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**High sequence specificity of micrococcal nuclease**

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**ABSTRACT**

The substrate specificity of micrococcal nuclease (EC 3.1.4.7) has been studied. The enzyme recognises features of nucleotide composition, nucleotide sequence and tertiary structure of DNA. Kinetic analysis indicates that the rate of cleavage is 30 times greater at the 5' side of A or T than at G or C. Digestion of end-labelled linear DNA molecules of known sequence revealed that only a limited number of sites are cut, generating a highly specific pattern of fragments. The frequency of cleavage at each site has been determined and it may reflect the poor base overlap in the 5' T-A 3' stack as well as the length of contiguous A and T residues. The same sequence preferences are found when DNA is assembled into nucleosomes. Deoxyribonuclease I (EC 3.1.4.5.) recognises many of the same sequence features. Micrococcal nuclease also mimics nuclease S1 selectively cleaving an inverted repeat in supercoiled pBR322. The value of micrococcal nuclease as a "non-specific" enzymatic probe for studying nucleosome phasing is questioned.

**INTRODUCTION**

Micrococcal nuclease digestion of nuclei generates a repeating pattern of DNA fragments which are multiples of 200 bp (1). Further digestion of this material leads to the production of a discrete particle, the nucleosome core, containing approximately 146 base pairs of DNA (2,3,4). Each nucleosome core in chromatin is separated from the next by a length of "linker" DNA which is degraded by the nuclease in the liberation of the core particle. The nucleosome core particle has been the subject of intense study (for reviews see 5,6,7) and micrococcal nuclease has become one of the major enzymatic probes of chromatin structure.

There is great interest in the question of whether nucleosomes are arranged randomly with respect to the DNA sequence or aligned precisely with respect to sequence, i.e. phased. There have been many reports recently that nucleosomes are indeed phased; for example, in tRNA genes in chicken chromatin (8), in 5S genes in Xenopus (9) and Drosophila (10), in heat shock genes

in Drosophila (11), and in satellite DNA in rat (12). The locations of the nucleosomes have been mapped to the nucleotide in the histone genes of Drosophila (13).

The dominant experimental strategy used to demonstrate phasing is to cleave nucleosomal DNA with restriction enzymes which cut the sequence of interest at a unique location. If the nucleosomes are arranged randomly, digestion will produce a very large number of fragments of different lengths, giving the appearance of a smear when analysed on polyacrylamide gels. If instead nucleosomes were arranged precisely with respect to sequence, a small number of fragments will be produced which reflect the location(s) of the nucleosomes. This experimental design depends critically on the assumption that cleavage by micrococcal nuclease is essentially random with respect to DNA sequence.

The fact that micrococcal nuclease cleaves DNA in "A-T rich" regions of the duplex is known (for reviews see 14,15). The enzyme degrades denatured DNA considerably faster than it does duplex DNA (16,17). When native DNA is the substrate the acid-soluble products are rich in adenylate and thymidylate residues suggesting preferential attack at "A-T"-containing regions of the DNA (18). Nearly all di- and tri-nucleotides produced during digestion have an "A" or "T" residue at their 5' OH terminus (18,19). Digestion of dinucleotides indicated a strong base preference, dTpTp being hydrolysed 100 times more rapidly than dGpGp (20). These data led the early workers to consider the enzyme as a possible candidate for DNA sequencing (21). The base preference of the enzyme, its preference for single stranded DNA and the fact that duplex DNA containing high concentrations of A and T residues is more readily thermally denatured (22) led to the postulation of a mechanism of enzyme action (23) in which the enzyme preferentially attacks AT rich regions of the duplex due to their transient single strandedness.

We show here that the magnitude of the previously known A-T preference has not been fully appreciated and that there are two additional levels of substrate recognition. Furthermore attempts to reconstruct nucleosome phasing by assembly in vitro (24) revealed that the sequence preference of the enzyme is the same for DNA in nucleosomes as for naked DNA.

### MATERIALS AND METHODS

Enzymes. Micrococcal nuclease preparations from Boehringer Mannheim, and Millipore Ltd. were used without further purification and were indis-

tinguishable in behaviour. Digestions were carried out in Buffer 'A' of Hewish and Burgoyne (25) with and without polyamines at 25°C in 2 mM calcium chloride unless otherwise stated. DNAase I and S1 nuclease were obtained from Sigma Ltd. Restriction enzymes were obtained from New England Biolabs and digestions were carried out under the recommended conditions. E.coli DNA polymerase I - large fragment (26) was from Boehringer Mannheim.

DNA substrates. Synthetic oligonucleotides from P-L. Biochemicals Ltd. were dissolved in buffer (see below) and pre-incubated at 37°C for 90 minutes to ensure optimal annealing. The tetranucleotides d(pT-A)<sub>2</sub>, d(pA-T)<sub>2</sub>, d(pC-G)<sub>2</sub>, and d(pG-C)<sub>2</sub> were purchased from Collaborative Research Ltd.

Supercoiled form I pBR322 was the generous gift of D. Melton.

Supercoiled molecules were relaxed by treatment with DNA topoisomerase I from wheat germ as described (27).

The double stranded replicative form of plasmid molecules mp2, an M13 derived vector, and mCet1S were prepared by growth in E.coli K12 71-18 (28). mCet1S contains a 273 bp fragment of DNA from the nematode C.elegans bearing a proline tRNA gene.

Restriction markers. Restriction markers for polyacrylamide gels were end labelled fragments produced by digestion of pBR322 with HpaII followed by incubation with  $\alpha$ -<sup>32</sup>P dCTP and E.coli DNA polymerase I - large fragment. Restriction markers for agarose gels were produced by digestion of pBR322 with Eco RII and labelled as above.

Determination of 5' terminal nucleotides of micrococcal nuclease digestion products. The 5' terminal nucleotides of nuclease digestion products were determined by labelling with  $\gamma$ -<sup>32</sup>P ATP and polynucleotide kinase. The labelling reaction mixture was treated with glucose and hexokinase to convert any residual ATP to glucose 6-phosphate, digested to exhaustion with DNAase I and snake venom phosphodiesterase and the products electrophoresed at pH 3.5 on Whatman 3 MM paper. The mononucleotides were identified by their characteristic mobilities, they were cut out of the paper and the incorporation was determined by liquid scintillation counting. Any base preference of the polynucleotide kinase was corrected for by labelling an equimolar mixture of the tetranucleotides d(pT-A)<sub>2</sub>, d(pA-T)<sub>2</sub>, d(pC-G)<sub>2</sub> and d(pG-C)<sub>2</sub> and determining the incorporation into each terminal nucleotide. The results show that in a mixture the proportion of C termini will be over-estimated by 50%. The proportions of A, T and C termini will be correct. DNA extracted from beef kidney chromatin was digested (10 units of enzyme/50

$\mu\text{g}$  DNA in 100 mM Tris Cl pH 7.0 10 mM  $\text{MgCl}_2$ , 1 mM  $\text{CaCl}_2$  37°C) to 10% and 50% acid solubility (5% perchloric acid) and the fragments in the total digest labelled as described above. The 5' terminal nucleotides of mononucleosomes prepared as described (29) were similarly determined.

Rate of digestion of homopolymers and random sequence DNA. DNA was isolated from beef kidney chromatin and used as a source of "random sequence" DNA. The rate of digestion, as percent acid solubility as a function of time was investigated for the polymers poly {d(A-T)}.poly{d(A-T)}, poly {d(A)}.poly {d(T)} and random sequence DNA in 100 mM Tris Cl pH 7.0 10 mM  $\text{MgCl}_2$  1 mM  $\text{CaCl}_2$  and for the polymers poly {d(G-C)}.poly {d(G-C)} and poly {d(G)}.poly {d(C)} in 100 mM Tris Cl pH 8.0 10 mM  $\text{MgCl}_2$  1 mM  $\text{CaCl}_2$  using 1 unit of enzyme/5  $\mu\text{g}$  DNA.

Gel electrophoresis and autoradiography. Polyacrylamide gel electrophoresis was carried out at 1 volt  $\text{cm}^{-1}$  for 16 hours in 40 mM Tris pH 8.3, 20 mM sodium acetate and 2 mM EDTA. Gels were stained in ethidium bromide (2 mg  $\text{L}^{-1}$ ) and photographed under ultra violet light. Gels were autoradiographed for 12 hours at -80°C using pre-fogged film (Kodak XH-1) and an intensifying screen (31).

## RESULTS

### 1. Nucleotide preference

(1.1) 5' terminal nucleotide. The terminal nucleotides of the micrococcal nuclease digestion products from naked DNA and chromatin indicate the nucleotide preference of the enzyme. The 5' terminal nucleotides of mononucleosomes prepared from beef kidney (29) and of DNA fragments produced by digestion of DNA extracted from beef kidney chromatin were quantitated. The DNA from both sources was labelled at the 5'-OH terminus with  $\gamma$ - $^{32}\text{P}$  ATP and polynucleotide kinase followed by degradation of the labelled DNA to mononucleotides, analysis of the products and quantitation of the incorporation into each nucleotide monophosphate. The results are shown in Table I. The values for DNAase I (32) and DNAase II (33) are given for comparison.

The products of digestion of naked DNA at 10 and 50% acid solubility contained the same relative proportions of T, A, C and G residues at their 5' termini. The proportions of T, A, C and G residues at the 5' termini are the same for naked DNA and DNA in nucleosomes. An estimation of the rate of cutting at each nucleotide indicates that the phosphodiester bond at the 5' side of the most favoured nucleotide (p - Tp) is cut at least 30

Table I 5' Terminal nucleotides as percentage of total

5' Terminal nucleotide	Micrococcal nuclease		DNAase I	DNAase II
	Nucleosome DNA	Naked DNA	Naked DNA	Naked DNA
T	60	60.4	59	13
A	36	35.6	22	22
C	1.9	2.3	11	31
G	2.1	1.7	8	34

times faster than the equivalent bond at the least favoured nucleotide (p - Cp).

These results indicate that micrococcal nuclease has a very marked nucleotide preference and that the preference is not altered when the DNA is in the form of chromatin.

(1.2) Digestion of homopolymers and random sequence DNA. The digestion of homopolymer and alternating copolymer molecules can confirm the nucleotide specificity of the enzyme. Random sequence DNA was isolated from beef kidney chromatin and digested for comparison. Each DNA sample (50 µg) was digested with enzyme (10 units) and the percentage of the DNA made acid soluble as a function of time was determined. The rate, as the time taken to render the DNA 50% acid soluble is given in Table II.

As no detectable degradation of the G-C polymers occurred at pH 7.0, the pH was raised to 8.0 for the digestion of these substrates.

These results show an extremely rapid digestion of A-T containing polymers and a marked resistance to digestion of G-C containing polymers. In poly {d(G)}.poly {d(C)} the poly {d(C)} strand is completely degraded and

Table II

Substrate	Time (minutes to reach 50% acid solubility)
Random sequence DNA	3.6
poly {d(A)}.poly {d(T)}	2.5
poly {d(A-T)}.poly {d(A-T)}	0.3
poly {d(G)}.poly {d(C)}	40
Poly {d(G-C)}.poly {d(G-C)}	100 minutes to reach 20% acid solubility

the poly {d(G)} strand persists. This could be due to the ability of poly {d(G)} to form self-complementary structures (34,35) which may be resistant to further hydrolysis. The slower digestion of poly {d(A)}.poly {d(T)} compared to poly {d(A-T)} may reflect the lower melting point of the alternating polymer. Whatever the explanation for these differences the A-T preference of the enzyme is marked.

2. Sequence specificity. To investigate whether micrococcal nuclease has a sequence preference in addition to its nucleotide preference, end labelled linear DNA molecules of known sequence were digested.

(2.1) Digestion of end labelled linear mCet1S (36). Circular mCet1S was digested with SmaI to give a linear duplex molecule and then labelled at both 3' termini (i.e. both strands) with  $\alpha$ -<sup>32</sup>P dCTP and DNA polymerase I - large fragment. This end labelled material was digested with micrococcal nuclease, samples were taken at different times and analysed on denaturing and non-denaturing polyacrylamide gels. Both gels showed the same pattern of fragments, the autoradiograph of the denaturing gel is shown in figure 1.

A striking pattern of labelled fragments is observed. Some fragments

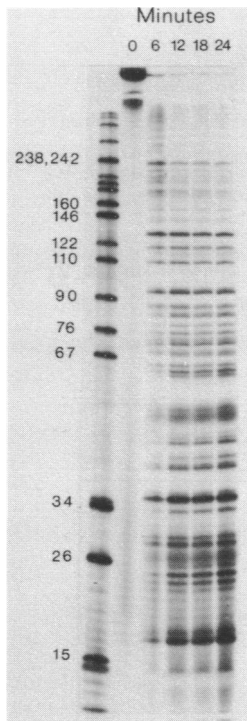
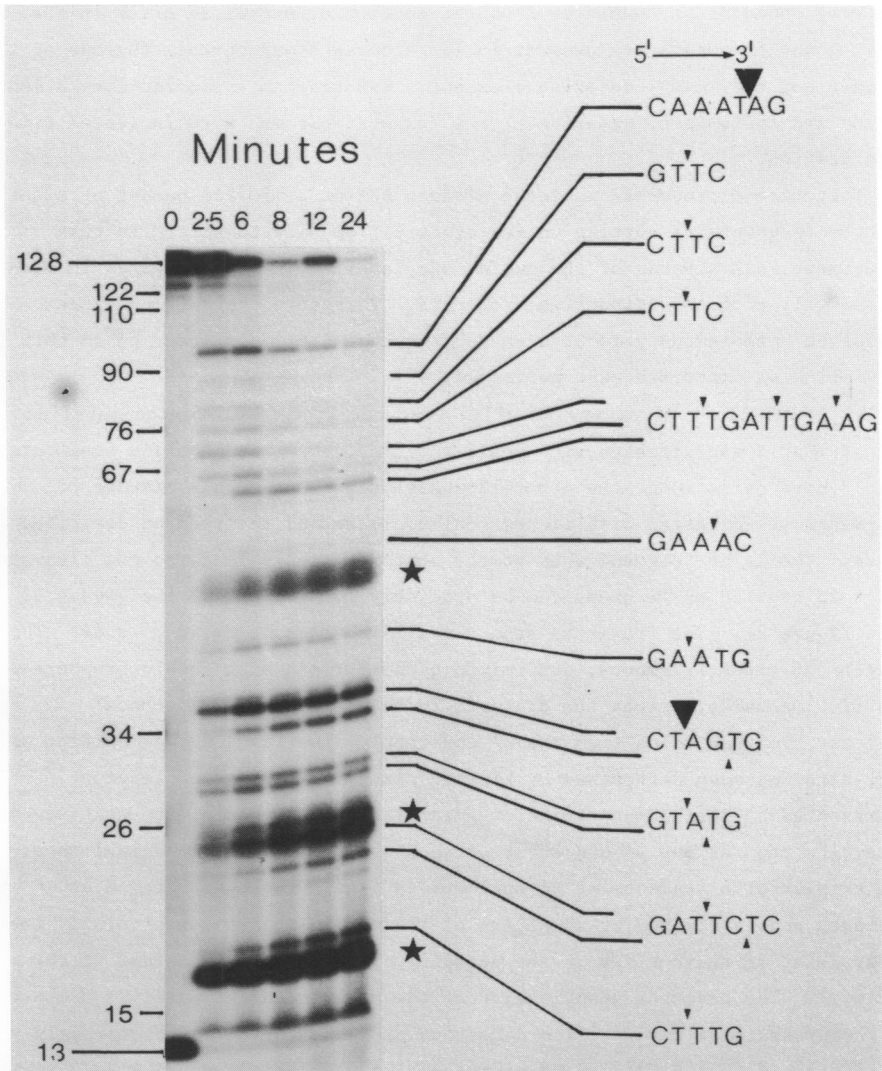


Figure 1. Autoradiograph of a time course of micrococcal nuclease digestion of end labelled linear duplex mCet1S. Samples (5  $\mu$ L) were removed from a reaction mixture (30  $\mu$ L) containing 2  $\mu$ g end labelled mCet1S and 0.06 units of enzyme, quenched with EDTA (1  $\mu$ L of 10 mM) and analysed under denaturing conditions on a 10% polyacrylamide gel. In this and all other micrococcal nuclease digestions the "zero" time point was taken from the reaction mixture after 5 minutes pre-incubation and immediately before the addition of the enzyme. The reaction was timed from this addition. The left hand lane shows an end labelled HpaII digest of pBR322.

are very abundant, produced by frequent cutting at preferred sites in the DNA. Other fragments are present in low abundance and certain regions of the DNA are not cut to any detectable extent. The fragments persist over a long period and there is no smearing of the fragment pattern with increased time of digestion.

Therefore micrococcal nuclease cleaves DNA at a limited number of sites and the frequency of cutting varies greatly from site to site. In this experiment both strands of the duplex are labelled, hence cleavages in each strand will produce radioactive fragments. Therefore the cutting sites cannot be unambiguously located in the sequence (36). In order to do this the following experiment was performed.

(2.2) Digestion of an end labelled fragment from mCet1S. End labelled linear mCet1S was digested with HpaII which produces two labelled fragments, one 13 base pairs long, the other 128 base pairs long. This mixture of fragments was digested with micrococcal nuclease and analysed as described above. The 13 bp fragment runs at the bottom of the gel and so the cleavage sites in the 128 bp fragment can be precisely located within the sequence (see figure 2). The fragments seen are all labelled at their 3' ends. The lengths of these fragments, determined by comparison with co-electrophoresed restriction markers gives the distance in nucleotides of micrococcal nuclease cleavage sites from the 3' end label. The frequency of cutting at each site has been determined by kinetic analysis (29). The cleavage represented by the 95 nucleotide fragment is the most rapid, the next seven cleavages towards the 3' end occur at about 5 to 15% of this maximal rate. The triplet of A residues at 56 nucleotides is flanked by several G and C residues and is cleaved at about 13% of the maximal rate. Surprisingly the cleavage at 36 nucleotides in the sequence CTAG is very rapid (94% of the maximum). The accurate quantitation of the other cleavage sites is hampered by the presence of unidentified digestion products which resist deproteinisation or further digestion by micrococcal nuclease and DNAase I (asterisks in figure 2). They probably represent small limit digest products which electrophorese anomalously slowly. They can be greatly reduced by fixing the gel in 10% acetic acid (see for example fig. 3). In spite of this complication the cleavages marked in the 30 to 15 nucleotide region can be estimated to occur at about 15 to 20% of the maximal rate. There was no detectable cleavage at G and C residues nor at single A and T residues in GC rich regions. Thus, while the enzyme prefers to cut on the 5' side of thymidine residues there is a sequence constraint which overrides this



**Figure 2. Autoradiograph of a time course of micrococcal nuclease digestion of an end labelled 128 bp fragment from mCet1S.** A fragment 128 bp long labelled on the 3' terminus of one strand was prepared as described in the text. The DNA (2  $\mu$ g) was digested with enzyme (0.1 units) and samples (5  $\mu$ L) were removed from the reaction (35  $\mu$ L), quenched with EDTA (1  $\mu$ L of 10 mM) and analysed under denaturing conditions on a 10% polyacrylamide gel. The positions of end labelled fragments produced by HpaII digestion of pBR322 is shown on the left. Asterisks mark anomalous digestion products which are discussed further in the text.



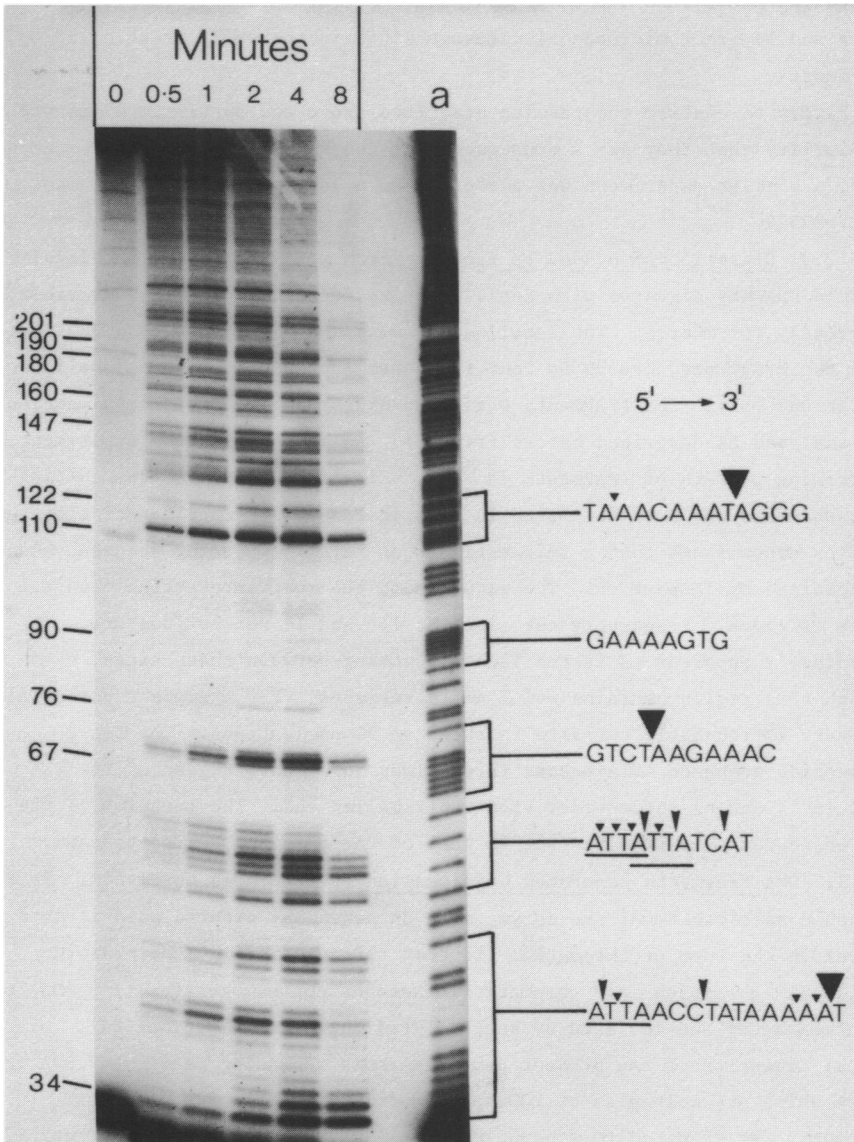


Figure 3. A time course of micrococcal nuclease digestion of an end labelled 4329 bp fragment from pBR322. A 4329 bp fragment, labelled at the 3' terminus was prepared as described in the text. The DNA (1.25 µg) was digested with enzyme (0.03 unit) and samples (4 µL) were removed from the reaction (30 µL) and analysed under denaturing conditions on a 8% polyacrylamide gel. Lane a shows a Maxam and Gilbert (43) pyridinium formate reaction (which cleaves the DNA at G and A residues) of the starting material.

nucleotide preference and so we see that there are probably only 2 or 3 major and 14 minor micrococcal cleavage sites in the whole of this 128 bp fragment.

Partly to confirm the results described above and partly to eliminate the possibility that they are a consequence of the short length of the fragment used, a similar experiment was performed on a long end labelled fragment from pBR322.

(2.3) Digestion of a 4362 bp fragment from pBR322 (37). The circular plasmid DNA was digested with EcoRI labelled at both 3' ends as described previously for mCet1S. The labelled DNA was then digested with Hind III to give two fragments, one 29 bp long the other 4362 bp long, each labelled on one strand only. The fragments were then digested with micrococcal nuclease and analysed as described before (fig. 3). As in the previous experiment, a striking pattern of fragments is seen. Kinetic analysis of the cutting frequency has not been attempted in this instance, but consideration of the results above shows that a fair estimate of relative cutting frequency can be obtained by inspection. The sequence of the most interesting cleavage sites is given. Frequently cut sites at 115 and 71 nucleotides are immediately apparent as is the lack of cutting between these sites, even though this region contains 64% T and A residues. For example a potential, but very infrequently cut site is shown at 90 nucleotides. The tetranucleotide sequence ATTA occurs three times in a short region of the DNA between 34 and 67 nucleotides from the labelled end. The location of the preferred cut site is different in each of the tetranucleotide repeats.

3. DNA fragments generated by digestion of assembled chromatin. Is the sequence specificity of the enzyme seen on naked DNA altered when it acts on DNA in the form of chromatin? To test this possibility the plasmid mCet1S was assembled into chromatin in Xenopus egg homogenates (1  $\mu$ g DNA/60  $\mu$ l homogenate) and isolated by sucrose gradient centrifugation (24). Micrococcal digestion of the product gave rise to a repeating pattern of DNA fragments which are multiples of 200 bp. A similar preparation of mCet1S chromatin was digested to mononucleosomes (60 units of enzyme/ $\mu$ g of chromatin for 6 minutes). The DNA was extracted and cleaved with SmaI which cleaves mCet1S at a single site in the tRNA gene. The 3' ends produced by SmaI cleavage were specifically labelled since the 3' termini produced by micrococcal nuclease digestion are phosphorylated and are not substrates for DNA polymerase I - large fragment. The fragments produced were analysed on non-denaturing polyacrylamide gels. The autoradiograph of the gel showed 10

prominent bands corresponding to the most abundant fragments seen in the micrococcal digestion of end labelled linear mCet1S. In parallel the M13 vector, mp2 which lacks the SmaI site, was analysed in an identical way. No labelled fragments were seen. Similar results were obtained when the locations of other restriction sites (e.g. EcoRI) were mapped in the mono-nucleosome DNA. Therefore the sequence preference of micrococcal nuclease is unchanged by the presence of nucleosomes on the DNA.

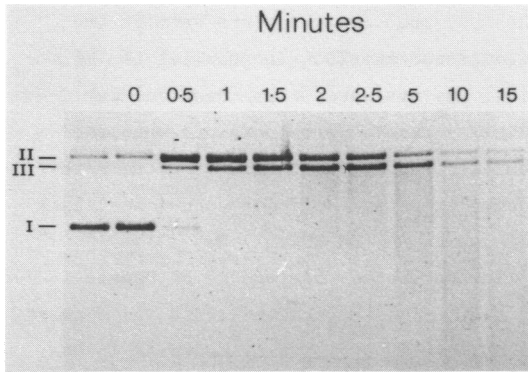
4. Specificities of DNAase I. The nucleotide preference of DNAase I (32) shown in Table I is similar to that of micrococcal nuclease and suggests that it may also recognise a restricted number of sequence features in DNA. Therefore end labelled linear mCet1S was digested by DNAase I in the presence of manganese to promote double stranded cutting by the enzyme (38). This gave rise to a distribution of fragments very similar to those seen with micrococcal nuclease but with less variation in intensity between bands and only the 36 nucleotide fragment is prominent. This means that DNAase I and micrococcal nuclease recognise many of the same sequence features but that DNAase I cleaves them at less variable frequency.

5. Duplex melting and S1 digestion. One possible mechanism of micrococcal nuclease action on duplex DNA was described in the introduction. The suggestion was that the enzyme cleaves AT rich regions preferentially because of their transient single strandedness. In order to test this the end labelled linear mCet1S was partly denatured (40% formamide at 40°C) and digested with S1 nuclease. A pattern of bands was obtained which bore only a slight correspondence to those seen in micrococcal nuclease digestions.

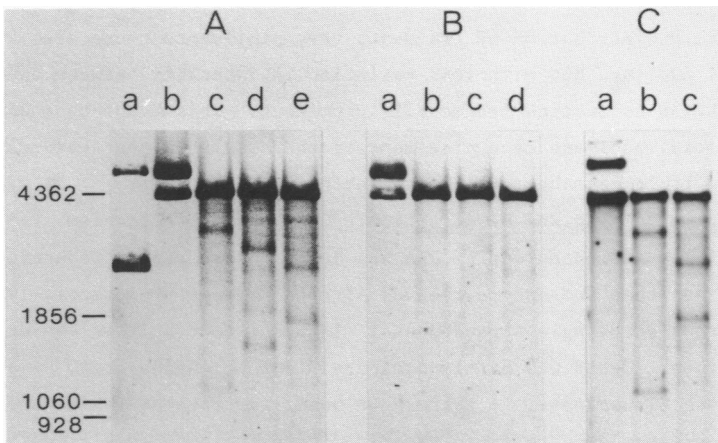
This suggests that micrococcal nuclease is not simply cleaving at AT rich regions because they are partly single stranded, but that some additional features of the substrate are being recognised.

6. Recognition of inverted repeats in supercoiled molecules. A time course of micrococcal nuclease digestion of supercoiled (form I) pBR322 showed a rapid transformation to a mixture of nicked circles (form II) and linear molecules (form III) followed by a slower digestion of these to small fragments (fig. 4).

To ask if this rapid cleavage occurs at a specific site a large scale preparation of lightly digested supercoiled pBR322 was divided into aliquots and digested with restriction enzymes which cleave the plasmid at a unique location. Two major fragments were produced as follows, EcoRI, 1.1 kb and 3.3 kb; Bam H-I, 1.6 kb and 2.8 kb; Ava I, 1.8 kb and 2.6 kb. These fragments were greatly reduced (though not completely abolished) when



**Figure 4.** A time course of micrococcal nuclease digestion of form I pBR322. Supercoiled form I pBR322 (5  $\mu$ g) was digested with enzyme (0.03 unit) and samples (5  $\mu$ L) were removed from the reaction (50  $\mu$ L) quenched with EDTA (1  $\mu$ L of 10 mM) and analysed on a 1% agarose gel. Supercoiled form I pBR322 is shown in the left hand lane. The ethidium bromide stained gel was photographed under U.V. light and printed with reversed contrast for clarity.



**Figure 5.** Determination of the location of the micrococcal nuclease cleavage site in supercoiled form I pBR322. Supercoiled form I pBR322 and relaxed pBR322 (5  $\mu$ g of each) were digested with micrococcal nuclease (0.03 unit) for 1 minute in a 50  $\mu$ L reaction. The reaction was stopped by the addition of EDTA (2  $\mu$ L of 100 mM) and the DNA was isolated. The purified DNA was divided into four aliquots, three of the aliquots were digested with restriction enzymes which cleave pBR322 at a unique location. The samples were analysed on a 1.2% agarose gel. **Panel A.** Form I pBR322 treated as described. A form I pBR322 marker is shown in lane (a). Lane (b) shows form I pBR322 digested with micrococcal nuclease without restriction enzyme digestion; lane (c) digested with EcoRI; lane (d) Bam H-I; lane (e) Ava I. The ethidium bromide stained gel is printed with reversed contrast. **Panel B.** Relaxed pBR322 treated as described. Lane (a) without restriction enzyme digestion; lane (b) digested with EcoRI; lane (c) Bam H-I; lane (d) Ava I. The ethidium bromide stained gel is printed with reversed contrast. **Panel C.** An autoradiograph of form I pBR322 cleaved with micrococcal nuclease and labelled with  $\gamma$ - $^{32}$ P ATP and polynucleotide kinase prior to restriction digestion. Lane (d) without restriction enzyme digestion; lane (b) digested with EcoRI; lane (c) digested with Ava I. The position of fragments from an EcoRII digest of pBR322 are shown on the left.

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relaxed (form Ir) DNA was used (fig. 5). In a parallel experiment the micrococcal digested material was radioactively labelled with  $\gamma$ -<sup>32</sup>P ATP and polynucleotide kinase prior to restriction and then analysed similarly.

These results show that micrococcal nuclease cleaves supercoiled pBR322 preferentially at a structural feature located at 3.2 kb clockwise from the EcoRI site. This is the location of an inverted repeat recognised by S1 nuclease (39,40). Supercoiled SV40 is similarly rapidly cleaved but the cleavage site is not at a unique location.

### DISCUSSION

The established "A-T" preference of micrococcal nuclease is confirmed and quantitated by the analysis of the 5' terminal nucleotides and the digestion of homopolymers and alternating copolymers.

That the preference of the enzyme is the same when DNA and chromatin are substrates is shown by the 5' terminal analyses. This is further supported by the fact that the same fragments are produced in digestion of reconstituted chromatin and of end labelled DNA. From the sequences studied so far it has not been possible to formulate firm sequence preference rules although some generalisations are possible. Cleavages at single A or T residues which are surrounded by G and C residues occur so infrequently that they are not detected. Short stretches (2-3) of contiguous A and T residues are cleaved slowly and the helix stabilising influence of nearby G and C residues may be important. The most rapid cleavage sites tend to occur in stretches of more than three contiguous A and T residues. In addition it was noted by A. Klug that the phosphodiester bond between T and A residues is often the most frequently cleaved site in these stretches. For instance, the sequence CAAATAG occurs in both fragments studied and in both cases cleavage occurs exceptionally efficiently in this oligonucleotide and at the same T-A bond (figs. 2 and 3). This could be explained by the poor base overlap in the 5' T-A 3' stack (41), and may partly account for the rapid cleavage in the ATTA repeats in pBR322 (figure 3), but the reason for the difference in the precise location of the cleavages is unclear. The rapid cleavage at the T-A bond in the sequence CTAG 36 nucleotides from the SmaI site in mCet1S is surprising in that it does not occur in a long AT stretch, but we note that in addition to the possible poor base overlap this site lies in the D loop of the tRNA gene raising the question of whether the enzyme is recognising tertiary structure features in the DNA. An example of the recognition of tertiary structure is the cleavage at the inverted repeat in supercoiled

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pBR322. S1 nuclease cleaves supercoiled pBR322 at a unique location which maps at exactly the same position as the preferred micrococcal nuclease site and has been shown to be the position of an inverted repeat or potential cruciform structure (39,40).

In the production of nucleosome core particles the nuclease probably cleaves the DNA at a preferred sequence close to the point where the DNA leaves the nucleosome. This partly explains the size heterogeneity of monomer length DNA. This is consistent with the fact that nucleosomes produced by digestion of chromatin reconstituted from poly {d(A-T)} · poly {d(A-T)} show a much narrower distribution of sizes (42).

Since the specificity of the enzyme is the same on naked DNA and on DNA in the form of chromatin, the previously described technique which has been widely used to demonstrate nucleosome phasing appears prone to serious artifacts. In particular it would seem impossible to determine precisely the positions of nucleosomes on DNA using this enzyme.

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