Volume 10 Number 2 1982 Nucleic Acids Research

Avian erythrocyte chromatin degradation: the progressive exposure of the dinucleosomal repeat by bovine-pancreatic-DNAase-I-armed probes and free DNAase-I

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Received 3 December 1981; Accepted 11 December 1981

ABSTRACT

Bulky probes composed of glutaraldehyde-cross-linked-complexes of ferritin or bovine serum albumen armed with bovine pancreatic DNAase-I have been used to generate the typical dinucleosome-based series of native or denatured DNA fragments from chicken erythrocytes. Double stranded DNA fragments have shown that the 2N series extends to at least 10N without any clearly defined end-point. The susceptible nucleosomes were observed to be nucleolysed by two major, different methods of attack that generated multiple peaks in the dinucleosomal size class and some characteristics of the digestion patterns have been taken as indications of the presence of another level of structure above that of the alternating structure. The chromatin structure becomes resistant to the probes when the average fragment length falls to approximately 4N but this 'core' is not a single integral multiple of nucleosomes.

INTRODUCTION

It is now generally accepted that the bulk of eukaryotic chromatin has a basic repetitive, nucleosomal, substructure and there is a growing body of evidence (1, 2, 3) indicating that many of the nucleosomes are organised into 'zig-zag' nucleofilaments.

Electron microscope studies of the various structures formed during chromatin assembly first suggested that the nucleofilament may be formed of nucleosomes arranged in some alternating fashion (1, 2) although shape and linkage number considerations have also suggested this (4).

However, the biochemical evidence for a nucleofilament of zig-zag-arranged nucleosomes mainly comes from the observation that DNAase-I-armed probes produce a dinucleosomal repeat (8, 3, 9).

This paper presents evidence from studies that have used DNAase-I-armed probes and 'free' bovine pancreatic DNAase-I to further explain this peculiar selectivity of DNAase-I with respect to chromatin superstructure while describing further features of this structure.

Nuclei, probes and digestion conditions

Chicken erythrocyte nuclei were used because of their low level of contaminating endonuclease activities (7). Bovine pancreatic DNAase-I-armed probes were prepared and digestion conditions set up as previously described (3).

0305-1048/82/1002-0665 $2.00/0

Preparation of load-zone gel blocks and electrophoresis conditions

DNA fragments were prepared from digests by standard salt-phenol procedures then were usually pre-embedded in small blocks (2 mm x 4 mm x 11 mm) of 0.75% Agarose. These small oblong blocks were used instead of load wells as high concentrations of DNA loaded from wells ran, disproportionately, at the sides of the tracks.

The 1.5% agarose running gels were approximately 2 mm x 10 cm x 17 cm. Denaturing gels were prepared and run in NaOH as previously described (3) at 10 MA per squ.cm and native fragments were run in (20 mm Na_4Pyrophosphate, 2mm EDTA-acid, final PH is 9.0) at 14 MA per squ cm.

Gels were neutralised, if necessary, and stained with ethidium bromide by standard procedures. During the staining procedures the main gel was usually accompanied by a set of standard gel slices that had embedded in them known amounts of calf thymus DNA. The standard gel slices were pre-equilibrated with the appropriate electrophoresis buffer so that the whole process of stain-adsorbtion and background-clearing would be as parallel to that occurring in the main gel as possible. The scanner output from the standard slices was used to generate a standard curve and this standard curve was used to convert the scanner output from the main gel to DNA concentrations. The DNA concentrations were then 'normalised' by being expressed as a proportion of the average DNA concentration of the gel.

The double-stranded fragments produced with bound DNAase-I

The first phase of the digests with ferritin-bound DNAase-I shows a 2N-based series that appears to be traceable to 10 to 12N and in which a 2N -∿ 70Bp peak only appears later in the digest (Fig 1). We also observe the intermittent existence of a weak peak at approximately 2N + 70Bp. In any one experiment it is usually in the same order as the 'noise' variations but averaging experiments have indicated its reality.

This also appears to be broadly true for BSA-bound DNAase-I. In both cases the latter part of the digestion is characterised by a loss of the species above ∿4N with a very pronounced limit at 2N-70Bp and 2N although these do not seem to constitute a definite 'core' structure, as all our digests appeared to be stopping while there were still 4N and 6N peaks present. Double stranded 1N-sized pieces have never been seen as a significant class in any digestion of chicken erythrocyte nuclei with bound DNAase-I.

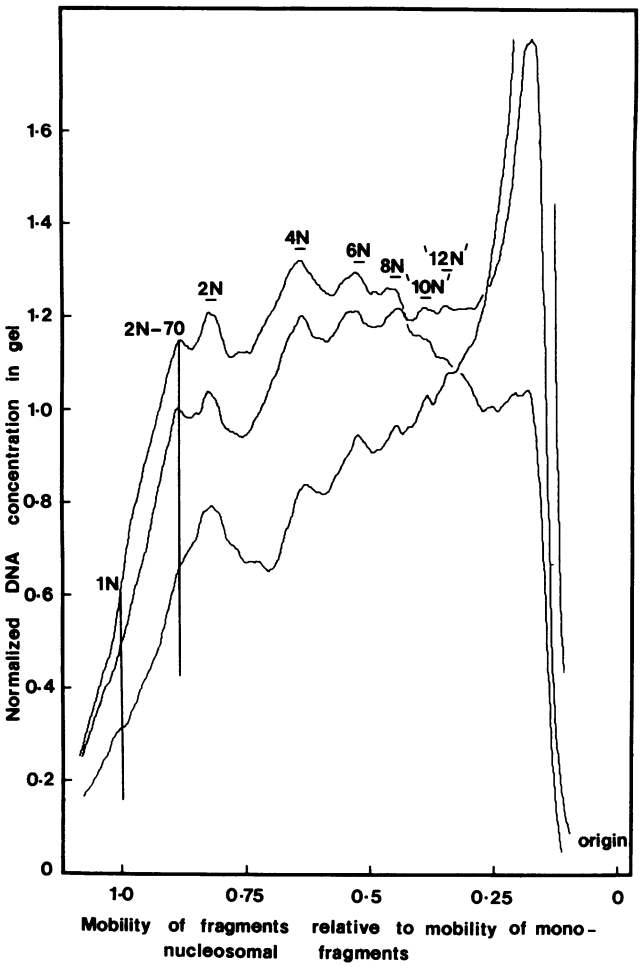


FIG 1.

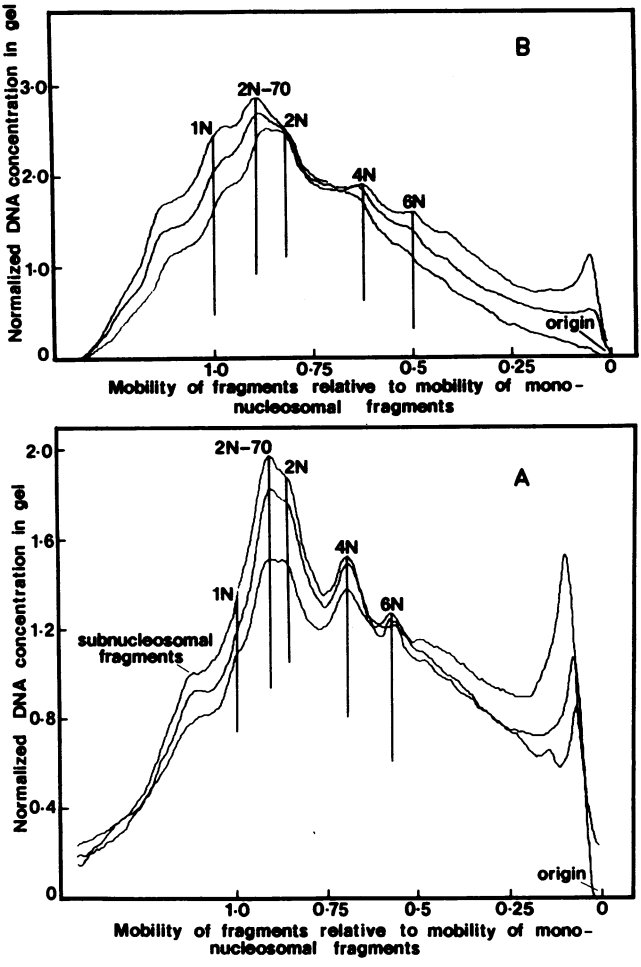
Native fragments from ferritin-DNAase-I.

Chicken nuclei equivalent to 690 µg/ml DNA, digested with ferritin-DNAase (KD=0.0 on Sepharose -2B) equivalent to 6.5 µg/ml free DNAase-I activity assayed on free DNA.

Curve Identification At the 2N position of the x axis, from the bottom up, in order - 90 mins digest, 120 mins, 180 mins. After the crossover region this order is reversed.

The single-stranded fragments produced with bound DNAase-I

A comparison of figures 2A, 180 min digest and 2B, 7 min digest shows that bound DNAase-I produces neglible amounts of 1N-sized pieces whereas



FIGS 2A and B

A comparison of denatured fragments of DNA produced by high molecular diameter ferritin-DNAase-I and free DNAase-I.

A. - Chicken nuclei equivalent to 742 µg/ml DNA were digested with ferritin-DNAase-I. Activity equivalent to 3 µg/ml free DNAase-I per ml assayed on free DNA, KD on Sepharose 2B ∿ 0.0. Curve identification - at the 2N position on the x axis, from the bottom up, in order - 90 mins digest, 120 mins, 180 mins. After the cross over region this order is reversed.

B. - Chicken nuclei equivalent to 1115 µg/ml DNA were digested with, nominally, free bovine pancreatic, DNAase-I (15 µg/ml) for 7, 11 and 20 minutes. Curve identification - At the (2N-70Bp) position on the x axis, from the bottom up, in order - 7 mins digest, 11 mins, 20 mins. After the crossover region this order is reversed.

'free'DNAase-I produces relatively large amounts of 1N-sized pieces. In other respects these two patterns are similar, being based on a 2N series with a 2N-70Bp peak and the two digests cited, have similar average fragment sizes of approx. 600 Bp as shown on fig 3. The 2N-70Bp peak never separated well on 1.5% agarose but could be clearly resolved on 4% agarose and seemed to be a major feature of DNAase-I-type digestions whether bound or free.

Using bound DNAase-I, the 2N-based susceptibility appears to be quite similar in both the single stranded fragments and the double stranded fragments, although there are roughly twice as many single stranded breaks as double stranded breaks. If the single stranded 'nicks' were distributed on the basis of some other pattern of susceptibility it would have caused the single stranded pattern to be obviously blurred or other peaks to have appeared in the pattern on denaturation. Although this was observed, to some degree, with free DNAase-I, effects of this type were not observed with bound DNAase-I. With bound DNAase-I the digestion limit species for single stranded fragments was virtually the same as for double stranded fragments; that is to say 2N-70Bp and 2N.

Digestion with free DNAase-I

High concentrations of free DNAase-I were much less structurally selective than low concentrations of free DNAase-I even after the digestion times had been adjusted to give the same degree of overall digestion. i.e. the same average fragment size. The lowest concentrations of DNAase-I we used were nearly as structurally selective as bound DNAase-I.

Digest time courses

The time courses of digests were observed by plotting the average fragment size for digestions against time of digestion. It is apparent that all digestions show a strong deceleration with time and our results indicate that this deceleration may be explainable in terms of the production of a true, sterically restricted, 'core' with weighted-average size of approximately 4N (native) as in fig 3a and 3c. However, this putative 'core' does not have a single integral multiple of nucleosomes.

It is noteworthy that the average length of double stranded fragments always appeared to be approximately twice that of the average length of single stranded fragments during the part of the digestion that showed the 2N-based series. It is not clear to us why this should be so and it is possible that it is to some degree a coincidence.

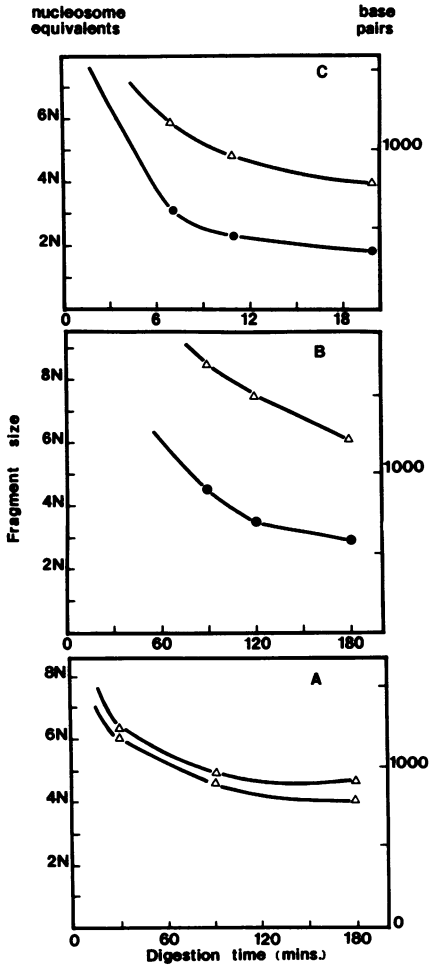


FIG 3, A. B and C.

The *average* fragment sizes tend to a limit of 2N for denatured fragments and 4N for the native fragments.

Each point in these figures marks the average fragment size in a curve of the type shown in figures 1 and 2.
Symbols - (Δ) denotes native fragments, (o) denotes denatured fragments.

A. - The average fragment sizes produced during an experiment of the type shown in Fig. 1. Lower curve is a ferritin-DNAase-I digest. Upper curve is a BSA-DNAase-I digest.

B. - A comparison of native and denatured fragments produced in a ferritin-DNAase-I digestion similar to that in A.

C. - Comparison of native and denatured fragments produced with nominally free DNAase-I in the latter stages of a digestion of the type shown in figure 2B.

However, even a ratio of approximately 1 : 1, double strand : single strand breaks indicates that the enzymes are showing a very strong tendency to make closely paired or grouped breaks rather than randomly dispersed breaks.

DISCUSSION

Any coiled nucleic acid generates a system of surfaces where it is vulnerable to attack and if the coiled structure is small, such as a nucleosome, these surfaces are mainly on its exterior. However, if the structure is large (e.g. a nucleus) the surfaces are mainly the cavity walls of the interior. Thus when a nucleolytic probe attacks the DNA it generates fragments that reflect the distances between sites that are accessible to the probe. This 'structural-signature' is a function of the structures present and the molecular diameter of the probes. So any level of coiling *tends* to produce a strong structural signature when a nuclease just enters the cavities that are generated by the next level of coiling above the one generating the signature.

Passive penetration studies (3, 5) have indicated molecular diameters considerably greater than 4 nM might be expected to selectively 'see' structures above the level of the nucleosome and the results presented in this paper are all compatible with this explanation of the nuclease digestion's specificity.

We would like to further suggest that there are actually two effects controlling the access of DNAase-I to chromatin. There is the effect of its own molecular diameter interacting with intranuclear cavities, as previously discussed, but when it is used at relatively low concentrations, it slowly binds to non-histone proteins, such as actin, which cause it to have a much higher effective molecular diameter. Actin is a common contaminant or component of chromatin and it is worth noting that the actin-DNAase-I complex has intrinsic nuclease activity (6).

Indications of variations in susceptibility near the 2N level

Although the main 2N-based repeat is the most obvious level of susceptibility there does appear to be evidence for sub-classes of susceptibility within the class of susceptible 2N-spaced sites. For example, the ratio of double stranded breaks as to single stranded breaks indicates that either some of these 2N-spaced sites are more susceptible than others or that attack on one strand renders the other preferentially susceptible. In addition to this, the presence of a (2N minus $\sim$ 70Bp)

and a very weak (2N plus ∿ 70Bp) peak besides the 2N peak seems to indicate that the susceptible nucleosomes have two attack sites spaced approximately 70Bp apart. At present one simple interpretation of these is to suggest they are generated by the two turns of DNA around the nucleosome each having one region of maximal exposure.

Indications of structure above the level of the 2N repeat

Our digestions with bound DNAase-I have shown a significant and persistent deficiency of mononucleosome size fragments. At first, the diffuseness of our 2N-based peaks from bound DNAase-I made this absence of doubtful significance, but digestions with naked DNAase-I can give comparable patterns (e.g. fig 2B) that are just as diffuse as those obtained with bound DNAase-I, but which quite clearly *do* show a significant class of mononucleosomal-sized single stranded fragments. This near absence of 1N-sized pieces in digests from bound DNAase-I has important implications for a zig-zag nucleofilament model because it means that the folding or coiling of this putative zig-zag is in such a way as to *consistently* protect one side of the zig-zag from attack. Thus most nucleofilaments are accessible from one side and yet there cannot be any more than a very minor proportion of the presumptive zig-zag nucleofilaments that are readily accessible from both sides and this would seem to reflect the presence of some very definite rules determining the way the nucleofilament coils or folds because this one-sided protection almost certainly has to come from a self-protective effect of some sort. In other words, a chromatin mass that folds in such a way as to leave a high proportion of open space within it while at the same time, giving the nucleofilaments a pronounced unilateral protection, seems most unlikely to be composed of a random mass of uniformly or bilaterally sticky filaments.

We thus suspect that the pronounced lack of mononucleosomal fragments found with covalently bound DNAase-I reflects the existence of the next level of structure above the nucleofilament although it does not tell us very much about that structure's form.

ACKNOWLEDGEMENTS

This work was supported by the U.S. Dept. of Health and Welfare and the Australian Research Grants Commission.

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