The reaction of the Ca-Mg endonuclease with the A-sites of rat nucleoprotein

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Received 7 July 1975

ABSTRACT

A quantitative study has been made on the action of rat nuclear Ca-Mg endonuclease on rat liver nuclei.

In a standard 30 minute digest 0.5-1.5% of the DNA was rendered acid soluble, 1.5-4% of the chromatin was rendered buffer soluble, 50-60% of the potential cleavage sites were actually cleaved, and these cleavages were distributed evenly throughout the bulk of the genome.

During these standard digests there was no significant loss of histones either in aggregate or relative to each other.

Trypsin digestion of the nuclei to a trypsin resistant core did not lower the specificity of the Ca-Mg endonuclease cleavages or expose other sites to its action.

Evidence is presented that indicates Ca-Mg endonuclease and micrococcal nuclease attack the same sites rather than different sites with the same spacing.

INTRODUCTION

The Ca-Mg endonuclease that is found in mammalian liver nuclei mainly attacks the DNA of the nucleoprotein at one class of regularly spaced sites^{1,2}. This is the class of sites that is the main subject of this paper and they will be referred to as the A-sites. Other nucleases also appear to attack these sites³ and the sites appear to be approximately 200 base pairs apart^{2,3}. However, there are other sets of regularly spaced sites. Some of these sets appear to be preferentially susceptible to other nucleases^{3,4,9}; thus a nuclease that produces fragments with a 200 base pair spacing is not necessarily attacking the A-sites.

The action of various nucleases on these various sites is becoming an increasingly important tool in the study of chromatin structure. Nuclease studies have demonstrated that the regular basic chromatin substructure extends down to eukaryotes as low as the yeasts¹¹ and have demonstrated that shearing procedures that are commonly used in chromatin isolation

procedures damage this basic substructure⁸.

One of the aims of this paper is to supply some more detail about the way the Ca-Mg endonuclease attacks the A-sites on the chromatin and to describe some of the effects of this attack on the chromatin's state of aggregation.

The other main aim of this paper is to examine the relative frequencies of the fragment size classes for non-randomness of the type that would arise if there was gross and uneven clustering of A-sites with respect to their Ca-Mg endonuclease susceptibility. Either the presence or absence of such clustering would eventually have important implications for any model of chromatin structure.

METHODS

Animal strains and liver nuclei preparation procedure - As described in reference (2).

Radioactive labelling of animal liver DNA - Rats were hepatectomised and 24 or 48 hours later the animals liver DNA was labelled by giving intraperitoneal injections of 40-50 μCi of ^3H -thymidine, specific activity approx. 18-20 $\mu\text{Ci}/\text{mmol}$. The animals were allowed to recover for at least one week after the labelling. They were used any time after the recovery week. The degree of labelling varied between animal batches, but usual figures were 60,000 to 140,000 cpm per mg of DNA.

Electrophoresis systems and stains - As described in reference (2). The electrophoresis system was essentially the 2.5% acrylamide gel system of Loening⁷ and when denatured DNA was to be run the gel was made 7 M with urea. The acrylamide gels were stained in the ethidium bromide stain² but only stained for 5 to 10 minutes with no destaining³.

Counting procedure - Gel pieces were dried or, if urea had to be removed also, they were extracted in 90% ethanol, 10% 1 M Mg acetate. Trials with the ethanol-Mg acetate extraction have shown that no radioactive material was lost from the gels by extraction in it. Nucleoprotein samples or gel pieces were then dried and oxidized in a stream of oxygen with carrier water. The tritium was then counted as tritiated water and corrected for quenching by use of internal standards of tritiated water. The oxidizer used was a Harvey Instrument Cor. biological materials oxidizer.

RESULTS*Low frequency cleavages between the A-sites*

In both native and denatured DNA, a class of high mobility fragments are found in a zone very near the bromophenol blue marker. A comparison of Table I and Table II shows that this class of fragments is derived from low frequency endonucleolytic cleavages that occur in the regions of stable DNA between the regularly spaced A-sites. This is apparent because there is an extremely marked increase in the relative proportion of this class of fragments on denaturing the DNA.

TABLE I -- DISTRIBUTION OF DENATURED DNA INTO 4 MOBILITY CLASSES

Experiment	Digestion Period	Relative proportion of load DNA found in a zone			
		Fast frags.	Band 1	Band 2	Bands slower than 2
1	30'	0.051	0.30	0.34	0.39
2	30'	0.059	0.36	0.23	0.36
3	30'	0.054	0.32	0.23	0.43
4a	45'	0.120	0.51	0.19	0.15
4b	45'	0.041	0.29	0.24	0.40
5	45'	0.096	0.50	0.25	0.25
6	45'	0.086	0.47	0.24	0.25

The amount of DNA in a band or zone was expressed as a relative proportion of the DNA loaded onto the gel. Thus all figures in one row should sum to approximately 1.0. Each estimate is an average of a triplicate and the final. Standard deviation of estimates is 2.1%.

The average total recovery of radioactivity from acrylamide gels was 103% with an S.D. of 7.5%.

Nuclei from rat or mouse liver were incubated in Buffer A² 10⁻⁴ M EDTA, 1 mM CaCl₂, 10 mM MgCl₂ for 30 or 45 minutes as stated in the table. The nuclei were then lysed in 2.5% sodium dodecyl sulphate, 0.38 M K₄ pyrophosphate, and deproteinised by extractions with phenol-CHCl₃ (1:1, v/v). After heat denaturing the DNA was electrophoresed in 2.5% acrylamide gels, 7 M urea², and radioactivity estimated in gel zones as described in Methods.

Some of the nuclei from experiment 4 were incubated with 75 µg/ml trypsin added at T₀ and this is indicated as experiment 4b.

TABLE II - DISTRIBUTION OF NATIVE DNA INTO 4 MOBILITY CLASSES

Experiment	Relative proportion of load DNA found in a zone			
	Fast frags.	Band 1	Band 2	Bands slower than 2
4a	0.013	0.270	0.210	0.470
4b	0.028	0.070	0.110	0.900

Incubations and DNA preparation as for Table I. All digestions were for 45 mins. Expression of results and standard deviation of results as for Table I. The DNA was not denatured before electrophoresis and was electrophoresed in 2.5% acrylamide gels⁵. Experiment 4b contained 75 µg/ml trypsin in digest added at T₀.

The progressive production of these cleavages between the A-sites means that at longer incubation times a comparison between the theoretical predictions as shown in Figure 1 and the observed distribution of DNA into the band classes becomes progressively invalid. This is the reason that the experiments reported here are confined to 30 and 45 minute digestions.

FIGURE 1 - PREDICTED PROPORTIONS OF DNA IN 3 SIZE CLASSES PRODUCED BY RANDOM CLEAVAGE OF REGULARLY SPACED SUSCEPTIBLE SITES

N is the order of band mobility as used in reference (2). N = 1 for the band of fragments approximately 200 base pairs long if native and 200 bases long if denatured. N = 2 for the fragments 400 base pairs long, native, or 400 bases long, denatured. P is the probability of any given susceptible site in a linear polymer being cleaved. The curves in this figure have been obtained from the relationship - expected proportion of DNA in a band = $N(1-P)N^{-1}P^2$. Open circles - band 1; closed circles - band 2; broken line - bands slower than 2.

The rate of production of degraded chromatin and solubilized chromatin during the Ca-Mg endonuclease digest

Acid soluble fragments - A number of digests have now been carried out under the standard conditions of digestion, 37° in 0.34 M sucrose, 10⁻⁴ M EDTA, 1 mM Ca⁺⁺, 10 mM MgCl₂ in Buffer A² at pH 7.4. During an average

30 minute digest, approximately 50-60% of the potential single stranded sites were cleaved (see Table III) and between 0.5% and 1.5% of the DNA was rendered acid soluble.

TABLE III - VALUES OF P CALCULATED FROM BAND 1 AND THE ZONE OF BANDS SLOWER THAN 2

Experiment	Band 1			Bands slower than 2			Average P		
	P_{ss}	$(P_{ss})^2$	P_{ds}	P_{ss}	$(P_{ss})^2$	P_{ds}	P_{ss}	$(P_{ss})^2$	P_{ds}
1	0.55			0.57			0.56		
2	0.61			0.60			0.61		
3	0.57			0.54			0.56		
4a	0.72	0.52	0.52	0.76	0.58	0.52	0.74	0.55	0.52
4b	0.54	0.29	0.27	0.57	0.32	0.20	0.56	0.31	0.24
5	0.71			0.68			0.70		
6	0.69			0.68			0.69		

Experimental details and digestions as for Table I. The DNA content of band 1 and the zone of bands slower than 2 were applied to the relationship shown in Figure 1 to give the values of P presented in this table. P is the probability of any given A-site suffering cleavage. P_{ss} refers to the single stranded sites and P_{ds} to the probability of both single strands being cleaved at any one site, i.e. the probability of a double stranded cleavage. If single stranded cleavages are at random with respect to each other $(P_{ss})^2$ should equal P_{ds} .

Experiment 4b contained 75 μ g/ml trypsin in digest, added at T_0 .

Low release of soluble chromatin - During a digest of this type only 1.5% to 4% of the chromatin DNA was released from nuclei as slower sedimenting pieces. These were a mixture of acid soluble fragments and soluble chromatin.

A more precise estimation of the rate of production of chromatin that is soluble in Buffer A is not practical as centrifugation of digested nuclei at different speeds has shown that the small amounts of solubilized chromatin that are produced have a range of fragment sizes.

After standard 30 minute digests nuclei suspensions were deliberately lysed by the addition of 2 vols of 0.2 M tetrapotassium pyrophosphate-HCl to pH 7.4. Small amounts of insoluble debris were removed by centrifugation at 50,000 g for 45 min and then the clear, non viscous, lysate dialysed against Buffer A, 10^{-4} M EDTA under N_2 . 97% of the DNA in the lysate was reprecipitated as insoluble nucleoprotein by dialysis against the Buffer A, 10^{-4} M EDTA. One rather interesting aspect of this nucleoprotein precipitate was its shape. The precipitate consisted of small regular spheres of approximately the same size and shape as the original nuclei.

Relative stability of histones - During some digests proteolysis of the histones was monitored by acid extraction of the histones and electrophoresis in acid acrylamide gels⁶. There was no significant loss of any of the histones over a 30 min digestion period. The ratios of F_1 as to F_{2a1} as to $(F_3 + F_{2b} + F_{2a2})$ were followed in one standard digest over a 60 min period and found to be essentially constant. At 15, 30, 45 and 60 minutes the proportions of F_1 were 20%, 20%, 19%, 21% respectively and the corresponding proportions of F_{2a1} were 19%, 20%, 19%, 21%.

Lack of clustering of endonucleolysed sites

On Tables I and II the relative DNA content of bands 1, 2 and the remaining bands with mobilities less than band 2, were applied to Figure 1 and used to estimate corresponding P values that are shown in Table III. P is the probability that an A-site will actually be broken. Thus there are two different sets of P values. P_{ss} is the value for single strand breaks and P_{ds} is the value for double strand breaks.

The results shown in Figure 2 show a satisfactory agreement between the values of P when P was estimated from band 1 or the sum of all bands

FIGURE 2 - DEMONSTRATION OF THE DEGREE OF AGREEMENT BETWEEN P VALUES ESTIMATED FROM BAND 1 AND P VALUES ESTIMATED FROM THE ZONE OF BANDS SLOWER THAN BAND 2

This figure is a graphic presentation of the P values shown in Table III. Each point represents a comparison of two zones on the one gel. The P value obtained from band 1 was plotted horizontally and the P value from the zone of bands slower than band 2 was plotted vertically. Closed circles are results for gel runs with denatured DNA. Open circles are the results for gel runs with native DNA. The line at 45° is to mark the line that fits random breakdown.

slower than band 2. The fact that these two estimates of P generally agree closely within an experiment indicates that the endonucleolysis is spread relatively evenly throughout the bulk of the genome. Band 2 gave quite poor estimates of P which were discarded. This is because the relation between the proportion of DNA in band 2 and values of P greatly amplifies any error that is in the DNA estimation in the region near $P = 0.65$ (see Figure 1).

Lack of large positive or negative coincidence of single strand breaks

If the probability of a single strand breakage at one A-site is independent of the status of the other single strand in the same A-site, then P_{ds} should equal the square of P_{ss} . The results shown in Table III indicated that this was substantially the case.

Tryptic-core chromatin and the Ca-Mg endonuclease

Nuclei were exposed to the action of trypsin simultaneously with the action of Ca-Mg endonuclease, as shown in Tables I and II, and also exposed to trypsin for preincubation periods of 45 minutes before initiating the action of the Ca-Mg endonuclease with calcium and magnesium. The levels of trypsin were sufficient to digest the chromatin to a standard trypsin-resistant core^{4,5} within approximately 20 minutes or less. Either type of tryptic digestion definitely inhibited the action of the Ca-Mg endonuclease on its associated chromatin but did not inhibit the action of the endonuclease on exogenous naked rat DNA. However, the action of the Ca-Mg endonuclease on the trypsinized chromatin still produced the standard pattern of DNA fragments that is characteristic of attack on the A-sites. Moreover, and surprisingly, the tryptic digestion did not cause any obvious broadening of the DNA bands seen on electrophoresis.

Evidence confirming that micrococcal nuclease is attacking the A-site

The possibility that the two nucleases were primarily attacking two entirely different sites but with the same 200 base pair spacing was considered. If this was so, mixed digests with both nucleases would be expected to give complex patterns of fragments that were different to the patterns obtained with single enzymes. In fact mixed digests using both nucleases together at various comparable activities gave very similar patterns of fragments to the single enzyme digests.

ACKNOWLEDGEMENTS

This work was supported by the Australian Research Grants Committee and the University of Adelaide Anti-Cancer Foundation

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