sucrose 2.0 mm Na^{+} dithio-bis-nitro-benzoate at pH 7.4 in Hepes-A without its usual 2-mercapto ethanol (fig 1). The inhibition is not quite complete but it is sufficient for these studies and it is highly reversible with thiol agents. The Ca-Mg endonuclease is not present in chicken erythrocytes so no treatment is necessary.

Preparation of probes armed with DNAase-I.

Dialdehyde glycogen (8) reacted too slowly with DNAase-I to be useful as the high-molecular-diameter-base of the probe so Ferritin and other proteins were used to generate the probes base. An example preparation of a probe is as follows. 25 mg of Ferritin and 10 mg of DNAase-I in 2.5 ml Ca-

borate-glycol buffer (8). (20 mm $CaCl_2$, 10 mm boric acid, 0.4% ethylene glycol, 0.1 mm EDTA, KOH to pH 8.5), incubated at 0^oC. Approximately 70 µl neutral, 25% glutaraldehyde was added in 5-20 µl lots over approximately 40 minutes at 0^oC and the cross-linking was monitored by assaying the DNAase-I activity. The glutaraldehyde crosslinking was terminated when the effective DNAase activity fell to approximately 15% of its initial activity. The reaction was terminated by adding 0.25 ml of 0.1 M ethanolamine-HCl pH 8.5 and 50 mg sodium cyanoborohydride to reduce unstable adducts. This was stored overnight at 0^oC to allow reactions to go to completion.

Removing low molecular weight species from the crude probe preparations.

Sepharose-2B columns were equilibrated and run in the Hepes-A-buffer (without 2-mercapto ethanol) containing 1 mm $CaCl_2$, 10% ethylene glycol. To disrupt any traces of loose disulphide crosslinks the sample was reduced and reoxidised at the top of the preparative column as follows. The load was treated with 5 mg dithiothreitol for 10 minutes and loaded onto the column, immediately following a similar volume of 1%, oxidized 2 mercapto ethanol (dihydroxyethyldisulphide, K & K suppliers) in column buffer. Fractions were collected at $0-4^o$ and stored at -81^o. The product had a wide range of aggregates many of which were much larger than Ferritin.

DNA extraction and alkaline agarose electrophoresis.

DNA was extracted by standard SDS-salt-phenol procedures. The gels of McDonnell *et al.* (9) were used. 1.5% Agarose gels were prepared in (30 mm NaCl, 2 mm EDTA, pH 7.4), briefly prerun with (30 mm NaOH, 2 mm EDTA) to remove Cl^- from the load zone and then run in the same NaOH electrolyte. Gels were neutralised and stained with Ethidium bromide by standard methods.

The observed repeat and the appearance of the peaks.

Figures 2, 3 and 4 show the same simple observation of a 'repeat' based on ~400 base pairs (2N-based). This 2N-based repeat can be observed in both chicken red blood cells and in rat liver nuclei. It can be exposed by DNAase-I attached to Ferritin (fig. 2) or Bovine serum albumen (results

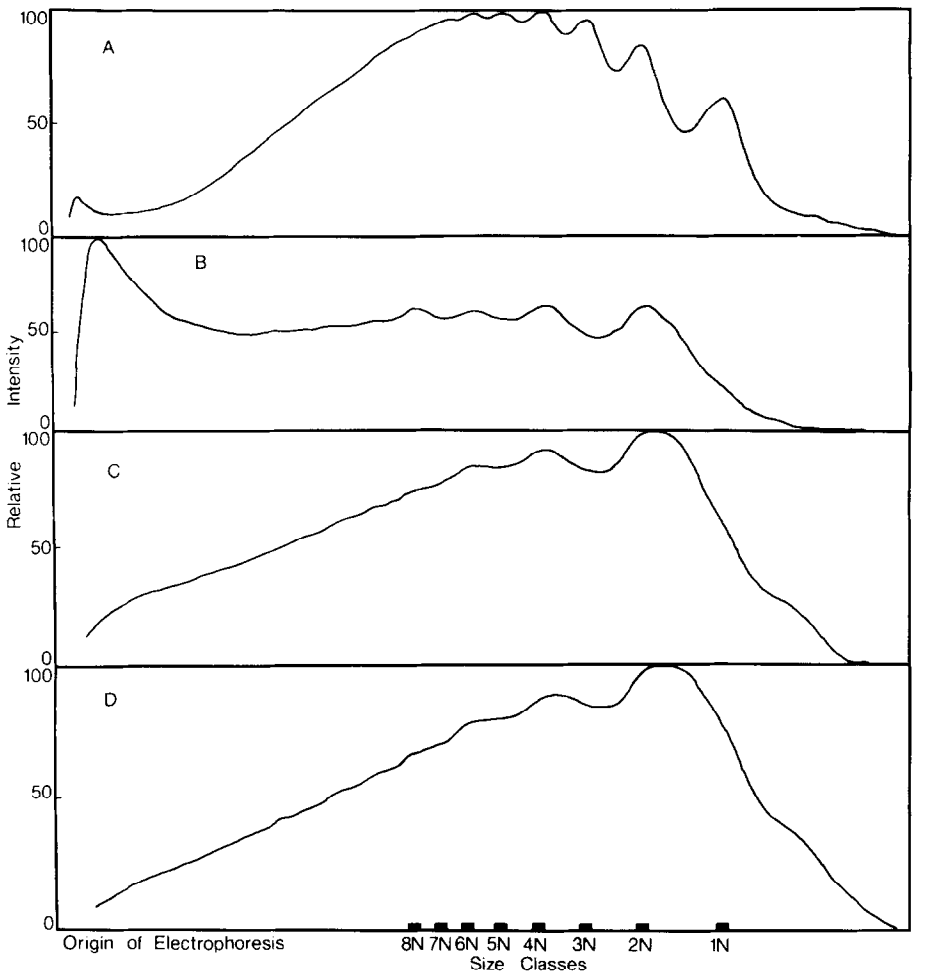

FIGURE 2 Ferritin based, DNAase-I armed, probe attack on chicken erythrocyte nuclei. Fractionation of fragments on alkaline gels.

Digestions contained 9.2 E_{260}* units/ml of nuclei and DNAase-I armed probe activity equivalent to that of 5 µg/ml free DNAase-I. The DNAase-I armed probe was taken from the void volume of a Sepharose 2B fractionation. Digestion conditions - 37°, 0.5 mm $CaCl_2$, 5.0 mm $MgCl_2$ in Hepes-buffered-A. Electrophoresis in alkaline 1.5% agarose (9) 2 mm thick with current densities of 10 MA/squ cm. Curve A - Standard micrococcal nuclease fragments from chicken erythrocyte nuclei. Curve B - 30 mins digestion Ferritin-DNAase-I complex. Curve C - as for B, 90 mins digestion. Curve D - as for B, 180 mins digestion. *One E_{260} unit of nuclei refers to the optic density generated when whole nuclei are lysed in 1% NaOH.

not shown) and can be weakly observed using free DNAase-I alone (fig. 4). Under the best conditions, the 2N-based repeat was the only repeat observable although the peaks were always much broader and lower than the corresponding peaks of standard 1N series generated by micrococcal nuclease or intrinsic

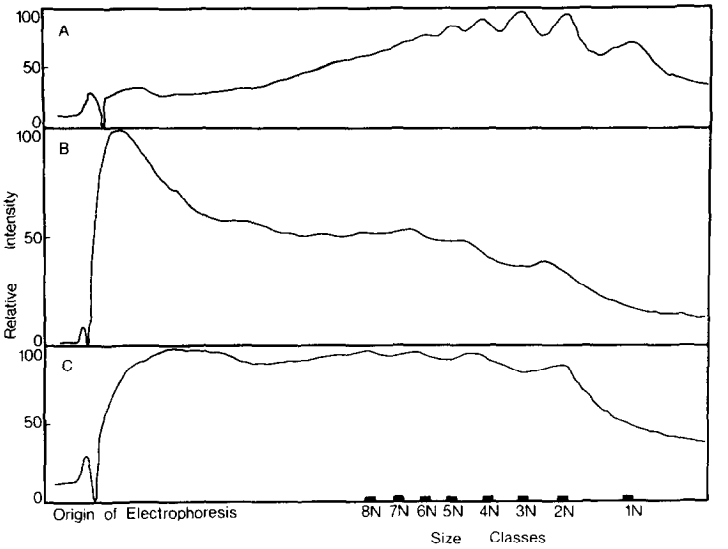

FIGURE 3 Ferritin based, DNAase-I armed probe attack on rat liver nuclei. All conditions as for the experiment shown in Figure 2. 25 mins digestion time. Curve A - Standard 1N, 2N, etc. series producted by autolysis of rat liver nuclei by their intrinsic Ca-Mg nuclease. Curve B - Rat liver nuclei digested with Ferritin-DNAase-I as in Fig. 2. 15 mins digestion. Curve C - As for Curve B, 30 mins digestion.

Ca-Mg endonuclease. The higher order bands (4N, 6N, 8N) were frequently observed although 8N and 6N were often so blurred that multiple tracks had to be examined to be confident of their presence. This spreading of the higher

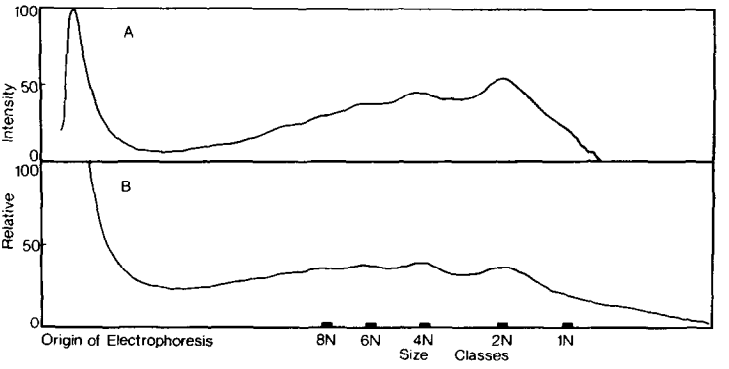

FIGURE 4 Comparable digestion of chicken erythrocyte nuclei with free DNAase-I. Chicken erythrocyte nuclei were exposed to free DNAase-I under the same assay conditions as Figure 2, but using free DNAase-I. Curve A - 0.5 min digest with 150 µg/ml free DNAase-I. Curve B - 5 mins digest with 3 µg/ml free DNAase-I.

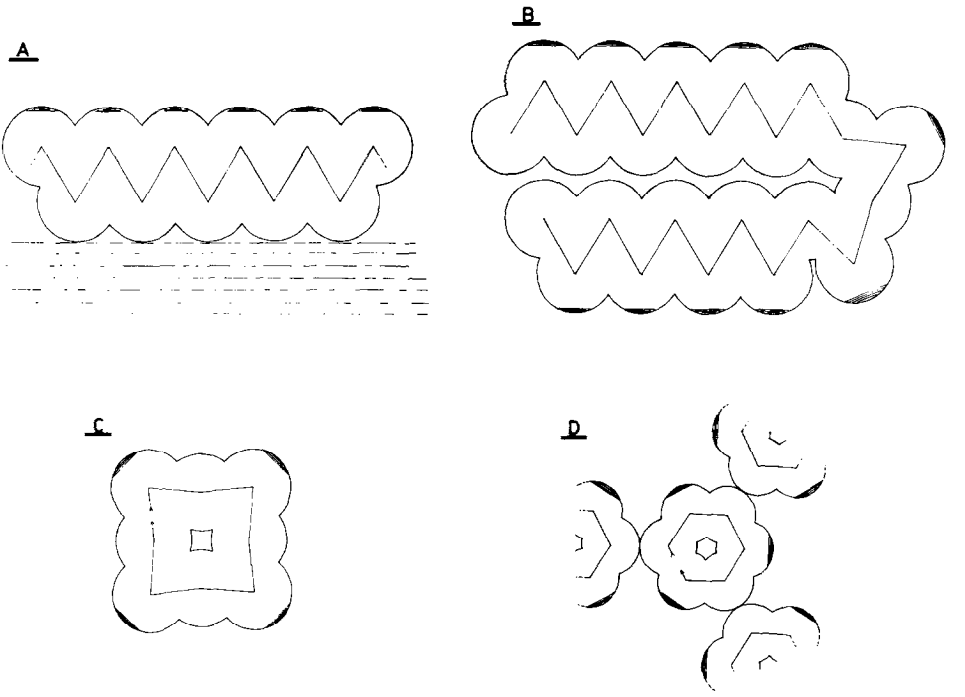

FIGURE 5 Nucleosomal folding patterns that would be expected to generate an alternating physical accessibility of nucleosomes.

Nucleosomes are represented as spheres in order to avoid detailed assumptions about their shape. The term 'nucleosome' is being used to refer to the whole complex containing the H1 histone and the bridge-DNA together with the core particle. The shaded zones on the accessible, outermost portions of the most approachable nucleosomes indicate the zones susceptible to nucleolysis by high molecular diameter probes. Attack on these zones will generate a 2N repeat in any of these four structures. Model a, b and c generate the alternation at the nucleofilament level. Diagram D generates it by the pattern of coil-stacking.

order peaks may indicate that the sites of nucleosomal susceptibility are zones containing two or more sites. This is supported by the observation that a number of the 2N peaks produced after long digestions, (e.g. fig. 2, curves C and D),were clearly not simple peaks symmetrical about the 2N mobility-position.

DISCUSSION.

The first and simplest way of explaining this repeat is to postulate an alternating physical projection or protection as shown in fig. 5. Another explanation, which could also be true, is to postulate that every second nucleosome has a different orientation so that a one-sided attack on a

nucleofilament or coil will result in an alternation of susceptibility. These two explanations are not mutually exclusive. The third possible explanation is to suggest that every second nucleosome is somehow compositionally different to its neighbours. We know of no evidence for this latter explanation and are inclined to consider it an unlikely explanation. The electron microscope studies of Thoma, Koller and Klug are quite compatible with the models shown in figures 5a, 5b and 5c (ref. 11). The simple zig-zag structure they suggest could easily generate either or both an alternation of nucleosomal orientation and an alternation of nucleosomal projection. However, it should be noted that there are other conceivable models (e.g. fig. 5d) although less supported by electron microscopy, that could also explain the alternation of nucleosomal susceptibility. If the diffuseness of our patterns is due to a mixture of degradation modes, then the 2N-based repeat would have to be a major one, implying that considerable 'inactive' DNA must be in this state. Although it is possible that there is considerable disorder disrupting the basic 2N-repeat, some stretches of it are at least 8 nucleosomes long as the 2N-based series of peaks has been followed to 8N.

Acknowledgements: This work was supported by the U.S. Department of Health, Education and Welfare, and the Australian Research Grants Committee.

REFERENCES:

1. Noll, M., (1974) Nucleic Acids Research 1, 1573-1578.
2. Noll, M., (1977) J. Mol. Biol. 116, 49-71.
3. Lutter, L.C., (1977) J. Mol. Biol. 117, 53-69.
4. Hewish D.R. and Burgoyne, L.A., (1973) Biochem. Biophys. Res. Commun. 52, 504-510.
5. Kornberg, R.S., (1974) Science 184, 868-871.
6. Igo-Kemenes, T., and Zachau, H.G., (1978) Cold Spring Harbor Symposium on Quantitative Biology XLII, 109-118.
7. Burgoyne, L.A. and Mobbs, J.D., (1975) Nucleic Acids Research 2, 1551-1558.
8. Burgoyne, L.A. and Skinner, J.D., (1979) J. Cell Sci. 37, 85-96.
9. McDonnell, M.W., Simon, M.M., Studier, F.W., (1977) J. Mol. Biol. 110, 119-146.
10. Sollner-Web, B. and Felsenfeld, G., (1977) Cell 10, 537-547.
11. Thoma, F., Koller, Th., and Klug, A., (1979) J. Cell Biol., 83, 403-427.
12. Zachau, H.G., Altenburger, W., Greil, W., Horz, W. and Igo-Kemenes, T., (1977) International Symposium on the Organisation and Expression of the Eukaryotic Genome. Tehran (1977) Bradbury, E.M. and Javaherian, K. (eds.) Academic Press.