Analysis of DNA double- and single-strand breaks by two dimensional electrophoresis: action of micrococcal nuclease on chromatin and DNA, and degradation in vivo of lens fiber chromatin

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ABSTRACT

We describe a novel system for two dimensional electrophoresis at neutral and alkaline pH for determining the double-stranded and single-stranded lengths of DNA. With this system we analysed the mode of micrococcal nuclease digestion of DNA in cellular and SV40 viral chromatin and of supercoiled SV40 DNA. The enzyme reaction occurred in two steps : the enzyme first introduced single-strand breaks, then converted these to double-strand breaks by an adjacent cleavage on the opposite strand. Digestion of cellular chromatin DNA occurred by a similar mechanism. Chromatin fragments produced by limited micrococcal nuclease action contained many single-strand breaks, which may be important when this method is used to prepare chromatin fragments for biochemical and biophysical studies. Nucleosome monomer to tetramer produced at later stages of digestion contained few if any single-strand breaks.

INTRODUCTION

The length of native and denatured DNA can be measured by sedimentation through neutral and alkaline sucrose gradients respectively (1, 2), or by electrophoresis in neutral or denaturing conditions (3-5). With the exception of the two-dimensional electrophoretic system (3), none of the existing methods allows a simultaneous determination of the double- and single-strand lengths of DNA molecules in a heterogeneous population. Recently, McDonnell et al. (4) have described electrophoresis of bacteriophage T7 DNA in agarose gels made in either neutral or alkaline buffers. The horizontal gel apparatus offers the advantage that one can construct low concentration agarose gels and thus study DNA of high molecular weight. We have combined the separate neutral and alkaline buffer systems of these authors, and we describe here a two-dimensional gel system which allows determination of both single- and double-strand lengths of the same DNA in a single resolving gel.

Micrococcal nuclease has been used widely to investigate the structure of chromatin. Since the studies by Hewish and Burgoyne (6), and Noll (7), a large body of evidence (see, for reviews 8, 9) has established that micrococcal nuclease, like the endogenous $\text{Ca}^{++}/\text{Mg}^{++}$ -dependent nuclease, cleaves chromatin between the nucleosomes to give rise to DNA fragments which in length are multiples of a monomeric unit. This process also occurs in vivo (10, 11) for instance in terminally differentiating lens fiber cells as nuclei degenerate and disappear. It has been suggested (12, 13) that micrococcal nuclease cleaves DNA by double-strand breaks, which seems to be in contrast with the situation for the endogenous nuclease from rat liver (3). Since the mode of action of micrococcal nuclease has not been studied by a simultaneous analysis of the double- and the single-strand length of the product, we have studied the action of this enzyme on a variety of substrates using the two-dimensional electrophoretic procedure described here. Similarly we have investigated the digestion in vivo of lens fiber cell chromatin by the endogenous nuclease during the terminal stage of lens differentiation.

METHODS

Digestion of Simian Virus 40 (SV40) chromosomes and avian embryo nuclei with micrococcal nuclease. SV40 chromosomes were isolated from virions by a modification (14) of the procedure of Christiansen et al. (15). SV40 DNA was extracted from lytically infected cells (16). DNA or chromatin samples were suspended in buffer containing 60 mM KCl, 3 mM NaCl, 15 mM Tris-HCl (pH 7.4), 0.34 M sucrose, 0.1 mM EDTA (17) which was supplemented with 0.1 mM EGTA, 5 mM 2-mercaptoethanol and 0.2 mM phenylmethylsulfonylfluoride (Sigma). Nuclei from 6-day old duck embryos and 15-day old chick embryo liver were isolated and purified as described before (18) and suspended in the buffer described above. Samples were treated with micrococcal nuclease (Worthington grade NFCP, electrophoretically pure) at 37° C (duck and chick nuclei), or at 0° C (SV40 chromosomes and DNA) in the presence of 2 mM CaCl_2 . The enzyme reactions were stopped by adding EDTA (2 mM final concentration), 10 ug/ml proteinase K and 0.1% sodium dodecyl sulphate (SDS). The lysates were then incubated at 37° C (1-6h). Samples were cooled on ice in the presence of 0.1M KCl and centrifuged (1 min., 1000 rpm, Beckman Microfuge) to remove the SDS:KCl complex (10).

The supernatants were subjected to electrophoresis.

Lens dissection. Lenses from 19-day old chick embryos were dissected to obtain lens fiber masses (10). Lens fiber masses were frozen on solid CO₂ and central (CF) and outer fiber (OF) masses were punched out as described (11). Samples were thawed, homogenized, suspended in the above buffer and treated with SDS-proteinase K (10). In some cases samples were phenol-extracted to purify DNA. At this developmental age, CF cells are nonnucleated but contain chromatin fragments with predominantly low molecular weight DNA, while OF cells are nucleated and transcriptionally active (10, 11, 19).

Apparatus for two-dimensional electrophoresis. The horizontal slab gel electrophoresis apparatus of McDonell et al. (4) was used. A single resolving gel (13.0 cm x 12.0 cm x 0.3 cm) was used for electrophoresis in both dimensions. A special comb was devised for making the wells (Figure 1). One set of wells is for sample and marker DNA to be electrophoresed in the first dimension. Another well is aligned immediately behind and at right angles to the innermost well of the first set, and is used for loading the marker DNA prior to electrophoresis in the second dimension. This system has the advantage of giving a uniform electrical field for migration of DNA from the first-dimension gel track through the gel bed in the second dimension.

Gel electrophoresis, staining and photography. 1.6% agarose resolving gels were made in Tris-acetate buffer (0.04 M Tris acetic acid, pH 7.8,

Figure 1. (a) design of the comb for horizontal slab gel electrophoresis in two dimensions. (b) electrophoresis in the first dimension; the sample is loaded in well A1 and A2, and size markers in A3. Using duplicate samples avoids ultraviolet irradiation of the DNA to be electrophoresed under denaturing conditions in the second dimension. (c) electrophoresis in the second dimension; markers are loaded in well B1.

0.02 M Na acetate and 2 mM EDTA). The resolving gel affords 9.5 cm pathlength for electrophoresis, sufficient to resolve DNA of molecular weight between 5×10^4 and 4×10^6 daltons. For SV40 chromosome DNA, 1.5 % agarose gels were used. DNA from nuclease digested chromatin samples was electrophoresed (4V/cm of the resolving gel) until the dye reached 7-8 cm distance. The agarose strip containing sample tracks A2 and A3 (Figure 1) was cut out, incubated with RNase A (25 ug/ml, 37⁰ C, 30 min), stained in ethidium bromide (2 ug/ml, 20 min) and photographed on an ultraviolet transilluminator plate (Model C-61, UV Products Inc., San Gabriel, Calif. USA) at 254 nm using Polaroid positive film type 667 or positive-negative film type 665. The gel slab containing tracks A1 and B1 was then rinsed in water (2 x 15 min) and soaked in 30 mM NaOH, 2 mM EDTA (2 x 15 min). The slab was then transferred to another gel support with 1% agarose bridges made in the alkaline buffer, and aligned with its original electrophoretic axis at right angles to the electrical field to be applied in the second dimension. The excess buffer along the edges was blotted out and fresh alkaline agarose poured around the slab to make electrical contact. Denatured marker DNA and the tracking dye were loaded in the well B1 and electrophoresis carried out in the alkaline buffer (4V/cm) until the dye front reached 8 cm (bromophenol blue) or 10 cm (phenol red). For staining, alkaline gel slabs were first rinsed in water (2 x 5 min) and then neutralized in 50 mM Na phosphate (pH 7.0) for 15 min. Acridine orange stain (15 ug/ml, 15 min) was used. Gels were destained in the phosphate buffer overnight at 4⁰ C or at room temperature for 4-5 h. Stained gels were photographed on a UV transilluminator plate as before. Densitometric scanning of Polaroid negatives was done with the Gilford model 2400-2 spectrophotometer and peak areas measured with the Hewlett Packard model 9825A/9864A. Molecular weight markers were SV40 DNA digested with restriction endonuclease Hind III (31).

RESULTS

The electrophoresis of DNA in horizontal agarose slab gels in neutral or alkaline buffers was described by McDonell et al. (4). The technique described here is a combination of the two buffer systems and offers the advantage that double-strand and single-strand size of DNA can be revealed

directly in a single gel. We adopted the acridine orange staining method developed in our laboratory (5) for staining native and denatured nucleic acids electrophoresed in polyacrylamide and agarose gels. This procedure is more sensitive than ethidium bromide staining for single-stranded nucleic acids (5) and in addition it allows to discriminate between double- and single-stranded DNA by their green and red fluorescence respectively. In Figure 2 we show a two-dimensional electrophoresis of a mixture of SV40 DNA cut by Hind III or Eco RI restriction nucleases. Upon denaturation, the DNA fragments migrate in the second dimension in such a way as to fall on a diagonal line. This system gives results superior to those obtained (3) using 2.5% polyacrylamide in the first dimension and 7.5% polyacrylamide in urea in the second. Using a single resolving gel for electrophoresis in both dimensions avoids the serious distortions (such as bending of the diagonal, 3) in the electrophoretic pattern of denatured DNA in

Figure 2. Two-dimensional electrophoresis of SV40 DNA restriction fragments. The pattern after first dimension electrophoresis is shown in the strip along the top of the figure. The faint bands not labelled with a letter are products of partial enzyme digestion. After electrophoresis in the second dimension, Eco RI-generated linear (T, 5224 base pairs) and Hind III fragments A to E (1772 to 447 base-pairs) lie on a diagonal line.

the second dimension.

Mode of digestion of SV40 DNA and SV40 chromosomes by micrococcal nuclease. Supercoiled SV40 DNA is a useful substrate for studying the mechanism of nuclease action. The introduction of one (or more) single-strand breaks converts the molecule to the relaxed circular form, and the introduction of a double-strand break gives the linear form. These three forms are easily separated by gel electrophoresis.

Supercoiled SV40 DNA was digested with increasing amounts of micrococcal nuclease and the reaction products were analysed by electrophoresis. From the densitometric scans of negative photographs, the amounts of supercoiled, nicked circular and linear DNA forms were estimated (Figure 3). Control experiments confirmed that the amounts of DNA in the three forms can be reliably quantitated by densitometry of the ethidium bromide fluorescence photographs (20). At low concentration of the enzyme, the major product was nicked circular DNA. With increasing enzyme concentrations, the proportion of nicked circular DNA decreased while that of the linear form increased. We also carried out time-course experiments in which SV40

Figure 3. Mode of digestion of SV40 DNA by micrococcal nuclease. DNA (0.12 ug) was incubated with micrococcal nuclease in 20 ul for 1 min. Left panel : photograph of the ethidium-stained gel : A, full length linear DNA marker ; B, supercoiled (lower band) and nicked circular (upper band) DNA ; C-F, digestion products of respectively 3.1, 6.2, 12.5 and 25 units/ml nuclease. Right panel : the proportion of each form of DNA determined by densitometry was plotted against nuclease concentration (units/ml). Δ supercoiled ; ○ nicked circular ; □ linear.

DNA and SV40 chromosomes (15) were digested with a constant amount of micrococcal nuclease for increasing lengths of time. Figure 4 shows the result of such an experiment using SV40 chromosomes as substrate. The SV40 chromosome preparation contained, before nuclease digestion, predominantly covalently closed circular DNA (supercoiled after removal of proteins) and about 20% of nicked circles. As we observed with the supercoiled SV40 DNA substrate, during digestion with nuclease the amount of nicked circular DNA at first increased. With longer incubation times this amount fell, accompanied by an increase in the amount of linear forms. Thus the initial mode of action of micrococcal nuclease on the DNA in SV40 chromosomes seems to be essentially the same as on supercoiled DNA.

These observations suggested that micrococcal nuclease might convert covalently circular DNA to the linear form by way of a nicked circular intermediate. But the result could arise in at least three ways. (a) micrococcal nuclease makes single-strand breaks in both DNA strands at random so that a double-strand break results from chance coincidence of breaks on opposite strands. (b) the nuclease first makes a single-strand break, then cuts the intact opposite strand near or at the site of the first break.

Figure 4. Time course of the introduction of single-strand and double-strand breaks into SV40 chromosomal DNA by micrococcal nuclease. Reactions (20 μ l) containing 0.3 μ g DNA in SV40 chromosomes and 1 unit of nuclease were incubated for A, 1 min; B, 2 min; C, 5 min; D, 10 min; E, incubated 10 min without nuclease. Left panel: the uppermost band is nicked circular DNA, the middle band is linear, and the lowest band supercoiled. Right panel: the data plotted as in Figure 3.

(c) the nuclease makes, quite independently, single-strand breaks at a high rate and double-strand breaks at a lower rate. These possibilities were distinguished by examining the products from SV40 DNA at three stages of the reaction, by two-dimensional gel electrophoresis.

Figure 5a shows the pattern obtained with a low concentration of nuclease which resulted in the formation of a small amount of linear DNA. The linear DNA, which would be expected to yield in alkali single-stranded linear molecules, gave rise to one band in the second dimension. The nicked circular DNA gave rise in the second dimension to two overlapping bands, of which the faster-moving comigrated with the band from linear DNA. We interpret the two bands from denatured nicked circular DNA as single-stranded circular and linear molecules respectively. Since the intensities of the two bands are similar most of the nicked circular DNA contained one single-strand break. With a nuclease concentration which converted about 30% of the DNA to the linear form (Figure 5b), many of the nicked circular DNA molecules still contained one single-strand break. With still higher amount of nuclease, when no supercoiled DNA remained (Figure 5c), the linear molecules contained both nicked (evidenced by a downward smear in the second dimension) and unnicked forms. The existence of a clearly delineated diagonal shows that many of the smaller DNA frag-

Figure 5. Two-dimensional electrophoretic analysis of SV40 DNA digested by micrococcal nuclease. As in Figure 2 the 1st dimension electrophoresis (neutral pH) is shown from left to right along the top of each panel. 2nd dimension electrophoresis (alkaline pH) is from top to bottom. The relative rates of DNA migration from the first dimension origin (upper strip, left) are nicked circular < linear < supercoiled. The extent of nuclease digestion increases in the order a, b, c and corresponds approximately to that in Figure 3 C, D and F respectively. For lengths of the 2nd dimension markers see Figure 6.

ments were not nicked.

The mechanisms (a) and (c) described above predict that the nicked circular DNA formed in the reaction would sustain a large number of single-strand breaks before a significant amount of the linear form were made. From the data in Figure 5 this is clearly not the case. Let us assume that staggered single-strand breaks on opposite strands, up to 10 base-pairs apart, give rise under the conditions used to an effective double-strand break. The DNA in Figure 5a contained, for the most part, either 0, 1 or 2 single-strand breaks. Of SV40-DNA molecules with two single-strand breaks on opposite strands, about 0.4% would be expected to show a double-strand break (i. e. be linear) on the basis of random coincidence. But the result observed (Figure 5a) was that, within the limits of detection, all the DNAs having two single-strand breaks were linear, because the nicked circles present had only one break (see above). So we think that micrococcal nuclease makes double-strand breaks in DNA by introducing successive single-strand breaks closely spaced on opposite strands.

Mode of action of micrococcal nuclease on nuclear chromatin.

Duck embryo nuclei were digested with micrococcal nuclease for 0.5 or 3 min. The deproteinised DNA products were electrophoresed in two-dimensional 1.6% agarose gels (Figure 6). In 0.5 min digests, double-stranded DNA migrated with a heterogeneous distribution although a discrete mononucleosome band was seen. In the second dimension, denatured DNA migrated along the diagonal and below it (Figure 6a). Thus the native DNA fragments contained many single-strand breaks which, upon denaturation, gave rise to single-stranded fragments moving below the diagonal. Single-stranded fragments from unnicked molecules are found on the diagonal. The single-strand breaks in at least nucleosome dimers and trimers were predominantly between nucleosomes since discreet bands below the diagonal could be seen (Figure 6a). With increasing time of enzyme treatment, the proportion of DNA found as short fragments from nucleosome oligomers increased (Figure 6b). An intermediate stage in the digestion of nuclei from chick embryo liver is shown in Figure 6c. Without nuclease digestion these DNAs were of high molecular weight (not shown). Nicks were present in nucleosome oligomer, except monomer, DNA only at early stages of digestion (Figure 6a) ; at the later time points,

Figure 6. Digestion of avian embryo nuclei by micrococcal nuclease. Duck embryo nuclei (a and b) (5×10^6 per 100 μ l) were digested with 6 units of micrococcal nuclease for 0.5 min (a), or 3 min (b). Chick embryo liver nuclei (c) were digested for 20 min. Electrophoresis was in two dimensions as Figure 2. SV40 Hind III DNA fragment lengths in base-pairs : A 1772 ; B 1169 ; C 1095 ; D 526 ; E 447 ; F 215.

monomer to tetramer DNA did not contain nicks in detectable amounts. We interpret these digestion patterns in terms of the mode of action discussed above whereby the micrococcal nuclease initially introduces single-strand breaks into chromatin DNA, and then in a second step converts most of these to double-strand breaks.

Degradation of differentiating lens fiber chromatin DNA in vivo in 19-day chick embryos. During the terminal differentiation of lens fiber cells nuclei degenerate and disappear with a concomitant loss of DNA (19). This has been correlated with the appearance of single-strand (21, 22, 23, 24) and double-strand (10) breaks in the DNA. The size distribution of native and denatured DNA from central and outer lens fibers (10) was analyzed on two-dimensional gels (Figure 7). In the first dimension, native DNA from central fibers (Figure 7a) migrated as two size classes, one comprising oligomers of 160 bp (10), and the other being long DNA migrating more slowly than the SV40 linear DNA marker (5224 bp). Upon denaturation and electrophoresis in the second dimension, the nucleosome monomer to pentamer fragments migrated along the diagonal indicating that these did not contain nicks. The long DNA did contain nicks since single-stranded fragments of size down to about 1000 nucleotides were seen

Figure 7. Two-dimensional electrophoresis of DNA from 19-day chick embryo lens fibers. (a) central fiber DNA ; (b) outer fiber DNA.

in the second dimension. There was little material migrating between the two DNA size classes. Most of the outer fiber DNA (Figure 7b) migrated as long DNA at neutral and alkaline pH. Outer fiber cells contained in addition a small amount of ethidium-stained material whose migration corresponded to DNA of size 440-800 bp. We do not know its nature or origin ; its denatured products were not detectable in the second dimension. Thus, degradation of DNA in differentiating lens fiber chromatin *in vivo* may well occur in a manner similar to that of chromatin digestion *in vitro* by micrococcal nuclease (Figure 6). By electrophoresing in 0.5% agarose gels, we have found recently that the long DNA in outer fibers has a molecular weight greater than 3×10^7 daltons, while that in central fibers has between 3×10^6 and 6×10^6 daltons (Modak, unpublished). Thus, the long DNA classes seen in Figure 7a and b are in fact of different size in outer and in central fibers.

DISCUSSION

We have studied the mode of action of micrococcal nuclease on SV40 DNA and on DNA in SV40 chromatin and cellular chromatin. The kinetics of

appearance of single- and double-strand breaks suggest a mechanism in which the enzyme cuts double-stranded DNA in two steps, firstly introducing a single-strand break then cutting the opposite strand near or at the site of the first break. Thus in the reaction time course with covalently circular SV40 chromosomes as substrate (Figure 4) the proportion of nicked circles increased at early time points, then fell. From the extent of accumulation of the presumed nicked circular intermediates (they reached a maximum level of about half the DNA present), we infer that the rate at which nicked circles are formed and the rate at which they are subsequently converted to linears are roughly similar under these conditions.

Nucleolytic cleavage of double-stranded DNA by way of successive single-strand breaks may well be a common mechanism. The endogenous $\text{Ca}^{++}/\text{Mg}^{++}$ -dependent nuclease from rat liver (3) and restriction enzymes Eco RI and Hpa II (27, 28) have been reported to work that way. S_1 endonuclease introduces a double-strand break into supercoiled DNA apparently by a two-step mechanism (29). Micrococcal nuclease (25), like S_1 nuclease (30), digests single-stranded DNA more rapidly than double-stranded. In native DNA, phosphodiester bonds adjacent to adenine or thymine residues, which are less strongly base-paired, are preferentially attacked (25). Therefore a reasonable model for micrococcal nuclease cleavage of double-stranded DNA is that the enzyme initially cuts one DNA strand in a transiently denatured region, then makes the second cleavage in the single-stranded DNA segment resulting from local denaturation, or exonucleolytic degradation of DNA ends, at the site of the first single-strand cleavage. From the present experiments alone we cannot be sure that micrococcal nuclease itself carries out all steps. But it has already been established (reviewed in ref. 25) that purified micrococcal nuclease can catalyse separately the reactions which we think occur in succession to give a double-strand break.

These conclusions on the mechanism of micrococcal nuclease action qualify the generally-held idea (see for example refs 12, 13) that the enzyme makes double-strand breaks in chromatin DNA. DNA in high molecular weight chromatin treated with the enzyme contains single-strand breaks. Micrococcal nuclease digestion has been used in the preparation of chromatin fragments (13); the presence of single-strand breaks

may be of importance when such preparations are used for biochemical and biophysical analyses. If some regions of isolated chromatin are under torsional stress (26) this would be relieved by nicks in the DNA. We do confirm, on the other hand, that DNA of single nucleosomes prepared in this way contains few, if any single-strand breaks (3, 12, 13).

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