Specific cleavage of chromatin by restriction nucleases

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ABSTRACT

Digestion of mouse and rat liver nuclei with a restriction nuclease from *Bacillus subtilis* (Bsu) is examined in continuation of previous work from this laboratory (Pfeiffer et al., 1975, *Nature* **258**, 450). The finding of more than 95% C in the 5'-termini of the DNA fragments generated during digestion with Bsu shows that the participation of endogenous nucleases in Bsu digestion is extremely small. The restriction nuclease Hae III, an isoschizomer of Bsu, yields identical degradation patterns. The patterns conform to what one expects from statistical calculations based on a nucleosome structure of chromatin with a region preferentially accessible to the nuclease of 40-50 nucleotide pairs per nucleosome. Integrity of the histones is maintained during digestion with restriction nucleases. Digestion of mouse liver nuclei with EcoRII shows that most if not all of the satellite DNA is organized in a nucleosome structure. Also in rat liver, much of the repetitive DNA appears to be present in nucleosomes.

INTRODUCTION

Digestion with endogenous nucleases, micrococcal nuclease, DNAase I and II has been one of the prime methods to elucidate the basic structure of chromatin (e.g. refs. 1-4). For the preparation of chromatin, slight digestion with micrococcal nuclease⁵ has largely replaced mechanical shearing methods. For both, analytical and preparative aspects, restriction nucleases should complement the nonspecific nucleases and open up new possibilities.

We have previously reported that the restriction nuclease Bsu can cleave the DNA in nuclei to yield chromatin of an average size suitable for preparative work⁶. Chromatin

Abbreviations: The nomenclature for restriction endonucleases follows the suggestions of Smith and Nathans⁷. A = deoxyadenylic acid, C = deoxycytidylic acid, G = deoxyguanylic acid, T = deoxythymidylic acid.

obtained by this method will be the starting material of choice for studies on particular coding sequences in chromatin because of the specificity of cleavage which leads to a much more defined product.

In the course of our work on the digestion of nuclei with restriction nucleases, some interesting analytical aspects had emerged concerning the organization in nucleosomes of the DNA and particularly of mouse satellite DNA⁶. Some questions of specificity and extent of digestion of nuclei as well as aspects of the distribution of nucleosomes on repetitive DNA had previously remained open. These questions have now been pursued further.

MATERIALS AND METHODS

Materials. Sepharose 2B was from Pharmacia, Frankfurt/Main, polyethyleneimine thin layer chromatography plates from E. Merck, Darmstadt, 2043 b Mgl paper from Schleicher Schüll, Dassel, Seakem agarose for gel electrophoresis from Marine Colloids Inc., Rockland, Mne., brewer's yeast tRNA and 5'-deoxymononucleotides from Boehringer Mannheim, (γ -³²P)-ATP and ³²P-phosphate from the Radiochemical Centre, Amersham.

Bacterial alkaline phosphatase and polynucleotide kinase were from Boehringer Mannheim, proteinase K from E. Merck, Darmstadt, DNAase I and micrococcal nuclease from Worthington Biochem. Corp., Freehold, N.J., T2 RNAase from Sankyo, Tokyo. Snake venom phosphodiesterase was prepared by M. Petrova⁸ according to Frischauf and Eckstein⁹ and generously donated. The purification procedure for Bsu previously described¹⁰ is similar to the method of Bron et al.¹¹. Hae III was prepared from *Haemophilus aegyptius*, obtained from H. Schaller, according to the method of Roberts et al.¹² and EcoRII according to Yoshimori¹³ from the *E. coli* strain 245 which was obtained from K. Murray.

Digestion of nuclei and gel electrophoresis of the DNA. Mouse and rat liver nuclei were prepared and digested as described⁶ with the following modifications: the NaCl concentration in the incubation medium was raised from 15 mM to 90 mM, and for digestion with EcoRII the pH was adjusted to

8.2 rather than 7.4. The same buffers were used for digestion of free DNA. In preparative digestions of nuclei, the concentration of nuclei was increased by a factor of 40 (equivalent to 1 mg DNA instead of 25 μ g in a total volume of 50 μ l). An increase in enzyme concentration by only a factor of about 2 sufficed to digest the DNA in the nuclei to the same extent as under the analytical conditions. Incubations were terminated by the addition of EDTA and SDS to final concentrations of 20 mM and 0.5%, respectively. Preparative digests were diluted 10-fold with 10 mM Tris-HCl, pH 7.4, 5 mM EDTA, and 0.5% SDS. The incubation mixtures were then digested with proteinase K (0.5 mg/ml) for 2 h at 37°, and the DNA afterwards extracted and analyzed on vertical agarose slab gels as described⁶.

Analysis of 5'-terminal nucleotides. The procedure follows closely that of Murray¹⁴ and Bron and Murray¹⁵. Only the modifications are detailed here. 0.5 mg DNA was isolated from nuclei which had been digested with Bsu or micrococcal nuclease and on electrophoretic analysis gave DNA distributions as shown in Fig. 2j and f, respectively of ref. 6, and Fig. 2 below. The DNA was dialyzed against 20 mM Tris-HCl, pH 8.0, 10 mM MgCl₂, dephosphorylated at a concentration of 0.25 mg/ml with bacterial alkaline phosphatase and re-phosphorylated with polynucleotide kinase. 1.0 ml of the phosphorylation reaction mixtures contained 0.4 mg DNA, 70 units polynucleotide kinase, and 8 nmol (γ -³²P)-ATP (specific activity 15 Ci/mmol). Excess ATP was removed by exhaustive dialysis against 0.4 M NaCl and finally 0.5 mM NaCl, 0.1 mM sodium citrate. The labeled material was separated on 2% agarose tube gels (25 μ g/gel). Afterwards the DNA was extracted from gel fractions by homogenization of the gel and hydroxyapatite chromatography¹⁶. The DNA fractions were digested to mononucleotides with DNAase I and snake venom phosphodiesterase. The mononucleotides were separated by high voltage electrophoresis at pH 3.5 on 2043 b Mgl paper¹⁷ after addition of the four unlabeled 5'-deoxynucleotides. Radioactive spots were detected by autoradiography, cut out, and quantitated by liquid scintillation counting.

Base composition analyses of fractions from rat liver DNA.

Rats were partially hepatectomized and labeled in vivo with ^{32}P -phosphate (15 mCi per rat). After liver regeneration, DNA was extracted from liver nuclei as described above, residual RNA removed by treatment with T2 RNAase, and the DNA digested with Bsu to completion. The digest was chromatographed on a Sepharose 2B column and collected in four fractions which gave DNA size distributions with maximal intensities around 300, 600, 1200, and more than 2000 nucleotide pairs on agarose gels. The DNA from these fractions was digested into mononucleotides as described in the preceding section and the mononucleotides separated by thin layer chromatography on polyethyleneimine plates developed with 0.5 M LiCl¹⁸. The radioactive spots were scraped off and quantitated by liquid scintillation counting.

Histone analysis of nuclei. Rat liver nuclei digested with Bsu for various times were extracted with 0.2 M HCl at 0° for 1 h and centrifuged for 10 min at 10 000 x g. The supernatant was dialyzed against 0.2 mM HCl, lyophilized, resuspended in 50 mM Tris-HCl, pH 6.8, 1% SDS, 10% sucrose, 5% β -mercapto-ethanol, 0.005% bromophenolblue, heated to 100° for 1 min, and analyzed on an SDS polyacrylamide gel (12% acrylamide, 0.8% bisacrylamide) according to Lämmli¹⁹. The gel was stained with Coomassie Blue G-250 according to Fairbanks et al.²⁰, scanned, and the peak areas of the histone H1 species determined relative to those of the other four histones.

Extraction of histone H1 from nuclei by tRNA. The procedure is based on a method designed for the extraction of H1 from chromatin by Ilyin et al.²¹. Rat liver nuclei were suspended in buffer A of Hewish and Burgoyne¹ at a DNA concentration of 1.5 mg/ml and yeast tRNA, purified by additional phenol extractions, added to a final concentration of 300 A₂₆₀ units/ml. After vigorously shaking the suspension for 1 h at 4° it was diluted sixfold with buffer A and the nuclear material recovered by a low speed centrifugation. The tRNA extraction was repeated once and the gel like nuclear material afterwards used for histone analyses and nuclease digestions. No histone H1 was detectable in this material.

RESULTS AND DISCUSSION

Specificity of digestion of nuclei with Bsu. Previous analysis of the digestion of nuclei with Bsu has concentrated on two points, the average molecular weight of the DNA fragments which could be most clearly determined in dilute agarose gels and secondly on the amount of nucleosomal DNA bands best resolved in more concentrated agarose or polyacrylamide gels (Figs. 1 and 2, of ref. 6). These analyses have been complicated by the presence of endogenous nucleases which always lead to some DNA degradation if nuclei are incubated in the presence of Mg^{++} . Even though we have previously shown that under the conditions used for digestion with Bsu and EcoRII, very little breakdown occurs in the absence of the restriction enzyme, we had not been able to eliminate the possibility of an activation of the endogenous nucleases in the course of digestion with restriction nucleases⁶. In order to do so conclusively, we analyzed the 5'-terminal nucleotides of the DNA fragments generated during incubation with Bsu. Any fragment resulting from cleavage with Bsu should have only C at the 5'-terminal positions, since Bsu introduces an even double strand break in the middle of the tetranucleotide sequence $5'GGCC3'$ ¹⁵ $3'CCGG5'$. Endogenous nucleases, on the other hand, would be expected to cleave fairly randomly and the 5'-termini of the fragments would reflect more closely the base composition of the DNA, equivalent to approximately 20% C. DNA from Bsu digested mouse and rat liver nuclei was resolved in agarose gels as shown for rat nuclei below in different context in Fig. 2. (The analogous pattern for mouse nuclei is shown in Fig. 2j of ref. 6). Nucleosomal DNA of the 600 and 800 nucleotide pair regions and the DNA larger than 800 nucleotide pairs were isolated and the 5'-termini determined. The results are shown in Table 1, together with values for DNA from control nuclei incubated without Bsu and a DNA fraction from a micrococcal digest of nuclei. For comparison an analysis of Bsu digested phage DNA is also shown. In the control nuclei, little breakdown of the DNA had occurred compared to the Bsu digested nuclei, and the resulting fragments did not have

Table 1. 5'-terminal nucleotides of DNA fractions in Bsu digests of mouse and rat liver nuclei. 5'-terminal nucleotides were determined for DNA fractions of Bsu digested nuclei as described in Methods. The values are listed for DNA of approximately 600 and 800 nucleotide pairs length and DNA larger than 800 nucleotide pairs (trimer, tetramer, and >tetramer, respectively). Also shown are values for trimeric DNA from a micrococcal digest of nuclei and for an unfractionated phage PM2 digest with Bsu. As a control, nuclei were incubated without nuclease under Bsu conditions and the DNA electrophoresed in 1% agarose gels after labeling the 5'-terminal nucleotides. Little degradation had taken place and due to the relative scarcity of termini the radioactivity was very low. The entire DNA was therefore taken from the gels, and the 5'-termini analyzed.

			T	G	A	C
Bsu	Mouse (Rat)	Trimer	2.6 (2.3)	2.0 (1.4)	1.3 (1.1)	94.1 (95.2)
		Tetramer	2.8	1.7	0.9	94.5
		>Tetramer	1.7 (2.1)	1.3 (1.3)	1.7 (1.1)	95.3 (95.5)
	PM2	Total	0.4	0.8	0.4	98.4
No added nuclease	Rat	Total	22.6	23.5	18.1	35.8
Micr.	Mouse (Rat)	Trimer	45.0 (51.2)	4.1 (0.7)	47.3 (44.7)	3.5 (3.4)

predominantly C at the 5'-terminal position. It is clear, therefore, that the contribution of endogenous nucleases during Bsu digestion of nuclei is extremely small.

The finding of only C at the 5'-termini does not completely exclude nonspecific cleavage by Bsu itself since it was found that with high Bsu concentrations, as used in the digestion of nuclei, the enzyme can also split free DNA at tetranucleotide sequences differing from GGCC in positions 1 and 4 which leads to the appearance of extra bands in phage DNA digests and finally to conversion of the DNA to low molecular weight material (K. Heininger, W. Hörz, and H.G. Zachau, manuscript in preparation). Even though λ dvl DNA

which had been added to the Bsu incubation of nuclei was cleaved exclusively into the bands characteristic of the limit digest, it is impossible to conclude rigorously that cleavage in the nucleus occurs with the same degree of specificity. We therefore turned to the restriction nuclease Hae III, which is an isoschizomer of Bsu and has been found to maintain specificity at high enzyme concentrations (K. Heininger, W. Hörz, and H.G. Zachau, manuscript in preparation). Hae III digests of rat liver nuclei were quantitatively compared to Bsu digests. When identical enzyme concentrations were used the two patterns were found to be indistinguishable in the average molecular weight of the DNA, the amount of discrete bands derived from repetitive DNA in the rat genome (see below) and the amount of DNA in the 200 nucleotide pair register.

Extent of restriction nuclease digestion and molecular weight distribution of DNA fragments. In both, the Bsu and Hae III digests, it was remarkable that elevation of the enzyme concentrations above the highest level previously used⁶ did not change significantly the molecular weight distribution in the DNA patterns to lower molecular weights. Instead, identical patterns were observed already after shorter periods of incubations. This suggests that digestion has reached a plateau value, and it seems reasonable to assume that under these conditions all the sites present in the more accessible regions of the nucleosomal array (see below) have actually been cleaved.

We have previously been concerned with the relative amount of DNA falling into the 200 nucleotide pair repeat pattern after digestion with the highest Bsu concentration (for the trimeric DNA, e.g. 1-4%) since we had expected lower values on a statistical basis (e.g. around 0.3% for the trimeric DNA)⁶. The calculations were based on the assumption that Bsu targets are randomly distributed in accessible segments of the nucleosome of no more than 20 nucleotide pairs length. We could confirm now the quantitations of the nucleosomal DNA using DNA radioactively labeled in vivo with ³H-thymidine. In an effort to explain the high amounts of DNA in the 200

nucleotide pair periodicity, we investigated as one possibility a nonuniform GC distribution in the DNA since this would lead to an increased probability of generating lower multiples of the nucleosomal DNA bands with Bsu. Rat liver DNA was digested with Bsu and separated into four size classes (see Methods). The GC content increased from 39.1% in the largest size class to 43.3% in the smallest one. This increase is more than one would expect on the basis of a relative enrichment of GC pairs among smaller fragments due to the Bsu generated termini. Calculations show, however, that this small degree of heterogeneity in the GC content would increase the calculated values by only about 10% and would thus not suffice to account for the difference between the experimental and the calculated amounts of nucleosomal DNA.

Results recently obtained with micrococcal nuclease (e.g. refs. 23-25) have suggested that the area more accessible to nucleases might comprise as many as 40-50 nucleotide pairs. With this value, instead of the 20 nucleotide pairs originally assumed, the amount of DNA in the Bsu digests falling into the 200 nucleotide pair repeat would be in perfect keeping with statistical calculations. One would calculate for example about 2% of the DNA in the 600 nucleotide pair region compared to 1-4% actually found.

Stability of histone H1 during digestion and effect of H1 extraction on the digestion patterns. Integrity of histones in chromatin prepared by restriction nucleases is a necessary requirement if this procedure is to be used for preparative purposes. In order to monitor possible proteolysis during incubations, histones were extracted with HCl and analyzed by SDS gel electrophoresis. Significant proteolysis could be excluded. Under the incubation conditions the amount of histone H1 decreased by no more than 10% relative to the other four histones over a digestion period of 3 h. This seems to be the case in control experiments without Bsu and may be within the experimental error. In another series of experiments it was investigated if removal of H1 would increase the accessibility of the nuclear material to Bsu without a major

change in the digestion patterns. To this end, H1 was extracted from nuclei by a procedure modified from Ilyin et al.²¹. Subsequent digestion with Bsu showed significantly altered digestion patterns containing more background DNA in the gel patterns with hardly any nucleosomal bands left. High backgrounds were found in the DNA gel patterns also after digestion of the same material with micrococcal nuclease. These results could be explained by the partial destruction of the nucleosome structure or by an increased accessibility of usually well protected DNA regions. H1 depleted chromatin was therefore not further investigated in this context.

Organization of repetitive DNA in nuclei. Digestion of eukaryotic DNA with restriction nucleases has been particularly fruitful for the analysis of simple sequence DNA components. Many of them form discrete bands after digestion with certain restriction nucleases and can thereby be directly differentiated from the rest of the nuclear DNA. A well characterized example is the mouse satellite DNA which forms a series of discrete bands upon digestion with a number of restriction nucleases^{16,26}. The patterns consist of fragments which are integral multiples of a unit length piece of approximately 245 nucleotide pairs. In digests with EcoRII, this unit length fragment is itself the most prominent band comprising 60-70% of the digest. This pattern is apparent in Fig. 1a where total mouse DNA is digested with EcoRII. It is superimposed on a background of heterogeneous cleavage products from the other nuclear DNA. We have previously reported that digestion of nuclei with high concentrations of EcoRII leads to the formation of the characteristic satellite bands⁶ indicating that satellite DNA in chromatin does not differ strikingly from the rest of the DNA in its accessibility for the restriction nuclease. A careful analysis of the EcoRII digestion patterns of mouse liver nuclei shows that bands of nucleosomal DNA in the 200 nucleotide pair register are also produced (Fig. 1b). They are always weaker, however, than in Bsu digests. This is consistent with the lower frequency of occurrence of the pentanucleotide recognition sequence of EcoRII compared to a tetranucleotide

Figure 1. Digestion of mouse liver DNA and nuclei with EcoRII. a) 1 μ g of mouse liver DNA was incubated for 3 h with an amount of EcoRII sufficient for complete digestion and resolved on a 1.5% agarose gel. A densitogram is shown with the numbers denoting the fragments derived from the satellite DNA. Additional bands migrating closer to the origin are derived from other repetitive components of mouse DNA²⁷. b) Mouse liver nuclei were incubated with a concentration of EcoRII 50 times higher than in a). The DNA was extracted and 5 μ g analyzed on a 1.5% agarose gel as in a). The shaded areas and numbers refer to the fragments from the satellite DNA, while mono, di, tri, tetra, and penta denote the respective bands of nucleosomal DNA. In the insert the periodicity of a nucleosomal array and the spacing of EcoRII cleavage sites on satellite DNA are depicted. The space between the circles represents in a schematic manner a region where DNA is more accessible to nucleases. This DNA stretch is assumed to be 40-50 nucleotide pairs of a 200 nucleotide pair nucleosomal periodicity. The EcoRII sites are designated by arrows with a spacing of approximately 245 nucleotide pairs¹⁶.

sequence. The expected amount of, for example, 600 nucleotide pair material can be calculated to be three times lower for EcoRII than for Bsu, consistent with the experimental values.

Superimposed on the broad bands of nucleosomal DNA are the discrete satellite DNA bands. It is remarkable, though, that these bands form a pattern which is quite unlike a

partial digestion pattern from free DNA. Instead, there are strong tetramer and pentamer bands, the trimer is weaker, there is no dimer detectable and only a trace of monomer (Fig. 1b). A pattern very similar to a partial digest of free DNA can be generated, however, if the nuclei are mechanically sheared in order to destroy nucleosomes prior to digestion with EcoRII. Those findings show directly that cleavage of satellite DNA in chromatin is subject to two periodicities, the spacing of the cleavage sites on the DNA and the spacing of the nucleosomes. Only where the two periodicities coincide, in what resembles an interference pattern, cleavage is possible, which is shown schematically in the insert of Fig. 1. This scheme is clearly an oversimplification. There are satellite fragments of trimeric length even though the corresponding cleavage sites in the scheme are in a "non-accessible" region. There might be some variation in the spacing of the nucleosomes which tends to lessen the effect especially with larger satellite fragments. It is clear though, that the majority of the satellite DNA is present in a nucleosome structure. Bokhon'ko and Reeder²⁸ have reached a similar conclusion when they digested mouse chromatin with micrococcal nuclease and by a hybridization assay found the satellite DNA sequences in the nucleosomal DNA.

In rat DNA the situation is more complicated since the repetitive components have not been characterized as thoroughly. Satellite components cannot be easily separated in density centrifugation, but the digestion patterns with restriction nucleases reveal a number of discrete bands^{29,30}. The pattern obtained with Bsu is shown in Fig. 2 together with a Bsu digest of rat liver nuclei. It is remarkable that certain of the discrete bands (see arrows) are present in high amounts in the digest of the nuclei while others are almost completely absent. Mostly fragments which are equivalent in their sizes to the 200 nucleotide pair register are present in large amounts in digests of nuclei and therefore appear as discrete bands within the broad nucleosomal DNA bands. This argues in favor of a nucleosome structure for many of the repetitive DNA components also in rat liver.

Figure 2. Digestion of rat liver DNA and nuclei with Bsu. 3 μ g rat liver DNA was digested for 3 h with an amount of Bsu sufficient for complete digestion. Analysis of the digest in a 1% agarose gel is shown (track 3). Rat liver nuclei were incubated with Bsu at a 100 times higher concentration and 10 μ g of the extracted DNA analyzed in the same way (track 2). The arrows denote bands in the digest of free DNA which are clearly visible also in the digest of the nuclei. In track 1, mouse satellite DNA partially digested with EcoRII is shown as a molecular weight marker with fragments of multiples of approximately 245 nucleotide pairs¹⁶.

Concluding remarks. These findings emphasize the potential value of restriction nucleases for the digestion of chromatin. Work in progress shows that preparative amounts of soluble chromatin can be prepared with restriction nucleases (T. Igo-Kemenes, W. Greil, unpublished). Clearly, questions concerning the organization in chromatin of gene sequences should be amenable to an analysis with this material. These studies will be complemented by further work on the organization of simple sequence DNA in the nucleus. Mouse may be the organism of choice in this respect since detailed information is available on the arrangement of cleavage sites in mouse satellite DNA for a number of restriction nucleases^{16,26}.

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