THE DISTRIBUTION OF NUCLEASE-SENSITIVE SITES IN CHROMATIN

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1. Introduction

Analysis of ratios of chromatin fragments containing from 1–4 nucleosomes produced from extensive degradation of chromatin by Ca–Mg-dependent endonuclease [1] suggested that by the most advanced stages of nucleolysis, at 20–30% degradation to mononucleosomes, the resultant overall pattern of inter-nucleosomal cleavage approximated a random distribution.

This paper examines whether interaction between Ca–Mg-dependent endonuclease and nuclease-sensitive sites is still random for the earliest detectable nucleolysis of chromatin remaining in situ in the nucleus. Apparent non-randomness was observed, and some of the structural and kinetic implications of this nucleolysis are discussed.

2. Methods

Polyamine-stabilised nuclei [2] were prepared from fully-regenerated liver removed from 4–8 week old Hooded Wistar rats (200 g) labelled by intraperitoneal injection of 150 μCi [^{3}H]thymidine, 20 h after initial hepatectomy. Endogenous Ca–Mg-dependent endonuclease was activated in purified nuclei by addition of 1 mM Ca^{2+}, 10 mM Mg^{2+} and incubation at 37°C. Nucleolysis was terminated after times ranging from 0.5–20 min by addition of equal volumes of 0.6 M NaOH, containing 1.4 NaCl and 2 mM EDTA.

Polyamine-stabilised nuclei were also degraded by activation of Ca–Mg-dependent endonuclease for 0.75–20 min after pre-treatment of nuclei with trypsin (75 μg/ml nuclei for 1 min at 37°C), or by addition of micrococcal nuclease for 0.5–20 min. (40 units/ml at 37°C). Nuclei were also lysed in 0.1 M NaOH, containing 5 mM EDTA, and the resultant gel X-irradiated using a Phillips RT 100 X-ray source with a 70 kV/1.25 Al filter, at 9 mA, at 10 cm distance from the source, for times from 5–75 min (surface dose rate approx. 1000 R/min).

Linear sucrose gradients, 4.3 ml, (2.0–2.5 M sucrose in 0.3 M NaOH, containing 0.7 M NaCl, and 1 mM EDTA) were poured using a gradient former, and allowed to equilibrate further for 4 h at 4°C before use. Samples of chromatin containing 30–60 μg DNA in 200 μl 0.3 M NaOH/0.7 M NaCl/ 1 mM EDTA were centrifuged at 240 000 × g (g_{av}), for 12 h at 4°C accelerating to the final g value over 30 min by increments of 80 000 × g at 10 min intervals to minimise possible occlusion affects in the chromatin sample. Samples, 150 μl were collected, diluted to 1 ml with ice-cold 5% trichloroacetic acid and filtered onto millipore membranes. ^{3}H was estimated by scintillation spectrophotometry using toluene–PPO scintillant.

Molecular weights of denatured DNA were determined using the formula of Burgi and Hershey [3] and converted to equivalent numbers of nucleosomes, assuming approximate nucleosomal DNA content of 0.12×10^6 daltons.

3. Results

Random breakdown of DNA is approximated by the equation:

$$I = N(p)^2 (1 - p)^{N-1}$$

where I is the intensity of a nucleosomal size-class

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containing N subunits, given a probability p of inter-nucleosomal cleavage. The expression yields two relationships, deviations from which can be used as a measure of non-randomness:

i. Random Distribution (1) $-I$ as a function of N for stated p.
ii. Random Distribution (2) – the maximal size-class of nucleosomes N_{max} as a function of increasing p. Examples of this second type are shown in figs. 1–3.

Analysis of sucrose gradients of the distribution of [^{3}H]DNA from X-irradiated chromatin gel suggests that this nucleolysis conforms to the expected random degradation in Random Distribution (1) (not shown), and Random Distribution (2) (fig.3). Similar ultra-centrifugal analysis of single-stranded [^{3}H]DNA from chromatin fragmented by intra-nuclear activation of endogenous Ca–Mg endonuclease, or added micro-coccal nuclease and then compared with Random Distribution (2) from predicted random/X-ray-induced nucleolysis (figs. 1–2) consistently suggests that the time-course of enzymatic cleavage deviates significantly from a random pattern. The ratio between rates of enzymatic degradation during incubation times less than 2.5 min (under stated conditions), and during 2.5–20 min incubation is far greater than that predicted from random breakdown. This initial, relatively more rapid, non-random rate of decrease in size of chromatin fragments begins to level after the initial decline to yield fragments in the molecular weight range equivalent to 30–40 nucleosomes, which are more resistant to further nucleolysis (figs. 1–2). Comparisons (not shown) between Random Distribution (1), and the molecular weight range of single-stranded [^{3}H]DNA fragments produced after limited (< 4 min incubation) nucleolysis fail to show non-random features as clearly as seen in figs. 1–2. However it was noted that the molecular weight range of [^{3}H]DNA produced after 20 min activation of Ca–Mg-dependent endonuclease ($p \simeq 0.08$) approximated the corresponding Random Distribution (1) more closely than did comparisons between molecular weight ranges of DNA for shorter incubation times of 2–5 min ($0.01 < p < 0.03$), and their corresponding values of Random Distribution (1).

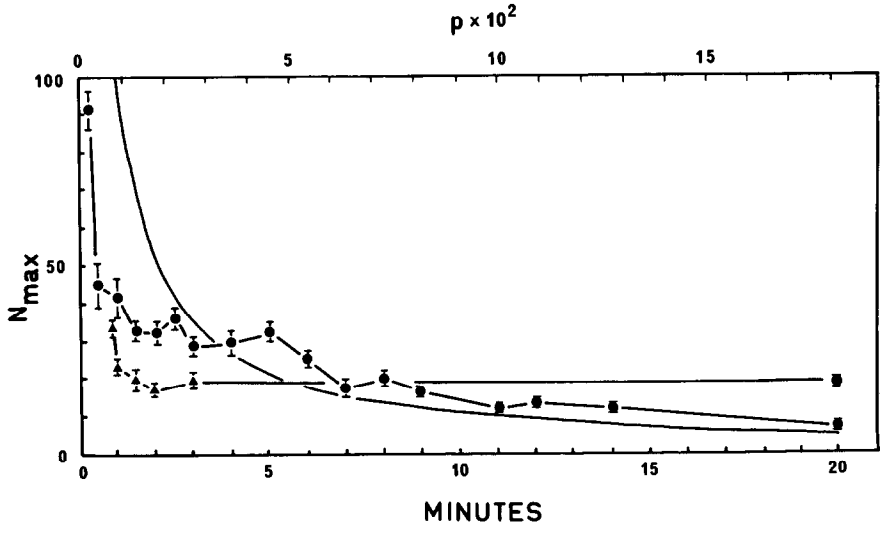


Fig.1. Time-course of decrease in value of N_{max} (most frequent size-class of single-stranded oligonucleosomal fragments equivalent to 1, 2, 3 . . . . N nucleosomes) produced during nucleolysis of intra-nuclear chromatin by Ca–Mg-dependent endonuclease. (•) Nucleolysis of chromatin by Ca–Mg-dependent endonuclease. (▲) Nucleolysis of chromatin, pre-treated with trypsin, by Ca–Mg-dependent endonuclease. (–) Expected relationship between N_{max} and probability of inter-nucleosomal cleavage (p) for random nucleolysis. The curve has been plotted as the best fit to the Ca–Mg-dependent endonuclease curve (-•-) given by the 'least squares' method. Assuming a relationship between incubation time (t) and p as $p = Kt$, the value $K = 0.9$ gave the best fit between observed and predicted data.

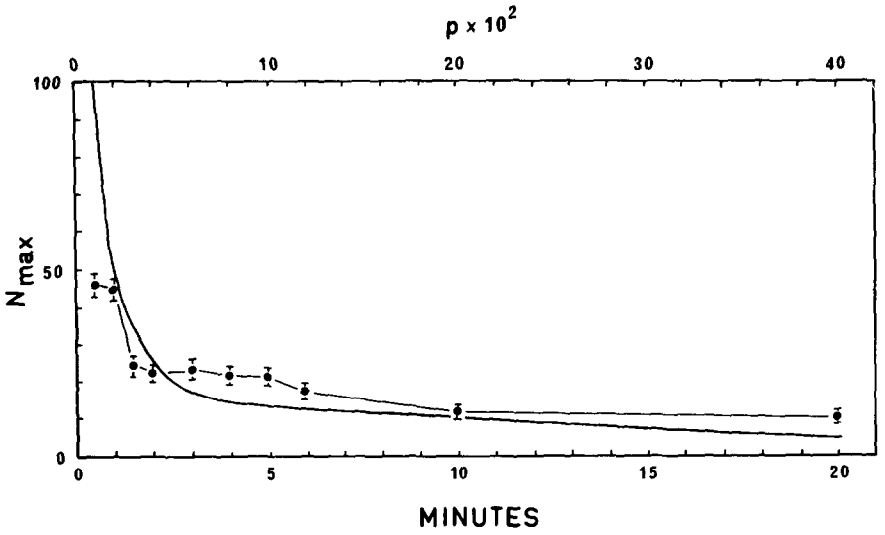
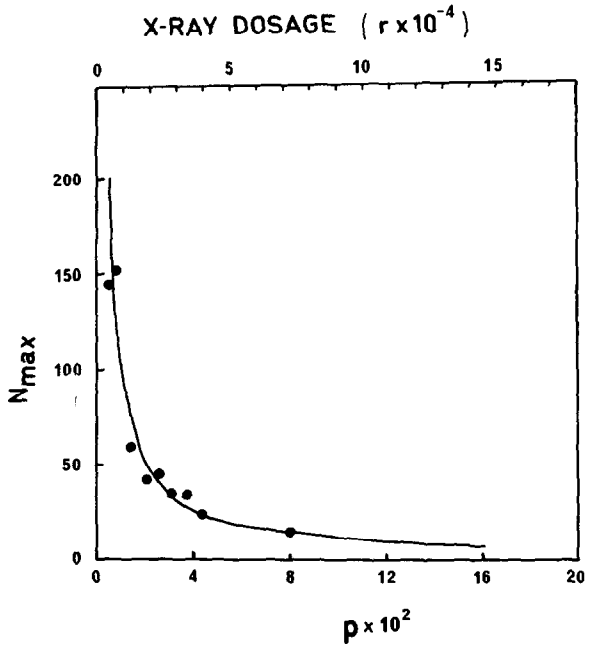


Fig.2. Time-course of decrease in value of N_{max} (most frequent size-class of single-stranded oligonucleosomal fragments) produced during nucleolysis of intra-nuclear chromatin by micrococcal nuclease. (•) Nucleolysis by micrococcal nuclease. (–) Expected relationship between N_{max} and probability of inter-nucleosomal cleavage (p) for random nucleolysis. The curve has been plotted as the best fit given by the 'least squares' method. Assuming a relationship between incubation time (t) and p as $p = Kt$, the value $K = 2.0$ gave the best fit between observed and predicted data.



Pre-treatment of nuclei with trypsin before activation of Ca–Mg-dependent endonuclease resulted in a rapid decrease in values of N_{max} during the first 2 min incubation, to approx. 15–25 nucleosome equivalents, and little further decrease in N_{max} occurred after another 18 min incubation (fig.1).

4. Discussion

Determination of the kinetics of nucleolysis of chromatin by Ca–MG endonuclease suggests that

Fig.3. Time-course of decrease in value of N_{max} (most frequent size-class of single-stranded oligonucleosomal fragments) produced during nucleolysis of denatured chromatin by X-irradiation. (•) Nucleolysis by X-irradiation. (–) Expected relationship between N_{max} and probability of inter-nucleosomal cleavage (p) for random nucleolysis. The curve has been plotted as the best fit to the X-ray data given by the 'least squares' method. Assuming a relationship between irradiation time (t) and p as $p = Kt$, the value $K = 0.06$ gave the best fit between observed and predicted data.

single-strand breaks are not distributed randomly between inter-nucleosomal sites, but are located such that single-strand DNA fragments, corresponding to 30–40 nucleosomes, are the major size-class initially formed.

This non-random pattern of nucleolysis could be caused by steric hindrance effects resulting from the differential accessibility of inter-nucleosomal cleavage sites to the Ca–Mg endonuclease [4,5], in a similar manner to the nuclease–DNA–protein interaction suggested for the preferential production of 10 base-pair DNA fragments from nucleosomes [6,7]. The distribution of histone H1, which appears to be external to the nucleosome [8], may be a factor, and some evidence, although mainly from sheared chromatin, suggests that H1-depleted and H1-enriched areas exist [9,10]. In addition to histone H1 [11], the distribution of non-histone proteins, especially if inter-nucleosomal, may also determine nuclease–chromatin interaction, and heterogeneity of nucleosomal types [12], may also contribute, as may differential distribution of inter-nucleosomal base-pair sequences. Since in intact nuclei the number of nuclease-sensitive sites are reduced compared with isolated chromatin [13], supercoiling of nucleosomes may induce higher orders of nuclease restriction. Pre-treatment of chromatin with trypsin, before activation of Ca–Mg endonuclease, results in a rapid decrease in values of N_{max} with increasing values of p to 15–25 nucleosomes, approximately half the value encountered when nuclei were not pre-treated with trypsin (fig.1). Since, under the incubation conditions used, trypsin selectively removes histone H1 [14] and results in extensive unfolding of nucleosome coiling [15], the 30–40 nucleosome average spacing of the most nuclease-sensitive sites may be either a function of H1 shielding of more nuclease-resistant sites (cf. ref. [1]), or of structural constraints imposed by its probably role in promotion of nucleosome packing.

Inhibition effects, such as progressive denaturation, are most unlikely to be important for the short incubation periods used, but the possibility of product inhibition of endonuclease by the production of single-strand breaks with a high affinity for the nuclease remains. The latter possibility however would still reflect the distribution of nuclease-sensitive sites during very limited digestion, unless binding of nuclease severely limited its unbound concentration.

It is also possible that the observed nucleolysis results in part from enzyme–chromatin binding properties. Ca–Mg endonuclease has been implicated in the initiation of DNA synthesis [16], and the sites of single-strand cleavage may be localised by the distribution of endogenous endonuclease receptor/stimulator proteins [17]. The endogenous endonuclease could remain highly adsorbed to localised sites, and the resultant initial nucleolysis could represent distribution of the enzyme, rather than distribution of nuclease-sensitive sites. This interpretation is suggested by the data since added micrococcal nuclease nicks the chromatin with much greater randomness (fig.2) than endogenous Ca–Mg endonuclease (fig.1), and the slight deviation of the former from randomness could be caused by residual Ca–Mg endonuclease activity under the conditions of the micrococcal nuclease incubation.

After activation of Ca–Mg endonuclease for 1 min under the stated conditions, ($p < 0.01$), over 90% of the chromatin is nicked at less than 60 nucleosome intervals, producing a single peak of DNA which, after further nucleolysis tends towards the 'early limit digest' interval of 30–40 nucleosomes. This suggests a distribution of sites of earliest detectable nucleolysis throughout the bulk of the chromatin, and not a zonal distribution. The results therefore suggest that the distribution of Ca–Mg endonuclease itself, or the most nuclease-sensitive sites, occur regularly throughout the chromatin, with a mean spacing of 30–40 nucleosome intervals, and maintenance of this distribution appears to be dependent in part on the distribution of trypsin-labile proteins, probably histone H1.

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